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Studies of interactions of staphylococcal and streptococcal peptides with a type-I module pair of human fibronectin

Thesis submitted for the degree of Doctor of Philosophy
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La perfection est atteinte non quand il ne reste rien à ajouter, mais quand il ne reste rien à enlever.

(You know you've achieved perfection in design, not when you have nothing more to add, but when you have nothing more to take away.)

A.M.R. de Saint-Exupery */The Little Prince/*

ACKNOWLEDGEMENTS

First of all I would like to thank my supervisors, Iain and Jen, without whom none of this would have been possible. You are both great and patient (especially Jen!) teachers and I hope I shall make good use of the knowledge I have gained thanks to you. Also a big thank you to Uli, Joern, Andy P., Minakshi and Elspeth; to all the rest of IDC and RR people, who made my time here both educating and fun; and the Wellcome Trust for funding this project.

As for strictly personal acknowledgements, I have decided against naming individuals. It would add another chapter to this thesis and could get too personal at times... You know I am thinking about you now! So I am just bowing low before St. John's, DP, PTCL and 'wherever else' teams of people uttering humble words of gratitude for putting up with my moods and ideas. We had a great time here, did we not?

And of course a huge thank you to my family and Polish friends, now spread out all over the Europe (good! I had lots of places to visit!), for their constant support. These pretty pictures inside are for you.

ABSTRACT

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A number of pathogenic bacteria, such as *Staphylococcus aureus* and *Streptococcus pyogenes*, can adhere to the protein fibronectin (Fn) in the human extracellular matrix. These host/pathogen interactions are likely to play an important role in the process of infection and have therefore been identified as targets for new therapeutics. Numerous proteins from gram-positive bacteria have been shown to bind to the N-terminal part of Fn, which contains five F1 modules ($^{1\text{-}5}$F1). Fn-binding proteins have several homologous binding units, which exist in an unfolded state under physiological conditions. The primary binding site for the D3 repeat from *S. aureus* Fn-binding protein A (FnbpA) is the ^{4}F1^{5}F1 module pair (Huff *et al.*, 1994).

The high-resolution structure of ^{4}F1^{5}F1 has been calculated *de novo* and the structure of ^{4}F1^{5}F1 in complex with D3 has been determined using nuclear magnetic resonance (NMR). Long-range orientational restraints derived from ^{15}N relaxation and H^{N}-N residual dipolar couplings have been added to conventional NOE and J-coupling data. The ensemble of the final structures of bound ^{4}F1^{5}F1 was used as a model in molecular replacement in attempts to solve the crystal structure of the ^{4}F1^{5}F1/D3 complex.

A new alignment of Fn-binding repeats (U Schwarz-Linek – manuscript in preparation) suggests the presence of conserved short motifs that bind specifically to individual $^{1\text{-}5}$F1 modules. This model was tested using residue-specific NMR binding studies; the ^{1}H^{15}N chemical shift changes induced by ligands from *S. aureus* and *S. pyogenes* were mapped on the structure. 'Dissecting' the D3 repeat has shown that it consists of smaller units that exhibit different binding specificities. Ligands from two different regions of the FnbpA protein from *S. aureus* were shown to induce similar chemical shift changes in Fn modules. Isothermal titration calorimetry and NMR were used to determine the binding constants of the module pair/ligand interactions. Peptides consisting of several binding motifs were shown to bind stronger than ones containing a single binding site. These studies lead to a better understanding of FnBP-Fn interactions.

WORK DONE IN COLLABORATION

Some of the work presented in this thesis was done in collaboration with other scientists. The *Pichia pastoris* clones, as well as a part of protein samples, were produced by Drs A. Pickford and J. Bright. The electrospray mass spectrometry (ESMS) was performed by Drs R. Aplin and N. Oldham. The ^{15}N-relaxation data for the unbound ^{4}F1^{5}F1 module pair (Chapter 4) were collected by Dr J. Werner. The collection of X-ray diffraction data (Chapter 6) was carried out by J. Murray and Dr E. Lowe. The studies of N-STAB (Chapter 7) were performed by H. Jenkins. The docking protocol (Chapter 7) was prepared and run by Dr A. Almond.

ABBREVIATIONS

CD - circular dichroism

COSY - correlation spectroscopy

DANI - diffusion anisotropy

ECM – extracellular matrix

ESMS - electrospray mass spectrometry

EUR - extended upstream region

F1 – type I module of fibronectin

F2 – type II module of fibronectin

F3 – type III module of fibronectin

^{n}FX – nth type X module of fibronectin

FID - free induction decay

Fn – fibronectin

FnBP - fibronectin-binding protein

FnbpA - fibronectin-binding protein A

FPLC - fast protein liquid chromatography

GST – glutathion S-transferase

HMQC - heteronuclear multiple-quantum correlation

HPLC - high performance liquid chromatography

HSQC - heteronuclear single-quantum correlation

ISAC - implicit Saupe tensor alignment constraint

ITC - isothermal titration calorimetry

M.W. - molecular weight

MAD - multiwavelength anomalous dispersion

MIR - multiple isomorphous replacement

MR - molecular replacement

NMR - nuclear magnetic resonance

NOE - nuclear Overhauser effect

NOESY - nuclear Overhauser effect spectroscopy

RDC - residual dipolar couplings

RMSD – root-mean-square deviation

SANI - Saupe anisotropy

SfbI - streptococcal fibronectin-binding protein I

SUR - short upstream region

TOCSY - total correlation spectroscopy

UR - upstream region

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1. INTRODUCTION

1.1. Modular proteins

Numerous medium to large sized proteins consist of repeated copies of smaller conserved sequences, that fold as independent units. These proteins are often referred to as mosaic or modular proteins. Within protein families, the tertiary folds of the individual modules are highly conserved, even though their sequences may be considerably different. Thus it appears that only a limited number of key residues is necessary to maintain a structural fold (Baron *et al.*, 1990, Bork *et al.*, 1996).

The major advantage of building proteins from modules is likely to be the simplification of the creation of new proteins during evolution. Although modules can already be found in prokaryotes, most likely they were acquired relatively late in evolution as they are only found in rather specialised bacteria. In eukaryotes, intracellular modules mostly occur in the cytoskeleton and in signal transduction pathways that do not seem to have an equivalent in bacteria. The majority of modular proteins are however, extracellular, such as numerous proteins of the mammalian extracellular matrix (Bork *et al.*, 1996).

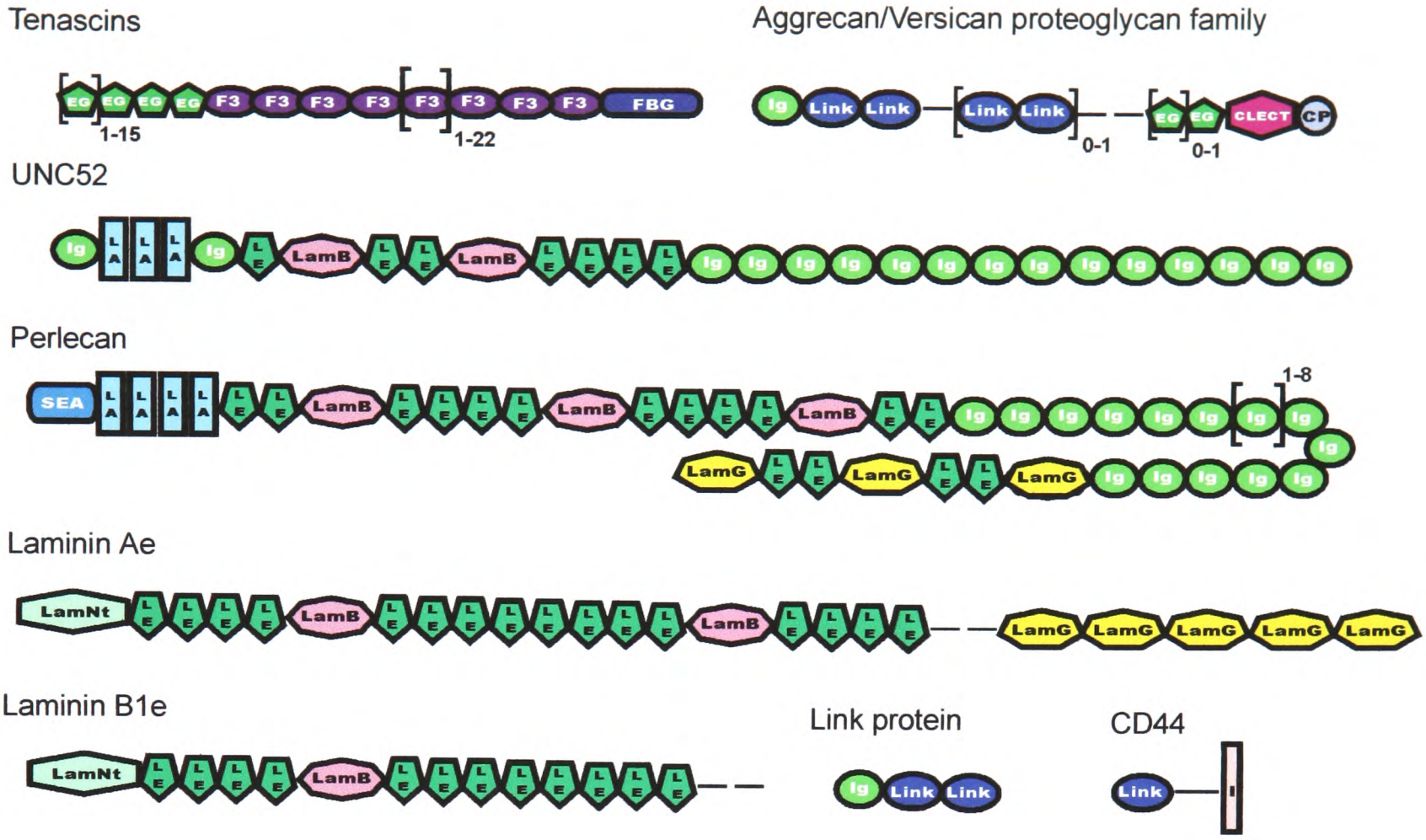
1.2. Extracellular matrix

The extracellular matrix (ECM) is a stable structure that underlies epithelial and endothelial cells and surrounds connective tissue cells. The major matrix components are collagens, highly glycosylated proteins such as laminin and fibronectin (Fig.1.1), and proteoglycans containing protein-bound glycosaminoglycan chains. Many of these macromolecules form complex interactions with each other, with minor components of the ECM and with cells. They also function as targets for bacterial adhesion (Kuusela, 1978, Switalski *et al.*, 1982). Bacterial pathogens that are primarily extracellular can adhere to components of the ECM of the host to initiate colonisation of specific tissue. Adherence is mediated by protein adhesins binding to ECM elements such as fibrinogen or fibronectin (Patti *et al.*, 1994).

1.3. Human fibronectin

Fibronectin (Fn - Fig.1.2a) is a 460 kDa mosaic protein, almost entirely composed of three different types of modules (F1, F2, F3). It is secreted into the extracellular environment as a dimer, where two monomers are joined in an antiparallel orientation near their C-termini by two disulphide bonds (An *et al.*, 1992). Fn has been found in a soluble, dimeric form in the plasma and also in an insoluble, fibrillar form as part of an ECM. Fn takes part in important physiological processes such as embryogenesis, wound healing, haemostasis and thrombosis (Hynes, 1990). Different biological activities of Fn have been ascribed to different regions using a fragmentation approach (digestion with trypsin, cathepsin D, G and plasmin – Venyaminov *et al.*,

a. Proteins of the extracellular matrix



b. Other modular proteins

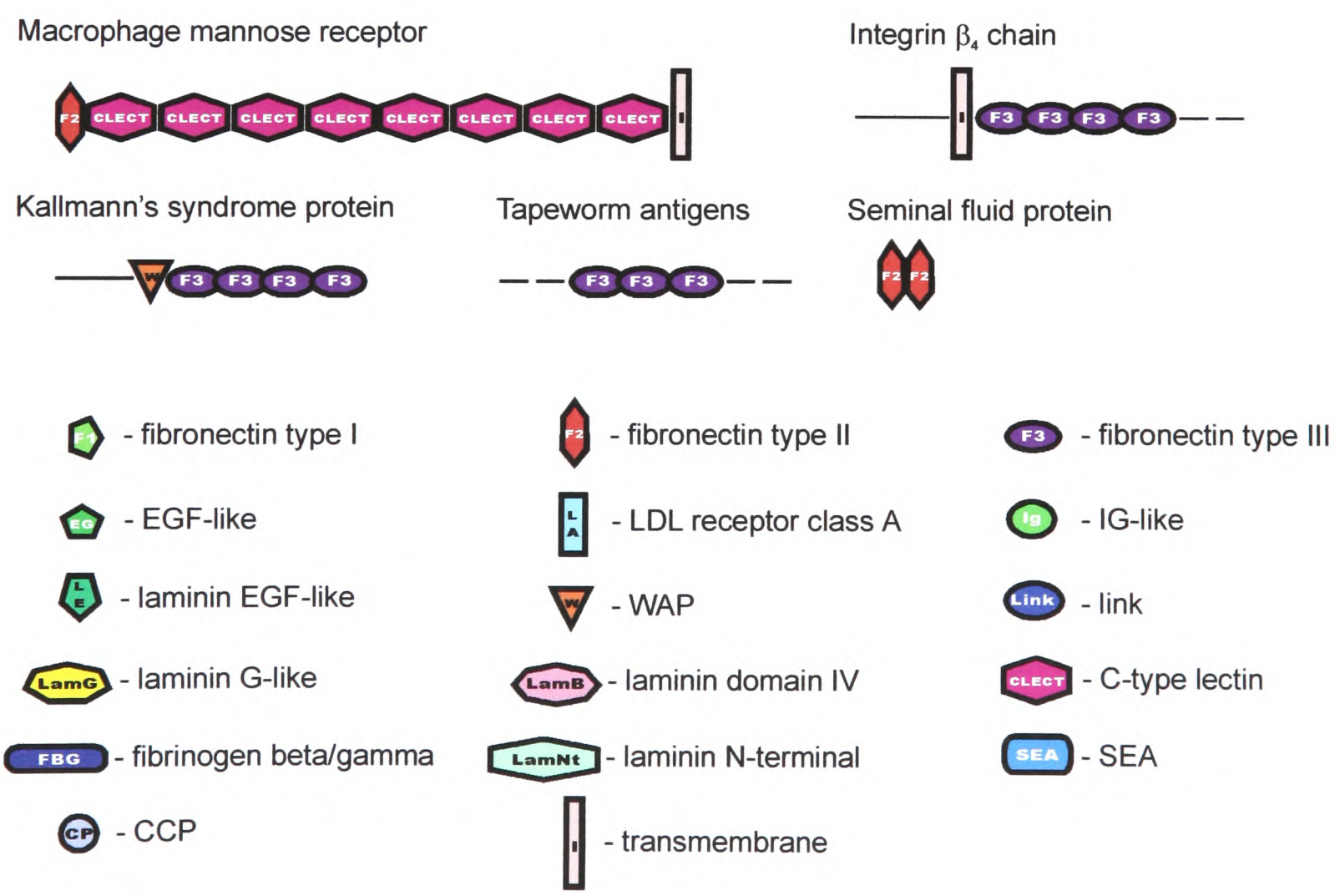


Fig.1.1. Selected modular proteins. Based on Bork & Bairoch (1995).

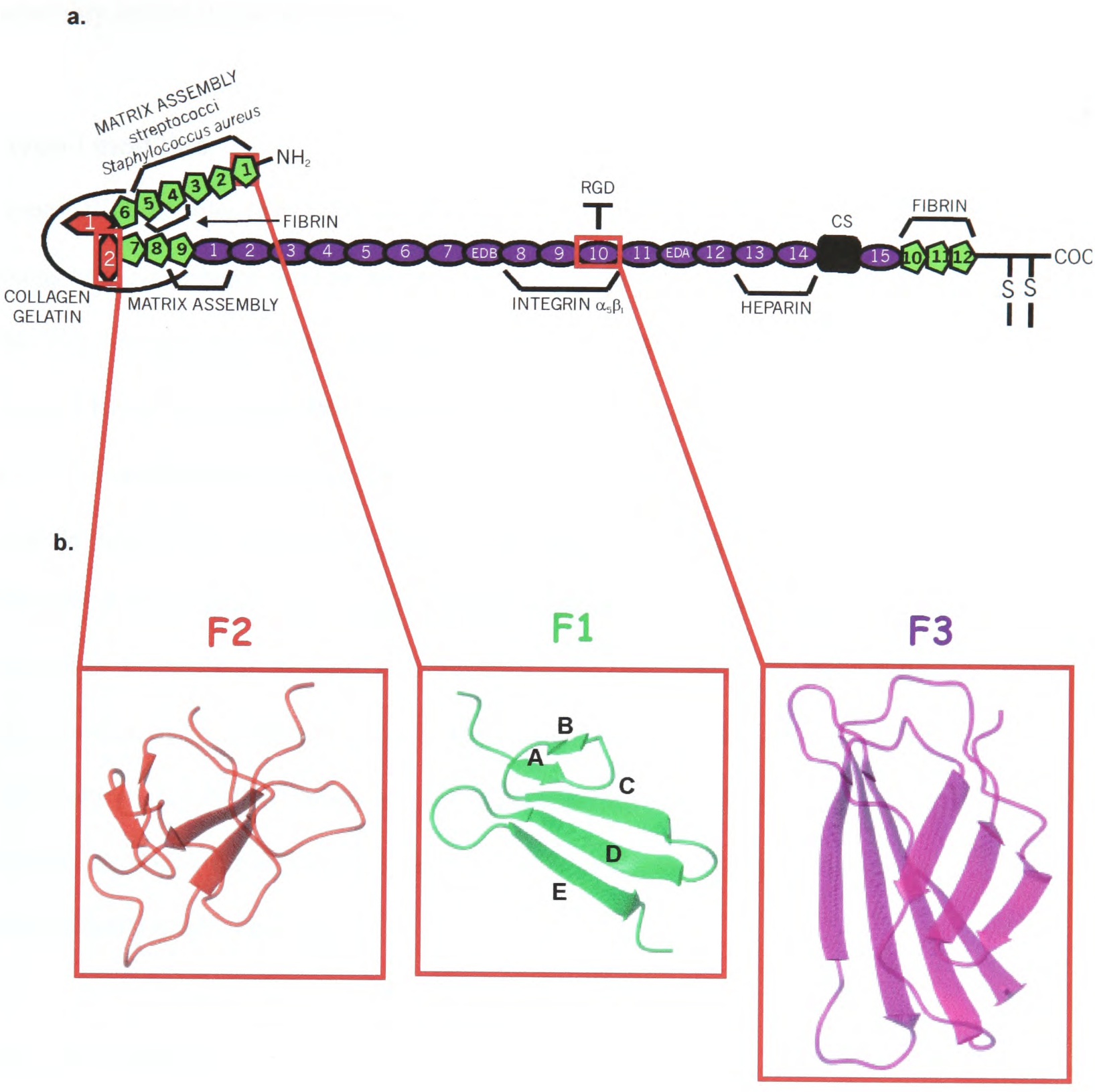


Fig.1.2.

a. The modular structure of Fn (Hynes, 1990). - F1, - F2, - F3, EDA, EDB, CS - - alternatively spliced regions (EDA and EDB - extra domains A and B, CS - connecting segment). RGD - $\alpha_5\beta_1$ integrin-binding motif (Pierschbacher & Ruoslahti, 1984). Other binding sites are also shown (Hahn & Yamada, 1979; Matsuka *et al.*, 1994; Mosher, 1993).

b. Three-dimensional structures of the three types of Fn modules: [1]F1 with labelled β-sheets (Potts *et al.*, 1999), [2]F2 (Sticht *et al.*, 1998) and [10]F3 (Main *et al.*, 1992).

1983). Unlike other ECM components, soluble Fn does not self-polymerise. Instead, its assembly occurs at specialized areas on the cell surface (Mosher, 1993).

Fn type-I modules

Fn type-I (F1) modules (Fig.1.2b) are found in the N- and C-terminal fibrin-binding domains and the collagen-binding domain of Fn, in tissue plasminogen activator and in the blood coagulation factor XII. Each F1 module consists of approximately 45 residues. F1 modules contain four cysteine residues which form two disulphide bonds in a 1-3, 2-4 configuration, except for the twelfth F1 module (^{12}F1) which has an extra disulphide bond. The tertiary structure of the module: a short, double-stranded antiparallel β-sheet folded over a triple-stranded antiparallel β-sheet, is stabilized by hydrophobic interactions between core residues. The ^{8}F1 module in the gelatin-binding domain of Fn is the only glycosylated F1 module in Fn, having two N-linked sugars (Potts *et al.*, 1994). Through this thesis, the β strands of the F1 modules will be referred to as A-B (forming the double-stranded β-sheet) and C-D-E (forming the triple-stranded β-sheet) – eg. Fig.1.2b.

Fn type-II modules

Fn type-II (F2) modules (Fig.1.2b) are found in the collagen-binding region of Fn and also in a range of other proteins such as type IV collagenases (Potts *et al.*, 1994). F2 modules consist of ~60 residues forming two double-stranded antiparallel β-sheets oriented approximately perpendicular to each other. A cluster of conserved aromatic residues enclosed by the β-sheets is partially solvent-exposed. The ^{2}F2 module in the

gelatin-binding domain of fibronectin is glycosylated by a N-linked sugar (Sticht *et al.*, 1998).

Fn type-III modules

The consensus sequence of Fn type-III (F3) modules (Fig.1.2b) has been identified in numerous proteins in both eukaryotic and prokaryotic organisms, as well as in both intra- and extracellular proteins (Patthy, 1991). In Fn F3 modules account for ~60% of the sequence. The largest among Fn modules, F3 consists of approximately 90 residues, folded into a sandwich formed by a triple- and a four-stranded antiparallel β-sheet enclosing the hydrophobic residues of the core (Main *et al.*, 1992). The ^{10}F3 specifically recognises and binds to the $\alpha_5\beta_1$ integrin (Ruoslahti & Pierschbacher, 1987) on the cell surface.

The N-terminal domain

The 29 kDa N-terminal domain of Fn, consisting of the $^{1\text{-}5}$F1 modules, contains both a covalent and a non-covalent fibrin-binding site (Matsuka *et al.*, 1994, Williams *et al.*, 1994). In matrix assembly, $^{1\text{-}5}$F1 appear to form a single functional unit. The deletion of any of these modules (Sottile *et al.*, 1991) or alteration of its structure by mutation of a conserved hydrophobic residue disrupts the function of the domain, causing a decrease in binding affinity for assembly sites (Schwarzbauer, 1991).

F1 modules are connected by linkers of various lengths (Potts *et al.*, 1999).

^{1}F1 ~ 6 aa ~ **^{2}F1** ~ 2 aa ~ **^{3}F1** ~ 3 aa ~ **^{4}F1** ~ 2 aa ~ **^{5}F1** ~~~

In ^{4}F1^{5}F1, where the intermodule linker is short, a well-defined intermodule interface was observed which stabilises the relative orientation of the modules (Williams *et al.*, 1994). A similar phenomenon might occur for ^{2}F1^{3}F1, which also contains a short intermodule linker, however the three-dimensional structure has not yet been determined. ^{1}F1 and ^{2}F1 have a long linker between them and lack an intermodule interface (Potts *et al.*, 1999).

Many bacterial pathogens have exploited Fn as a means to adhere, colonize, and invade host tissue (Kuusela, 1978, Patti *et al.*, 1994). Although the 70 kDa domain (containing the N-terminal and the collagen-binding domains) has been reported as the smallest full-strength binding site of Fn (Ozeri *et al.*, 1996), the gram-positive human pathogens *Staphylococcus aureus* and *Streptococcus pyogenes* have been shown to bind primarily to $^{1\text{-}5}$F1 (Joh *et al.*, 1994).

The collagen-binding domain

The collagen-binding domain of Fn has been identified by proteolytic studies as encompassing modules ^{6}F1^{1}F2^{2}F2^{7}F1^{8}F1^{9}F1 (Hynes, 1990). It was shown to bind either heat-denatured collagen (gelatin – Hahn & Yamada, 1979, Forastieri & Ingham, 1985) or isolated collagen α-chains (Guidry *et al.*, 1990). The solution structure of ^{6}F1^{1}F2^{2}F1 showed that these modules adopted a hairpin conformation, the first non-linear arrangement of modules observed in Fn (Pickford *et al.*, 2001). As shown in Fig.1.2, this may result in $^{1\text{-}5}$F1 having an antiparallel orientation with respect to the

rest of Fn molecule, and is also consistent with calorimetry studies, which suggested that ^{6}F1 and ^{7}F1 interact with each other (Litvinovich *et al.*, 1991).

1.4. *Staphylococcus aureus* and *Streptococcus pyogenes*

The clinical manifestation of *S. aureus* infection (Table.1.1) is widespread. In hospital settings *S. aureus* is present in most cases of post-operative wound infection and medical-device-related sepsis (Waldvogel, 1995). Effective treatment is becoming more difficult with the constant rise in the incidence of infections with the methicillin-resistant *S. aureus* (MRSA) (Panlilio *et al.*, 1992, Speller *et al.*, 1997), combined with the emergence of MRSA strains exhibiting reduced sensitivity to vancomycin (Hiramatsu *et al.*, 1997). Thus, there is a pressing need to understand the mechanism of pathogenesis of staphylococcal diseases, as a basis for developing novel therapeutic and preventative approaches.

Table.1.1. Diseases involving *S. aureus* (http://www.med.sc.edu:85/fox/strep-staph.htm)

bacteraemia*
endocarditis*
osteomyelitis*
septic arthritis
pneumonia
toxic shock
food poisoning
skin infections - boils, scalded skin syndrome in young children

* *S. aureus* is the primary cause of these infections

Adherence is but one of many virulence mechanisms that *S. aureus* possesses (Fig.1.3). *S. aureus* expresses several surface receptors belonging to the adhesins group: Fn-binding proteins A and B (FnbpA and FnbpB), collagen-binding protein Cna, fibrinogen-binding protein - clumping factor A, and proteins binding vitronectin and elastin (Foster & Höök, 1998, Hartford *et al.*, 1999).

Although *S. aureus* is primarily considered as an extracellular pathogen, it has been shown to invade a variety of non-phagocytic cells, e.g. human endothelial cells (Ogawa *et al.*, 1985, Peacock *et al.*, 1999), epithelial cells (Dziewanowska *et al.*, 1999), osteoblasts (Hudson *et al.*, 1995) and fibroblasts (Fowler *et al.*, 2000). Adherent staphylococci stimulate polymerization of the cellular actin of the host, then are enclosed within membrane-bound vacuoles and transported into the cell (Hudson et al., 1995). Some bacteria also escape from the phagosome into the cytoplasm, where they induce apoptosis (Bayles *et al.*, 1998).

Several independent groups of investigators, using similar molecular techniques, have recently shown that the main mechanism of staphylococcal invasion *in vitro* occurs by binding to host cells either *via* serum or cellular proteins which are adherent to both the cell and bacteria (i.e. bridging ligands) (Dziewanowska *et al.*, 1999, Sinha *et al.*, 1999). Presence of both, Fn bound to the $\alpha_5\beta_1$ integrin (Ruoslahti & Pierschbacher, 1987) and FnbpA are required for *S. aureus* internalisation (Sinha *et al.*, 1999). The interaction between FnbpA and Fn is the primary focus of the work described in this thesis.

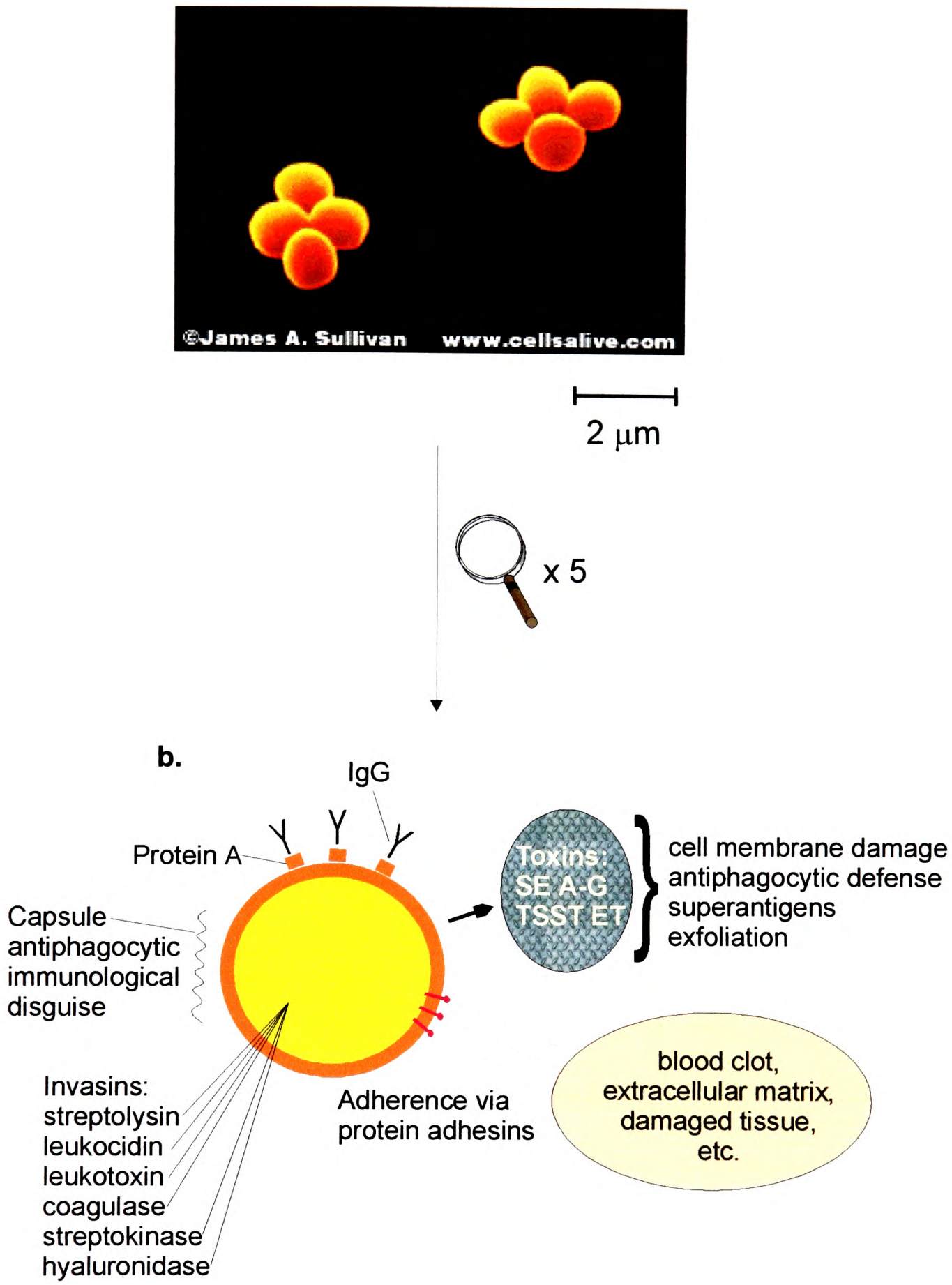


Fig.1.3. Features of *S. aureus*. **a.** *S. aureus* cells. **b.** Pathogenic mechanisms developed by *S. aureus* cell. Sources: www.cellsalive.com, www.bact.wisc.edu, www.liunet.edu, Voët & Voët, 1995.

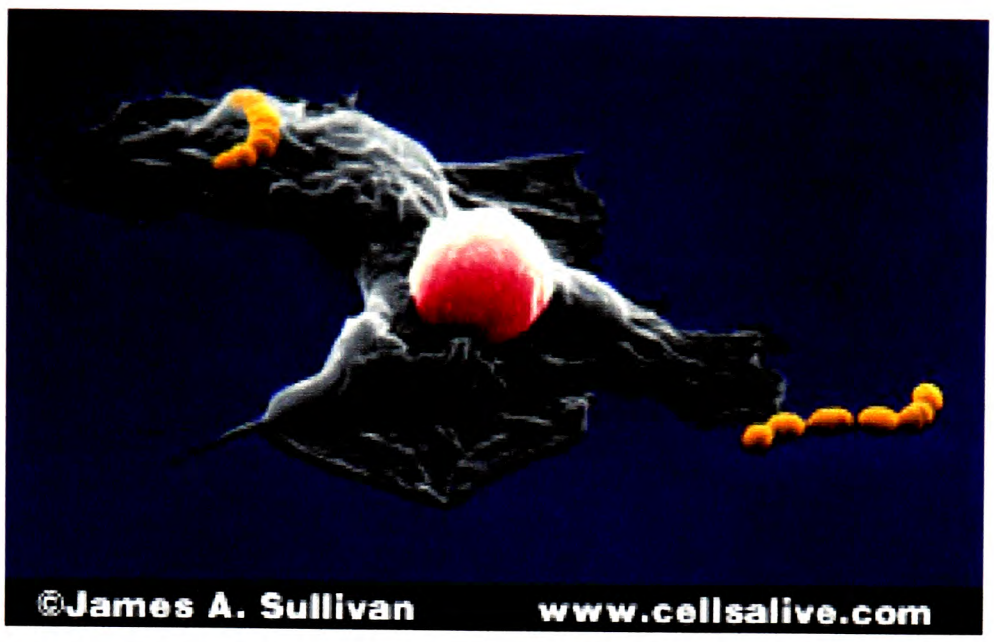
S. pyogenes (group A *Streptococcus*), also traditionally viewed as an extracellular pathogen, is responsible for many human diseases of varying severity (Table.1.2). Recent studies showed that *S. pyogenes* is capable of internalizing into several types of eukaryotic cell *in vitro* (LaPenta *et al.*, 1994, Greco *et al.*, 1995). Entry of *S. pyogenes* into host cells also occurs *in vivo* and may contribute to bacterial resistance to antibiotic therapy (Ensenberger *et al.*, 2001). Although *S. pyogenes* is still susceptible to β-lactam antibiotics *in vitro*, more and more frequently penicillin treatment of a patient is ineffective (Pichichero, 1998).

Table.1.2. Diseases involving *S. pyogenes* (http://www.med.sc.edu:85/fox/streptococci.htm)

pharyngitis
sepsis
toxic shock-like syndrome
scarlet fever
rheumatic fever
rheumatic heart diseases
acute poststreptococcal glomerulonephritis

Streptococcal cells (Fig.1.4) express a large variety of extracellular proteins that have been associated with its pathogenicity (for an extensive review see Cunningham, 2000). Fn-recognizing adhesins include: streptococcal Fn-binding protein I – SfbI/F1 (Okada *et al.*, 1994), Fn-binding protein F2 (Jaffe *et al.,* 1996), Fn-binding protein 54 - FBP54 (Courtney *et al.*, 1994), lipotechoic acid - LTA (Beachey & Ofek, 1976) and streptococcal Fn-binding protein II – SfbII/serum opacity factor (Kreikmeyer *et al.*, 1995). High-frequency invasion of *S. pyogenes* requires expression of protein M (Cue

a.



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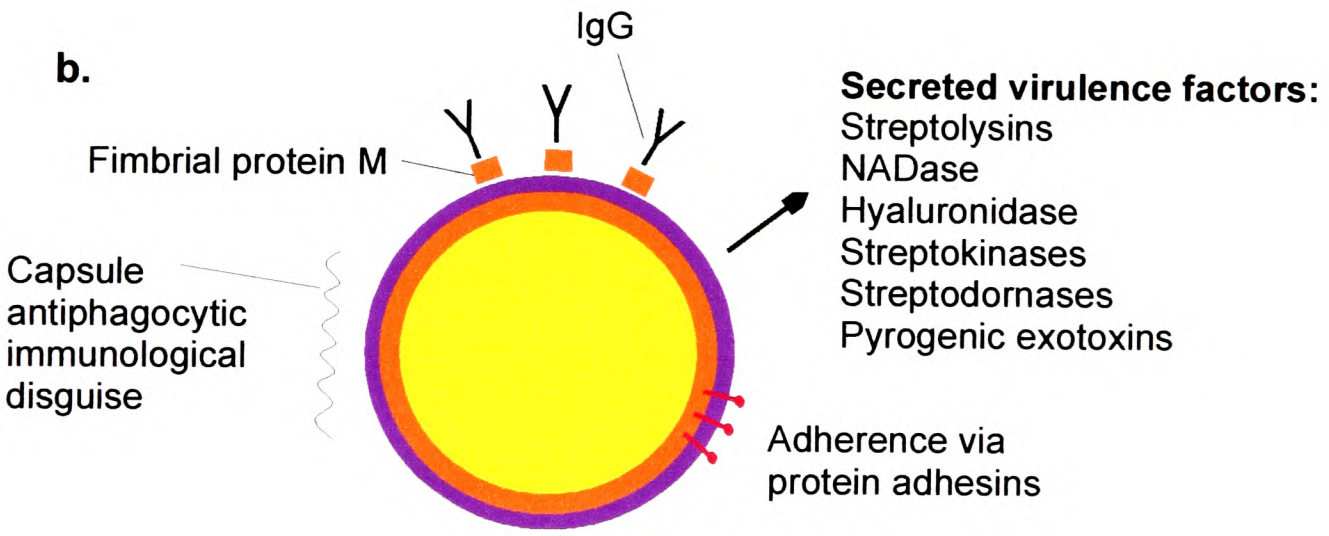


Fig.1.4. Features of *S. pyogenes*. **a.** Human macrophage ingesting the bacteria. **b.** Pathogenic mechanisms developed by *S. pyogenes* cell. Sources: www.cellsalive.com, www.bact.wisc.edu, Voët & Voët, 1995.

et al., 1998) and/or SfbI (Jadoun *et al.*, 1998). It was also demonstrated that rearrangements within the cytoskeleton of the host cells occur during the invasion process (Molinari *et al.*, 1997).

1.5. Fn-binding proteins

Fn-binding proteins (FnBPs) from different gram-positive bacteria share many characteristic features (Fig.1.5a). The N-terminal signal sequence is composed of 23-38 amino acids. This segment is followed by the A domain, shown to bind fibrinogen (Wann *et al.*, 2000), which is in some cases interrupted by two repeats of 20-35 residues. The major Fn-binding site lies within a region near the C-terminus of the protein, composed of a 37-50 residue-long motif that is repeated 3-5 times and is followed by a putative cell wall-spanning domain. An LPXTG motif, characteristic of cell-wall anchored proteins in gram-positive bacteria is situated between the cell-wall spanning region and the cytoplasmic membrane-spanning region. This motif is cleaved between the Thr and Gly residues and the carboxyl group of Thr is then covalently joined to the carboxyl group of a branch peptide in the peptidoglycan. In the case of *S. aureus* this is a pentaglycine sequence. A short stretch of positively charged amino acid residues is located at the C-terminus and may represent a cytoplasmic anchor (Schneewind *et al.*, 1995).

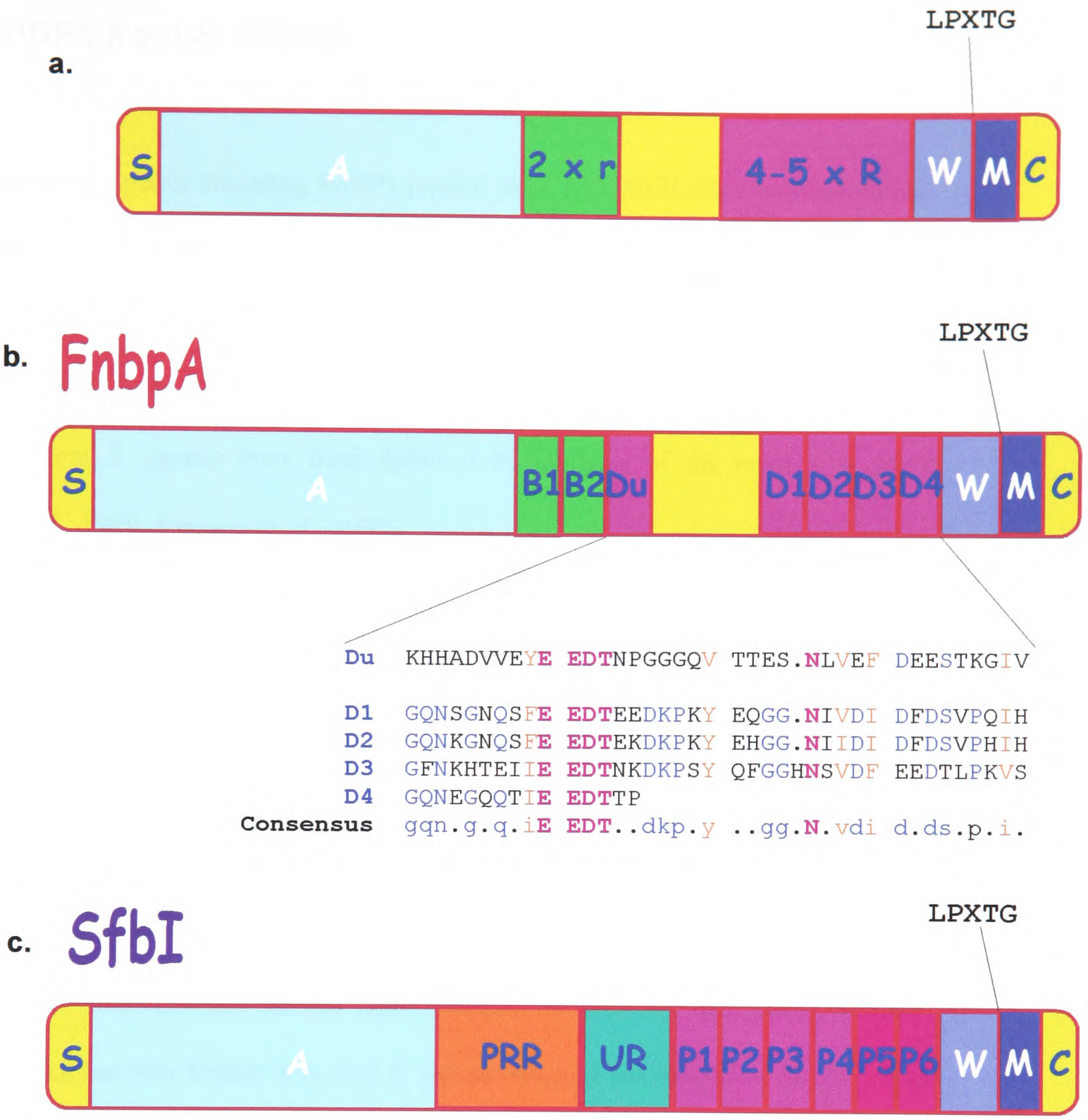


Fig.1.5.

a. Schematic representation of Fn-binding proteins from gram-positive bacteria. S - signal sequence, A - non-repetitive region, r - optional repeats, R - Fn-binding repeats, W - cell wall-spanning region, M - cytoplasmic membrane-spanning region, C - positively charged intracellular residues. For further explanation see text (**1.5**).

b. Fn-binding protein FnbpA from *S. aureus*. Symbols as above. B1, B2, Du, D1-D4 - Fn-binding repeats. In the sequence alignment conserved residues are indicated (MultAlin - Corpet, 1988). For further explanation see text (**1.6**).

c. Fn-binding protein SfbI from *S. pyogenes*. Symbols as above. PRR - proline rich region, UR - unique region recognising the collagen-binding domain of Fn, P1-P4 - Fn-binding repeats, P5-P6 - optional Fn-binding repeats. For further explanation see text (**1.7**).

1.6. FnBPs from *S. aureus*

In *S. aureus* two genes encoding FnBPs (called *fnbA* and *fnbB*) have been identified. The genes are separated by ~700 base-pairs of non-coding sequence, and each one is located in its own open reading frame. Some strains of *S. aureus* have both genes, whereas others carry only *fnbA*. The primary amino acid sequences of the Fn receptors from *S. aureus* have been deduced by analysis of the respective genes (Signäs *et al.*, 1989, Jönsson *et al.*, 1991).

FnbpA (Fig.1.5b) and FnbpB have four full-length (37-40 amino acid residues) Fn-binding units called Du, D1, D2 and D3, and an incomplete Fn-binding unit D4 (Signäs *et al.*, 1989). The Du repeat is found ~100 amino acid residues N-terminal of D1. All D repeats are highly conserved between the two proteins (94% amino acid identity). N-terminal regions of FnBPs are less similar and share only ~40% amino acid identity. Experimental studies have focused on the first discovered protein, FnbpA, which has two further so-called B repeats situated N-terminal of the D motifs (Signäs *et al.*, 1989). They also have been shown to recognize the N-terminal domain of Fn (Massey *et al.*, 2001, also see **Chapter 8**).

Each of the full-length D repeats in FnbpA can bind Fn and synthetic peptides encompassing the sequence of D1, D2, and D3 repeats inhibit Fn binding to bacterial cells (McGavin *et al.*, 1993). The ^{4}F1^{5}F1 module pair contains the primary binding site for the D motifs of FnbpA, but neither ^{4}F1 nor ^{5}F1 have been shown to bind D1-D4 as a single module (Huff *et al.*, 1994). Nuclear magnetic resonance (NMR)

spectroscopy was used to measure chemical shift changes that occur in ^{4}F1^{5}F1 upon D3 binding. The results suggested that the binding site for D3 is located on one face of the module pair, and primarily on the D-E loop and E β-strand of ^{4}F1, although other residues in both modules may also be involved (Penkett *et al.*, 2000).

A synthetic peptide, encompassing residues 17 to 37 of D3 ($D3_{17-37}$ – named here D3t) has been shown to have a similar binding-affinity for Fn as that of the larger D3 peptide (residues 2 to 38). D3t-Sepharose columns have been used to purify the N-terminal domain of Fn and also to purify full-length Fn from a mixture of N-terminally truncated fragments (Huff *et al.*, 1994).

1.7. Conformation of D-repeats in solution

A number of proteins involved in protein-protein interactions have been found to be essentially unfolded and yet functional, eg. the cyclin-dependent kinase inhibitor $p21^{waf/cip1/sid1}$ (review by Plaxco & Gross, 1997). It has been shown that D1-D4 from *S. aureus* exists in an unfolded state under physiological conditions (Penkett *et al.*, 1998). One possible advantage may be the evasion of the host immune system. Previous studies showed that antibodies, produced against various forms of D1-D4 in the unbound (unfolded) state, are ineffective adhesion-blocking agents and could even enhance binding to Fn (Speziale et al., 1996).

As unfolded proteins are usually very susceptible to proteolysis, D repeats show several protective features. The presence of local hydrophobic interactions

between side-chains may protect the sequence from proteolytic cleavage by enzymes specific for large hydrophobic residues in unfolded proteins (Penkett *et al.*, 1998). Additionally, there appears to be a lack of proteases in human cells directed against acidic residues, when compared to the large number of proteases specific for basic amino acids. This may also allow much of the polypeptide chain of D1-D4, with its very acidic nature, to remain largely protected from proteolytic cleavage (Penkett *et al.*, 1998).

Analysis of the amino acid sequence and NMR studies show that the C-terminal regions of D repeats have a preference for adopting main-chain φ and ψ angles consistent with a β (extended) conformation (Penkett *et al.*, 1997). Studies using circular dichroism spectroscopy (CD - House-Pompeo *et al.*, 1996) and NMR (Penkett *et al.*, 2000) suggest that, on binding to the N-terminal domain of Fn, at least part of the D1-D4 sequence forms a more ordered, extended structure. Although CD provides evidence for a conformational change on binding, detailed interpretation of the CD spectra of protein-protein interactions can be difficult as the signals from the different species overlap. However, the downfield shifted H^{α} resonances, which are observed in NMR spectra of D3t when bound to $^{4}F1^{5}F1$, also indicate a transition to an extended conformation (Penkett et al., 2000).

1.8. FnBPs from *S. pyogenes*

SfbI (also named F1 - Fig.1.5c) is a major Fn-binding adhesin of *S. pyogenes*. In addition to Fn recognition it binds to the Fc fragment of human immunoglobulin,

interfering with phagocytosis and antibody-dependent cell cytotoxicity of macrophages (Medina *et al.*, 1999). The N-terminal signal sequence in SfbI is followed by an A domain terminated by a proline-rich-repeat region, then the Fn-binding upstream region (UR – Sela *et al.*, 1993) followed by four-six (depending on the strain – Natanson *et al.*, 1995) ~37 residue P repeats and finished with a typical cell wall and membrane anchor at the C-terminus (Schulze *et al.*, 2001). Similarly to the D repeats of FnbpA of *S. aureus*, the P repeats of SfbI recognize $^{1\text{-}5}$F1 of Fn (Joh *et al.*, 1994). It has also been shown that the UR domain binds the 70 kDa N-terminal fragment of Fn (Jaffe *et al.*, 1996).

1.9. Summary

"*S. aureus* is killed efficiently by antibiotics and by neutrophils *in vitro*, however, the organism has a remarkable capability to persist in the human body and requires prolonged antibiotic treatment for eradication. Thus, there must be protected sites of *S. aureus* survival." (from Sinha *et al.*, 1999).

Increasing incidences of antibiotic-resistant bacteria have been observed for a number of years, the best example being provided by *S. aureus*, which has become less susceptible to methicillin and vancomycin (Hiramatsu *et al.*, 1997). There is a threat that soon the infections will no longer be treatable with currently available antibiotics. A better understanding of mechanisms of bacterial virulence will aid the development of new therapeutic approaches.

Both localized infections, such as soft-tissue abscesses, and life-threatening systemic diseases, such as infective endocarditis, result from the ability of *S. aureus* (and similar gram-positive pathogens) to colonize host tissue surfaces from the bloodstream, allowing an infection to spread. Pathogen elaboration of exotoxins and enzymes destroys tissue and protect bacteria from the immune response of the host (Lowy, 2000). Internalisation of bacteria into the cells of invaded tissue may have many advantages, e.g. escaping phagocytosis by professional phagocytes (neutrophil granulocytes, macrophages), gaining an environment rich in nutrients and protection from certain antibiotics (Patti *et al.*, 1994).

Fn-binding proteins from *S. aureus* and group A, C and G streptococci have been shown to promote the colonization of implanted medical devices (Huesca *et al.*, 2000). These proteins have also been shown to be responsible for bacterial attachment and internalization into human epithelial and endothelial cells *in vitro* (Greco *et al.*, 1995, Sinha *et al.*, 1999, Peacock *et al.*, 1999). Using mutagenesis studies it has been shown that *S. aureus* strains deficient in Fnbps A and B were significantly less efficient invaders of epithelial cells, endothelial cells and fibroblasts *in vitro* than wild-type strains (Dziewanowska *et al.*, 1999, Sinha *et al.*, 1999, Peacock *et al.*, 1999). An expression of FnBPs in non-invasive strains conferred invasiveness. This allows the classification of FnBPs as invasins, which is supported by the fact that the soluble isolated fibronectin-binding domain of FnbpA (D1-D4) can block invasion (Sinha *et al.*, 1999).

Latex beads coated with SfbI from *S. pyogenes* were efficiently internalized by epithelial cells (Molinari *et al.*, 1997), showing that no other bacterial factor is involved in this process. Mutations in the gene encoding this protein reduce the capacity of streptococci to invade cultured cells (Cunningham, 2000). Thus the importance of these proteins in the invasion process *in vitro* is clear. Recently it has been shown that SfbI induced a protective response in vaccinated animals. After treatment they were protected against homologous and heterologous serotypes of group A streptococci (Guzman *et al.*, 1999).

In recent years, the discovery that adhesion molecules are used by pathogens for binding and internalisation into host cells has fuelled much research. Although most bacterial or fungal pathogens possess multiple adhesins, it has been demonstrated that targeting a single specific adhesin-receptor interaction can be sufficient to prevent infection (Kelly & Younson, 2000). Thus, anti-adhesive strategies aimed at blocking these interactions offer an attractive means for preventing microbial infections at an early stage. Structural information about host/pathogen interactions can be very helpful in achieving this goal.

Specific aims:

Sequence analysis and binding studies using synthetic domains of Fn-binding repeats from *S. aureus* and *S. dysgalactiae* have identified conserved acidic, hydrophobic and glycine residues that appear to be important for ligand binding (McGavin et al., 1993). The primary binding site for D3 on ^{4}F1^{5}F1 has been identified (Penkett *et al.*, 2000). The next step was the determination of the three-dimensional structure of the

^{4}F1^{5}F1/D3 complex, combined with further binding studies. This was the main aim of this work.

As pH 4.5 (the pH at which the structural studies of ^{4}F1^{5}F1 were originally performed, Williams *et al.*, 1994) is not suitable for studies of the complex due to problems with sample solubility, the structure of ^{4}F1^{5}F1 at a new pH had to be determined. In addition, new calculation protocols were used to improve the precision of the structures to maximize the ability to detect the, perhaps subtle, changes in the structure of ^{4}F1^{5}F1 that might occur on peptide binding. Previously only the ^{4}F1^{5}F1 backbone and methyl groups were assigned for the ^{4}F1^{5}F1/D3 complex thus full side-chain assignments were also necessary. As the NMR spectra of the complex did not reveal the conformation of the bound peptide, trials to solve the crystal structure of the ^{4}F1^{5}F1/D3 complex have been undertaken.

The dynamics of the complex were studied using ^{15}N relaxation data for the ^{4}F1^{5}F1/D3 complex. To enhance the accuracy of the calculated structures, ^{15}N-labelled ^{4}F1^{5}F1 was also used to acquire sets of N-H bond residual dipolar couplings for the module pair in the free and D3-bound state. Isothermal titration calorimetry and NMR have been used to identify the shortest binding motifs specific for the F1 modules. As well as the D3 peptide and its fragments, peptides containing sequences from *S. pyogenes* FnBPs have been used. These studies lead to a better understanding of FnBP-Fn interactions.

2. SAMPLE PREPARATION

2.1. Expression and purification of $^{4}F1^{5}F1$

Chemical reagents

Except where stated otherwise, chemical and biochemical reagents used (analytical purity grade) were supplied by Sigma.

Expression

Unlabelled and uniformly ^{15}N-labelled, $^{13}C^{15}N$-labelled and $^{2}H^{15}N$-labelled $^{4}F1^{5}F1$ were expressed in *Pichia pastoris*. Preparation procedures are described in **Appendix 1**. Expression was carried out at 30°C in a 1 l fermentor (Electrolab Ltd., Tewkesbury), using 0.2% (w/v) $(^{15}NH_4)_2SO_4$ for ^{15}N labelling, 0.2% (w/v) ^{13}C-glucose and ^{13}C-CH_3OH for ^{13}C labelling and ^{2}H-glycerol and $C^{2}H_3O^{2}H$ for perdeuteration. The initially used samples were expressed and purified by Drs A. Pickford and J. Bright.

A typical fermentation included an inoculation with 24 h starter culture (ratio 1:20), approximately 40 h of yeast growth with glucose as the carbon source and then 4-5 days of growth on CH_3OH. The exception was the fermentation to produce $^{13}C^{15}N$-labelled $^{4}F1^{5}F1$. Here the cells seemed to have problems adjusting to the ^{13}C-glucose, therefore induction with $^{13}CH_3OH$ was carried out 7 days after the start of the fermentation.

P. pastoris grows slower in D_2O, therefore the starter culture was grown in three steps (48 h each) to condition cells to a fully deuterated medium. The growth on ^{2}H-glycerol took approximately 55 h from the inoculation (ratio 1:20) and the $C^2H_3O^2H$ feeding was continued for 10 days (supernatant samples from the duration of $C^2H_3O^2H$ feeding are shown in Fig.2.1a). The $^4F1^5F1$ module pair is relatively sensitive to oxidation, therefore the oxygen level during the CH_3OH/ $C^2H_3O^2H$ feeding was kept at approx. 20%.

SDS-PAGE

The proteins were separated on NuPAGE® Bis-Tris 10% polyacrylamide pre-cast gels (Invitrogen) with NuPAGE® MES-SDS running buffer (pH 7). 7 µl supernatant/protein solution samples were diluted with 7 µl of the 2 x loading buffer (0.13 M Tris-HCl pH 6.8, 0.05% bromophenol blue, 4% (w/v) SDS, 20% glycerol, 5% (w/v) β-mercaptoethanol) and heated at 95°C for 5 min. The samples were centrifuged for 30 s before being loaded into wells that had been pre-washed with the running buffer to remove non-polymerised acrylamide. Mark12™ molecular weight (M.W.) standards (10 µl) were used (Invitrogen). The protein separation was carried out at constant voltage of 200 V for 35 min. Gels were stained in Coomassie Blue (10% acetic acid, 50% CH_3OH and 0.2% (w/v) Coomassie®) for 15-30 min and, after rinsing, left in destain (7.5% acetic acid, 10% CH_3OH) overnight before transferring to deionised water.

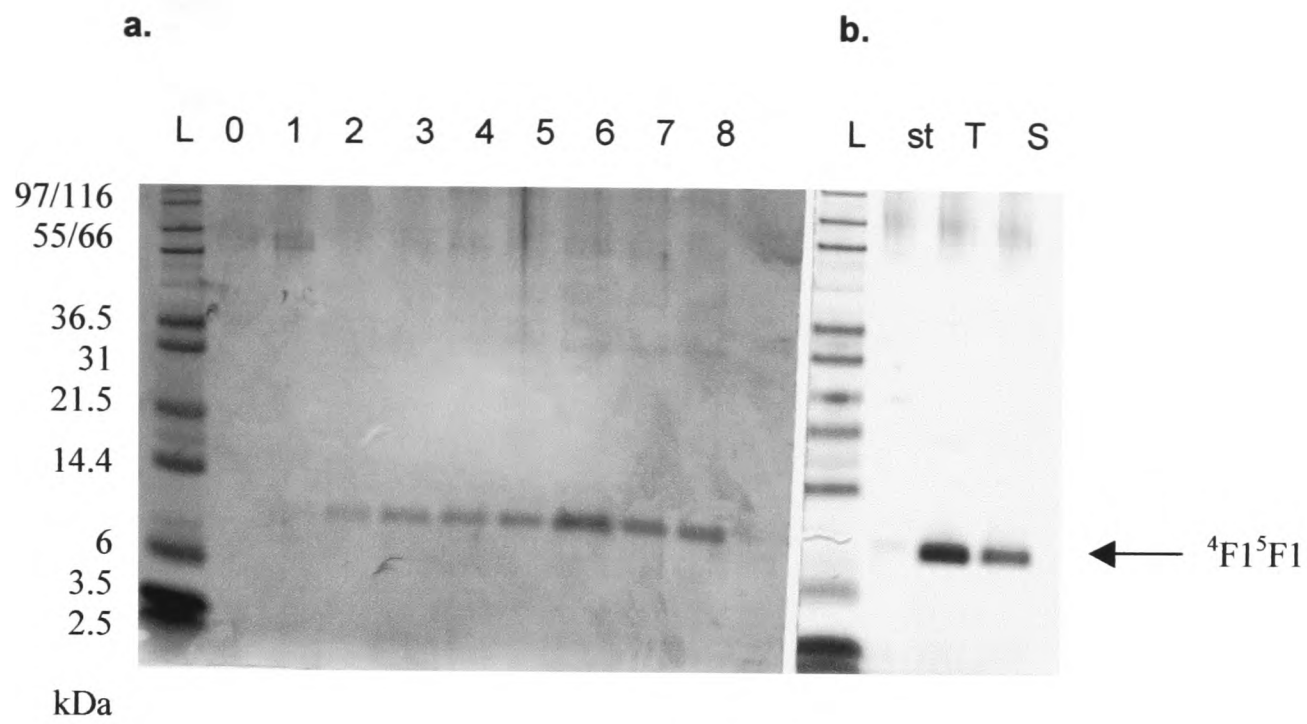


Fig.2.1.

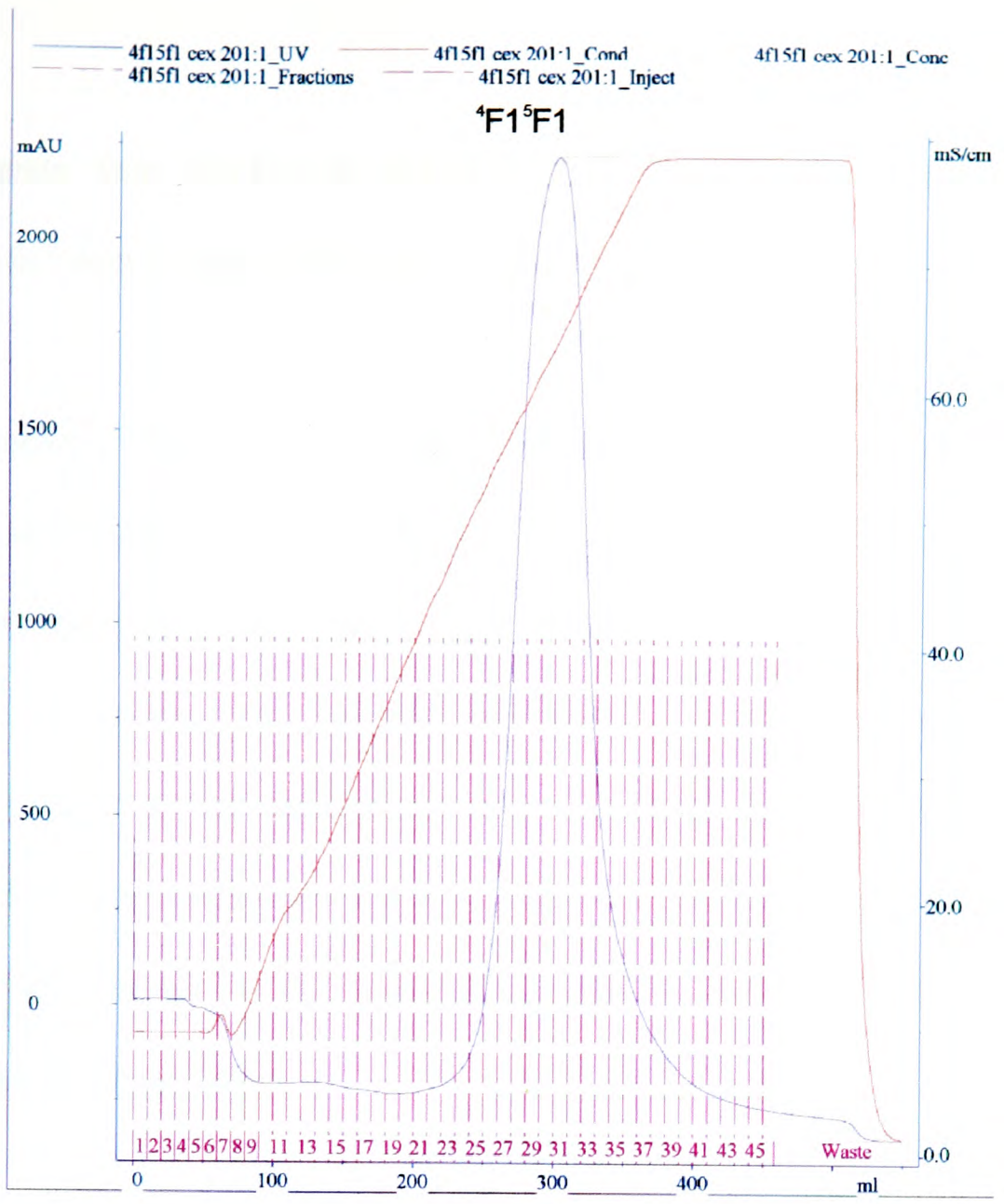
a. $^{2}H^{15}N$-fermentation: SDS-PAGE gel showing the protein level in the supernatant for 8 consecutive days after the $C^{2}H_{3}O^{2}H$ induction. L – ladder of M.W. standards, 0-8 – days after the $C^{2}H_{3}O^{2}H$ induction (0 – the day of the induction).

b. $^{2}H^{15}N$-$^{4}F1^{5}F1$ concentrated after FPLC (Fig.2.2a). L – ladder of M.W. standards, st – supernatant (diluted 5x), T – top of the retention peak, S – sides of the retention peak.

Purification

After harvesting the culture supernatant (the culture was centrifuged for 30 min to separate the cells), an initial fast protein liquid chromatography (FPLC; ÄCTA, Amersham Pharmacia Biotech, Bucks, UK) step utilized cation exchange on SP-Sepharose Fast Flow (Pharmacia, Milton Keynes, UK). The supernatant from the fermentation, after adjustment of pH to 3.0 and dilution to a total volume of 3 l to decrease the ionic strength, was flowed through the column (which had been equilibrated with 20 mM citrate, pH 3.0). The protein bound to the column was then eluted with 20 mM citrate 1 M NaCl, pH 3.0. Elution was monitored by measuring the absorbance at 280 nm. A typical chromatograph is shown in Fig.2.2a. Fractions of

a.



b.

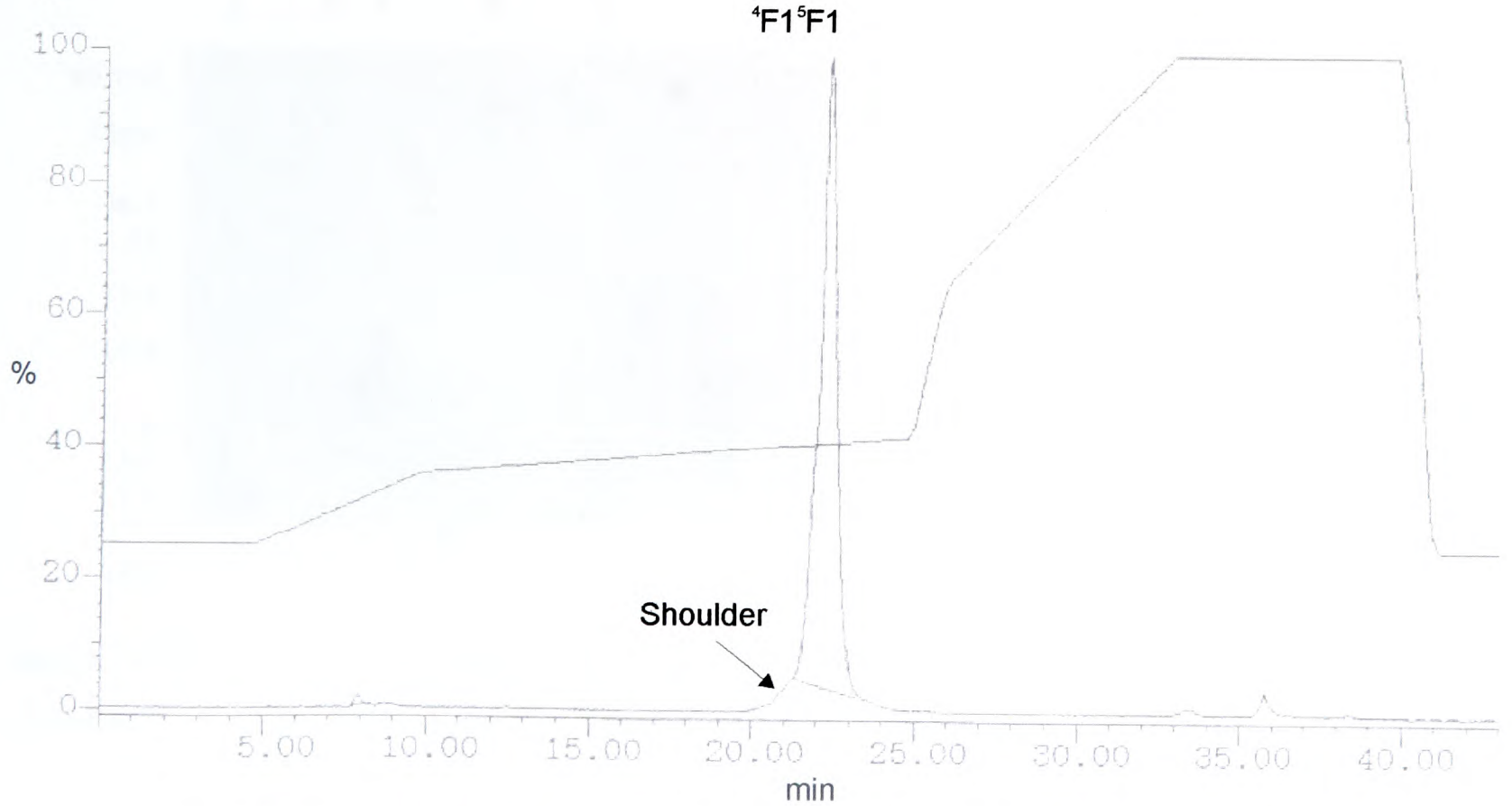


Fig.2.2. Chromatographs from $^{4}F1^{5}F1$ purification. **a.** FPLC on a cation-exchange column. **b.** Reverse-phase HPLC. The $^{4}F1^{5}F1$ HPLC profile typically contains a shoulder on the main peak. Mass spectrometry showed this shoulder contains the protein with two oxidised methionines while the main peak contains material with the expected M.W.

high absorbance were checked by SDS-PAGE (7+7 µl samples - Fig.2.1b, Fig.2.3) and afterwards taken to the second step of purification.

Final purification of the $^{4}F1^{5}F1$ module pair using reverse phase high performance liquid chromatography (HPLC – Gilson, Middleton, WI, US) was optimised and performed on a C8 semi-analytical column (Varian Analytical Instruments, Surrey, UK). Prior to loading, the column was washed with 100% buffer B (80% acetonitrile/0.1% TFA) and equilibrated with 75% buffer A (0.1% TFA)/25% buffer B. The samples were loaded through a syringe filter 0.2 µm (Whatman). The elution was monitored by measuring the absorbance at 280 nm. Fractions of high absorbance were collected and lyophilised.

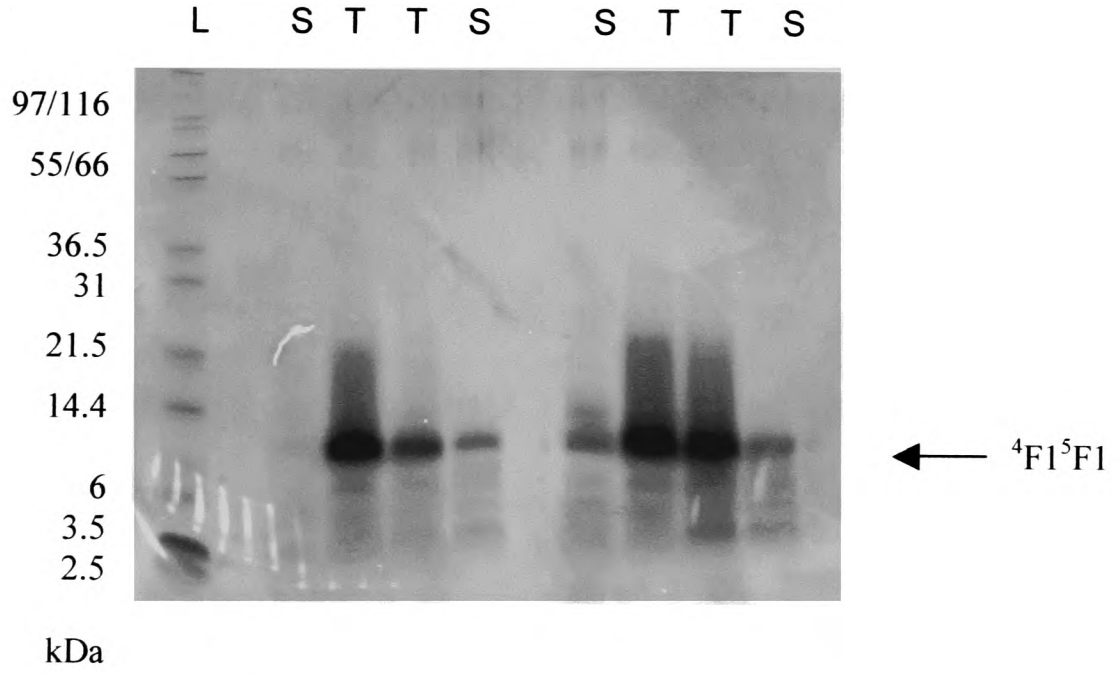


Fig.2.3. $^{4}F1^{5}F1$ SDS-PAGE gel showing the results of two runs of FPLC. L – ladder of M.W. standards, T – top of the retention peak, S – sides of the retention peak.

A typical chromatograph is shown in Fig.2.2b. The identity was confirmed by electrospray mass spectrometry (ESMS) performed by Drs R. Aplin and N. Oldham (Dyson Perrins Laboratory, Oxford University - Fig.2.4). The M.Ws were consistent with the predicted values, indicating ~100% labelling for isotope-labelled samples.

M.W.	Estimated	ESMS
$^{4}F1^{5}F1$	10512 Da	10509 Da
^{15}N-$^{4}F1^{5}F1$	10650 Da	10646 Da
$^{13}C^{15}N$-$^{4}F1^{5}F1$	11087 Da	11074 Da
$^{2}H^{15}N$-$^{4}F1^{5}F1$	11125 Da	11128 Da

2.2. Thrombin cleavage of GST-fusion proteins

S. pyogenes peptides UR, EUR (extended UR) and SUR (short UR) were supplied by M. Höök's laboratory (made by J. Kim, Houston, TX, US) as glutathion S-transferase (GST)-fusions. All the fusions were subjected to thrombin digestion. Each reaction was optimised on a small sample and the completion of digestion was checked by SDS-PAGE, as described below (Fig.2.5).

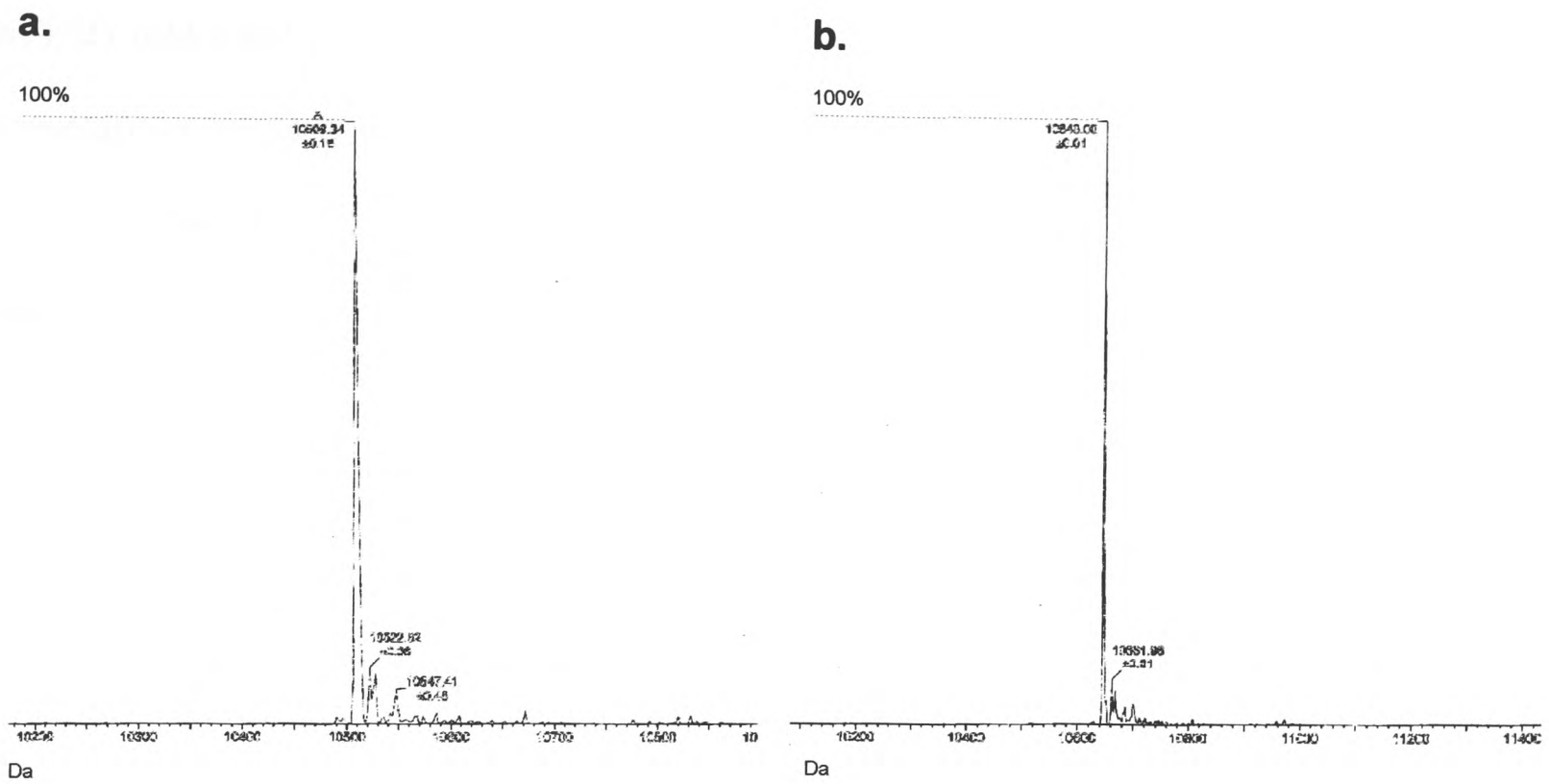


Fig.2.4. Results of ESMS. **a.** Unlabelled $^{4}F1^{5}F1$, **b.** ^{15}N-$^{4}F1^{5}F1$, **c.** $^{13}C^{15}N$-$^{4}F1^{5}F1$, **d.** $^{2}H^{15}N$-$^{4}F1^{5}F1$. Additional peaks in **c.** arise from K^{+} and Na^{+} adducts.

Each fusion was dissolved in thrombin cleavage buffer (50 mM Tris pH 8.5, 150 mM NaCl, 25 mM $CaCl_2$) to a concentration of ~25 mg/ml and 10 µl of thrombin solution (1 unit/µl) were added. Due to formation of secondary cleavage products the reaction was terminated after 50 min. The efficiency of the reaction, measured by comparison of expected and obtained masses of cleaved peptides was approx. 60%.

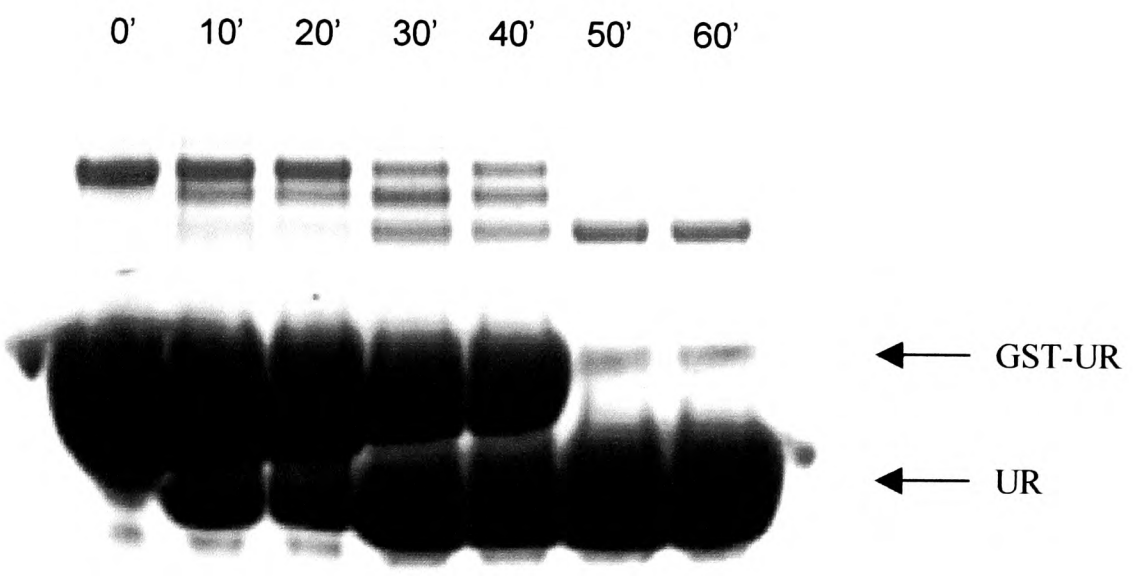


Fig.2.5. SDS-PAGE gel showing the UR thrombin cleavage progress. 5 µl samples were taken at 10 min intervals, 0 min indicates the starting solution. After 50 min the digestion was complete. A secondary cleavage product can be observed from 30 min onwards.

UR-GST (Fig.2.6a) and SUR-GST gave one correct cleavage product. EUR-GST digestion resulted in two protein species, one of correct M.W. and another, approx. 1 kDa larger, which could however be successfully separated (Fig.2.6b, Fig.2.7). The ESMS results are shown in Fig.2.8. Each cleaved peptide contained an additional N-terminal Gly-Ser sequence from the cleavage site.

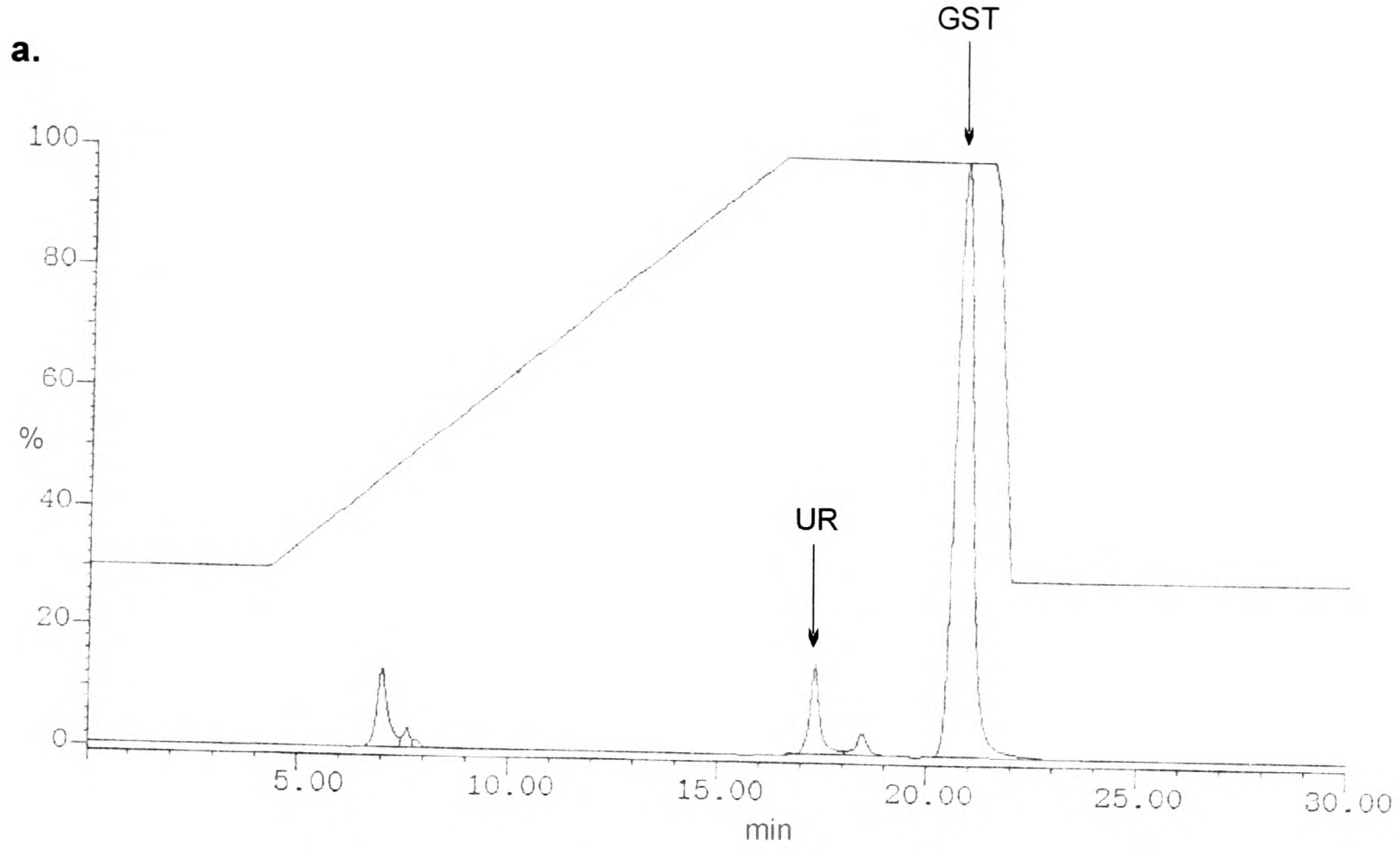


Fig.2.6. Reverse-phase HPLC chromatographs from UR and EUR separation. **a.** UR and GST after thrombin digestion. **b.** Two species of EUR after the re-purification of the single peak obtained after cleavage.

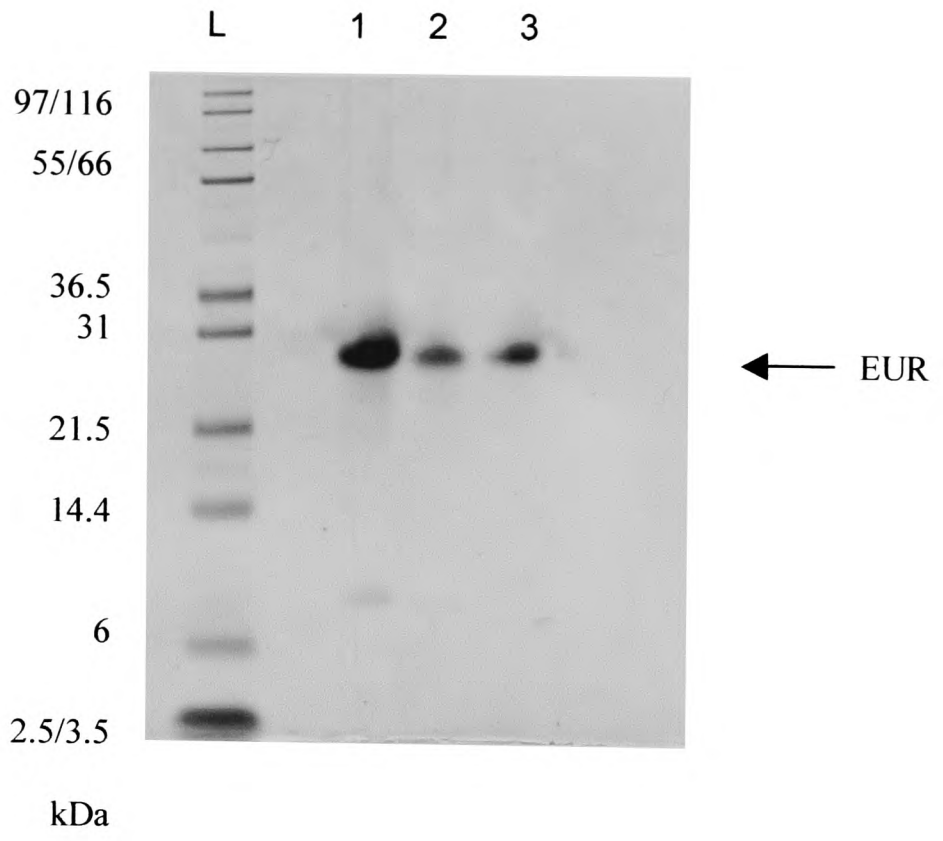
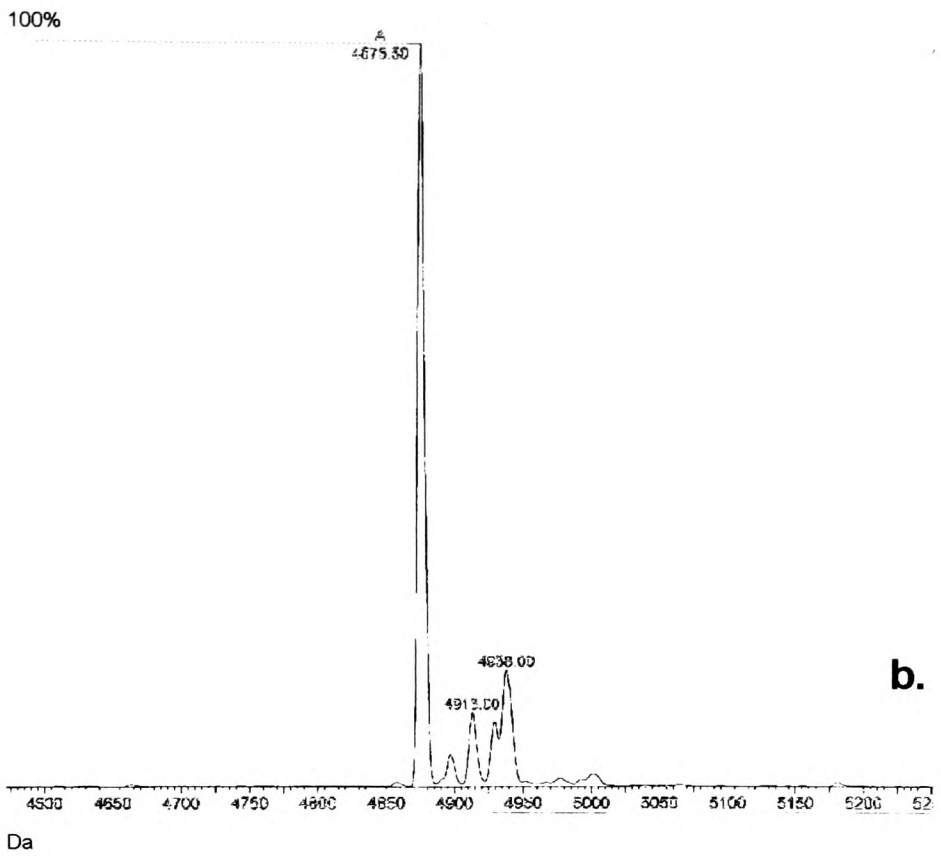
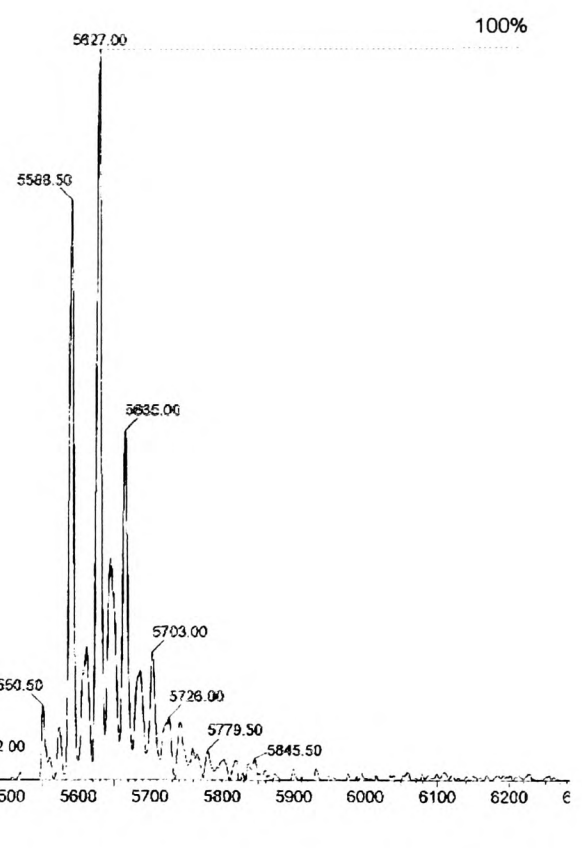


Fig.2.7. SDS-PAGE gel showing the products of EUR-GST thrombin cleavage. L – ladder of M.W. standards, 1 – mixture of products after the first HPLC purification, 2 – EURI M.W. ~10.7 kDa, 3 – EURII M.W. ~11.6 kDa





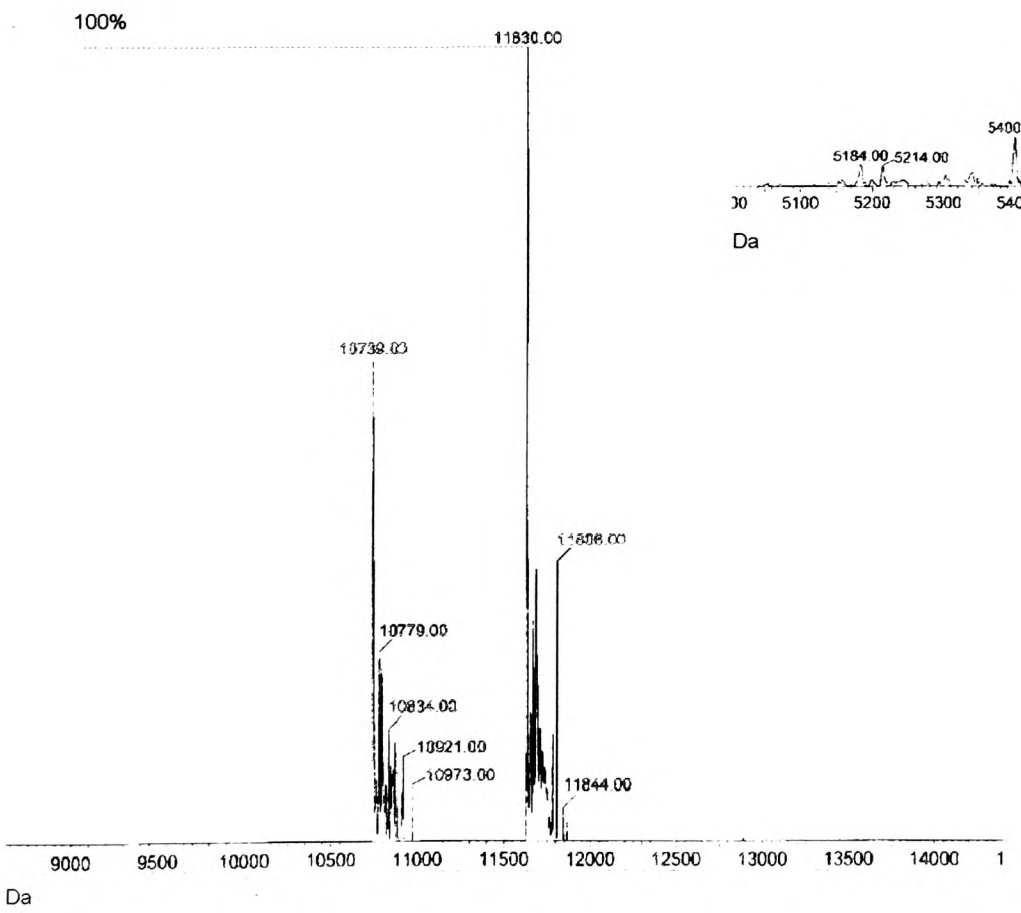


Fig.2.8. Results of ESMS. **a.** SUR, **b.** UR: first large peak indicates metal ion-free form, two others show binding of 1 and 2 K^+, **c.** EUR: there are two distinct species after the cleavage, corresponding to the EURI and EURII peaks in the HPLC profile, respectively (**Fig.2.6**).

3. NUCLEAR MAGNETIC RESONANCE – BASICS AND PROCEDURES

3.1. Introduction

Solution state nuclear magnetic resonance (NMR) is an alternative to the method of X-ray crystallography for solving molecular structures. Unlike crystallography, however, it also provides an insight into molecular behaviour in a solution. To elucidate biological processes involving mosaic proteins one might want to know not only how individual domains of proteins are internally structured, but also how they are oriented with respect to adjacent domains (Fischer *et al.*, 1999). A good example is ^{9}F3^{10}F3 and its interaction with the integrin $\alpha_5\beta_1$. The main binding site – the RGD motif, is situated in ^{10}F3 (Pierschbacher & Ruoslahti, 1984) and the synergy site, enhancing the binding, on ^{9}F3 (Aota *et al.*, 1994). Therefore the relative orientation of both modules is extremely important to preserve the binding capability.

In a strong constant magnetic field $\vec{B}_0$ the magnetic moments ($\vec{\mu}$ – Fig.3.1a) of nuclear spins align with the field. The averaged $\vec{\mu}$ of all the nuclei is called magnetization ($\vec{M}$). The sample is then subject to a short burst of radio frequency (rf) radiation, with a $\vec{B}$ field proportional to $\vec{B}_0$, which is called the rf pulse. This causes $\vec{M}$ to rotate towards the direction of the applied field. The carrier frequency of the rf pulse is chosen to match the precession frequency of the spins around the $\vec{B}_0$ axis (called the Larmor frequency – ω, Abragam, 1961). The return of $\vec{M}$ to the equilibrium state can then be

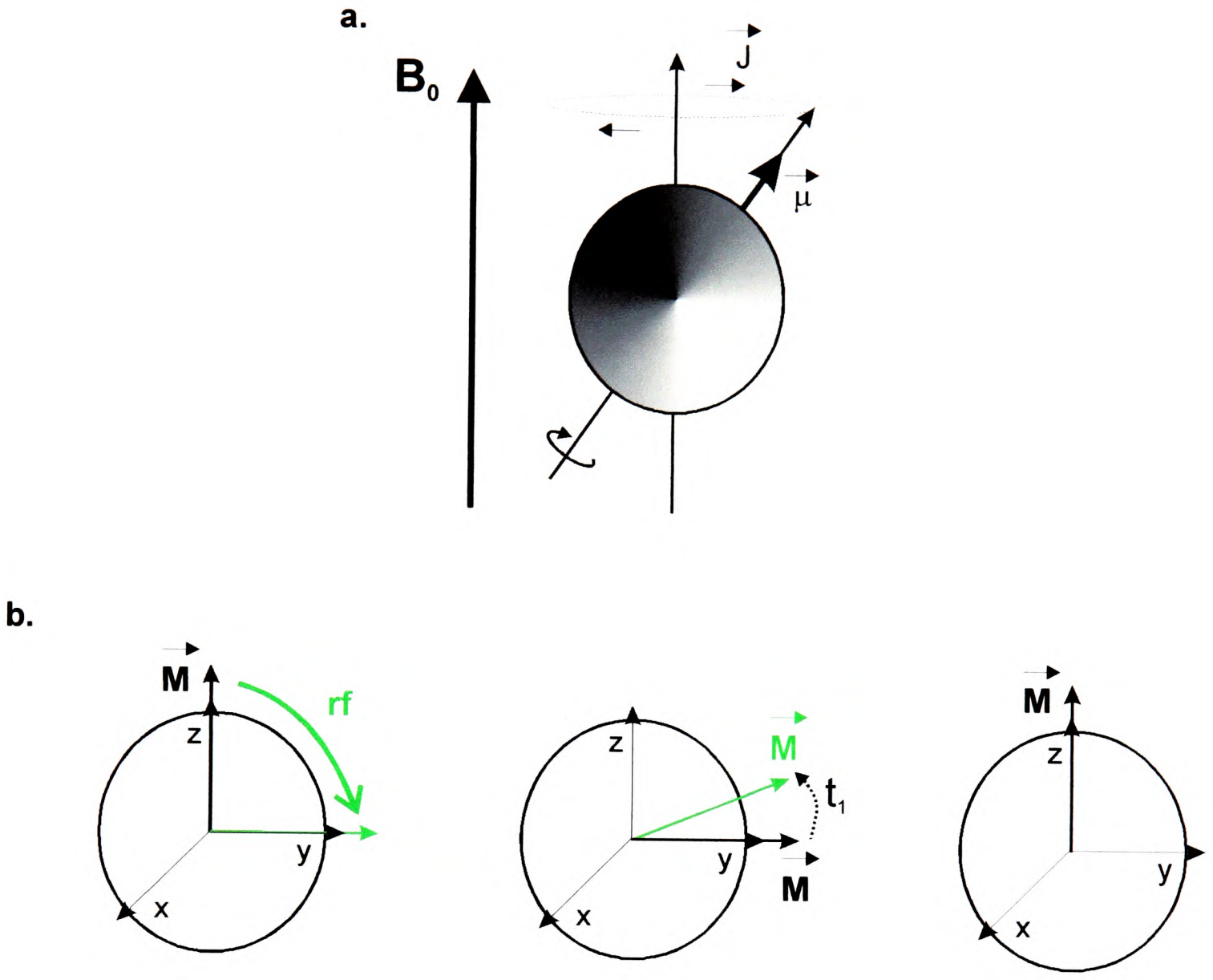


Fig.3.1.

a. Alignment of the magnetic moment $\vec{\mu}$ of a nuclear spin in a external magnetic field. The first scheme shows motion of the nucleus in a magnetic field ($\vec{J}$ - nuclear angular momentum).

b. The magnetization $\vec{M}$ aligns with the magnetic field. The green arrows represent the situation after application of an rf 90° pulse, when $\vec{M}$ is oriented perpendicular to the magnetic field. During the relaxation period the nuclei return to the equilibrium state. The last scheme shows fully recovered magnetization when no additional pulses were applied.

monitored by measuring of the induction of a current in the coil. The power and length of the pulse determine the rotation angle of $\vec{M}$ (Fig.3.1b). In the simplest one-dimensional (1D) experiment the data acquisition is carried out after application of a 90° pulse. In recording data after the pulse one measures an amplitude and phase of the signal as it evolves with time (in the time domain). The NMR response fades over a period following the pulse and is referred to as free induction decay (FID). The experiment can be repeated subsequently, so as to improve the signal-to-noise ratio. Application of a Fourier Transformation to the time dependent signal results in the frequency domain spectrum with Lorentzian line shapes.

Any two-dimensional (2D) NMR experiment can be divided into four periods: preparation, evolution, mixing and acquisition, shown in Fig.3.2. In the simplest case, the preparation period consists of a 90° pulse that aligns $\vec{M}$ perpendicular to the magnetic field. A sequence of pulses can be used for more complicated alterations of magnetization. During the evolution period the time evolution of the prepared magnetization is sampled at discrete time points t_1. In the mixing period the magnetization is transferred from one nucleus to another, and therefore the pulses and delays contained here are crucial for the information content in a spectrum. Finally the FID for the F2 dimension of a 2D spectrum is recorded during the acquisition time t_2. The variety of 2D experiments allows measurement of through-bond homonuclear interactions: homonuclear J-coupling - TOCSY (Total Correlation SpectroscopY - Hartmann & Hahn, 1962, Braunschweiler & Ernst, 1983, Bax & Davis, 1985) and COSY (Correlation SpectroscopY - Aue *et al.*, 1976), and through-space homonuclear interactions: Nuclear Overhauser Effect (NOE) - NOESY (Nuclear Overhauser Effect

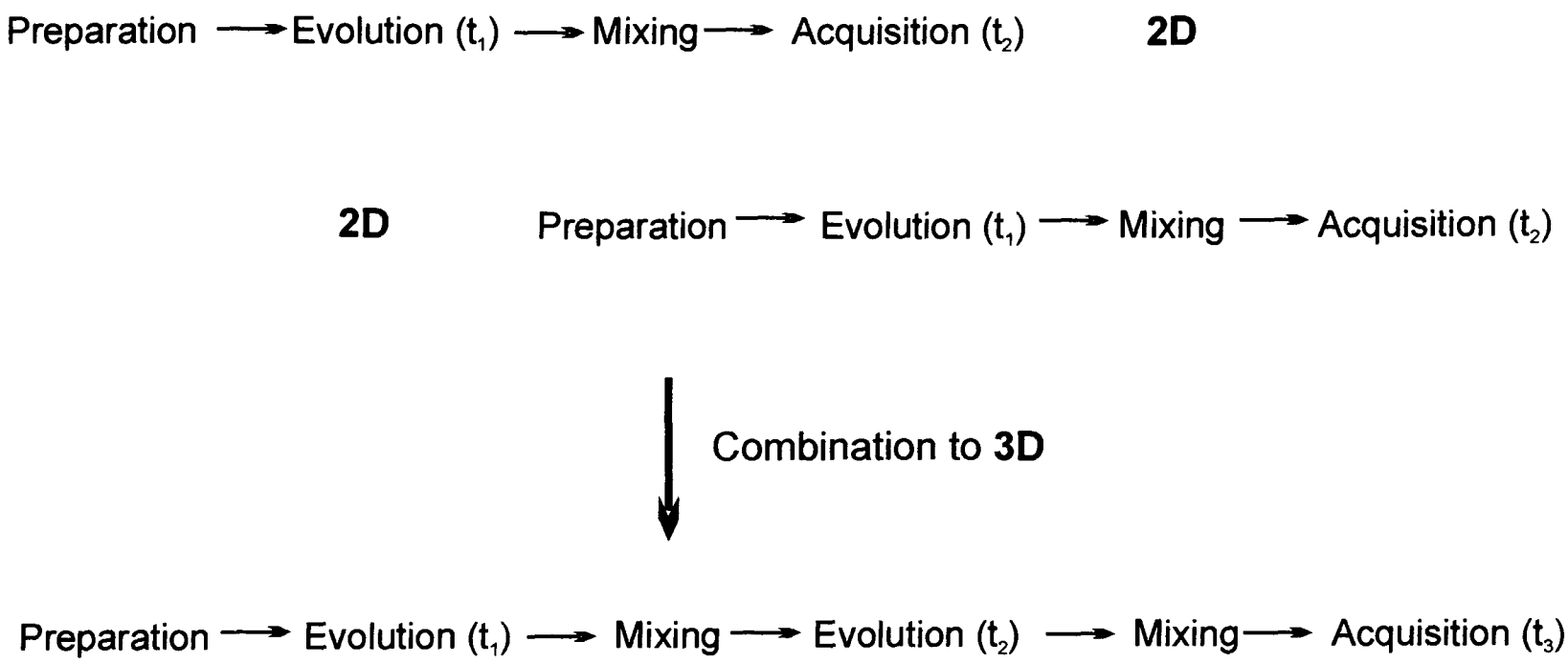


Fig.3.2.

The scheme of 2D experiment and the combination of two of them to obtain 3D spectrum. The evolution (t_1) period for the first 2D is combined with a preparation stage for the second 2D.

SpectroscopY - Overhauser, 1953, Solomon, 1955). Through-bond heteronuclear interactions can be measured using heteronuclear J-coupling - HSQC (Heteronuclear Single-Quantum Correlation - Bodenhausen & Ruben, 1980).

The three-dimensional (3D) experiment can be simply constructed as the combination of two 2D experiments (Fig.3.2). The evolution times t_1 and t_2 are incremented independently. The 2D experiments mentioned above and 3D experiments: HSQC-TOCSY (Marion *et al.*, 1989) and HSQC-NOESY (Schleucher *et al.*, 1994, Wagner & Berger, 1997) allow for assignment of the spectra and the intensity of each NOE (cross-peak in NOESY spectrum – Fig.3.3) can be transformed into distance information, which is useable in the calculation of the three-dimensional structure of the molecule. When the signal is processed and spectrum visualized, one can use NOEs to determine distances between atoms that are usually situated closer than 6 Å apart.

3.2. Assignments and local structural information

All NMR spectra were acquired at proton (^{1}H) frequencies of 500, 600 and 750 MHz at pH 5.0 and 37°C unless stated otherwise. The spectrometers consist of Oxford Instruments magnets with consoles and tri-axial gradient triple resonance probes built in-house at the Oxford Centre for Molecular Sciences, consoles running the GE/Omega software (Bruker Instruments Inc, Fremont, CA, US). For the purposes of ^{4}F1^{5}F1, D3 and ^{4}F1^{5}F1/D3 assignments, 2D and 3D spectra were recorded (Table.3.1, Table.3.2, Table.3.3) and the assignments were carried out at 37°C.

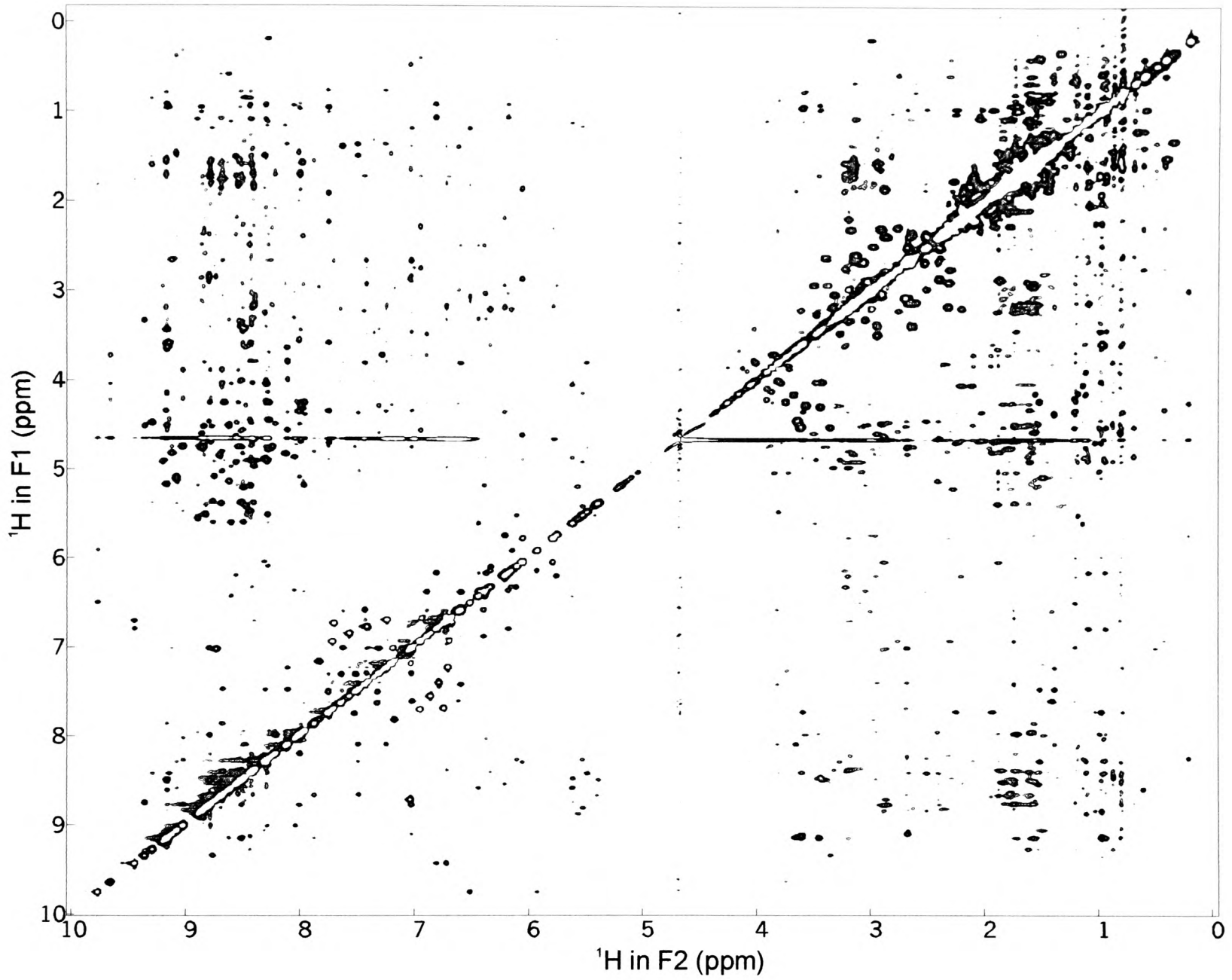


Fig.3.3. 2D NOESY spectrum of 1 mM unbound ^{4}F1^{5}F1 in water, acquired at 500 MHz, pH 5.0 and 37°C. The non-symmetrical appearance of the NOEs is due to the selective water signal suppression in F2. Most of the NOEs seen here are related to interproton distances lower than 5 Å, weaker cross-peaks can be visualised by decreasing the contour level of the spectrum.

Table.3.1. Unbound D3 – experiments used for assignments

1 mM unlabelled D3 in 90% H_2O/10% D_2O, pH 5.0		
Experiment	TOCSY/NOESY mixing time	Acquisition parameters: total points for sweep width (Hz)
^1H-^1H TOCSY 25°C, 37°C	46 ms	t_2: 2048 for 10000 Hz t_1: 1100 for 8000 Hz
^1H-^1H NOESY 25°C, 37°C	150 ms, 300 ms	t_2: 2048 for 8000 Hz t_1: 1100 for 8000 Hz

In the first stage of the assignment process TOCSY and COSY spectra, recorded in water and D_2O, are used to find the through-bond connectivities between protons within amino acid spin systems. Ten distinctive patterns for aliphatic side-chains plus four aromatic ring patterns have been determined for the twenty naturally occurring amino acids (Wüthrich, 1986). Once the spin systems are identified they can be put into sequential order by finding $H^N_{(i+1)}$ to H^{α}_i/H^{β}_i cross-peaks in NOESY spectra recorded in water. This method is called the 'sequential assignment approach' (Wüthrich *et al.*, 1982) and allows for identification of residues as they occur in a primary structure.

There is also an alternative assignment method called the 'main-chain directed approach' (Englander & Wand, 1987) that utilizes information about the protein fold and the resulting close proximity of residues situated far apart in the sequence. This approach is particularly useful when β-sheet structure is predicted. It has not, however, been used in these studies, as the 'sequential assignment approach' led to 99% complete assignments of $^4F1^5F1$ in both the unbound and bound state. Unassigned protons belong to side-chain amide groups of Gln and their identification

was impossible due to complete lack of visible cross-peaks connecting them to other protons.

Table.3.2. Unbound $^{4}F1^{5}F1$ – experiments used for obtaining distance restraints

1 mM unlabelled $^{4}F1^{5}F1$ in 100% D_2O, pH 5.0		
Experiment	TOCSY/NOESY mixing time	Acquisition parameters: total points for sweep width (Hz)
^{1}H-^{1}H TOCSY 37°C	46 ms	t_2: 2048 for 12500 Hz t_1: 1024 for 8000 Hz
^{1}H-^{1}H NOESY 37°C	100 ms	t_2: 2048 for 12500 Hz t_1: 1024 for 8000 Hz
1 mM ^{15}N-labelled $^{4}F1^{5}F1$ in 90% H_2O/10% D_2O, pH 5.0		
Experiment	TOCSY/NOESY mixing time	Acquisition parameters: total points for sweep width (Hz)
^{1}H-^{1}H TOCSY 25°C, 37°C	46 ms	t_2: 2048 for 12500 Hz t_1: 1300 for 6250 Hz
^{1}H-^{1}H NOESY 25°C, 37°C	100 ms	t_2: 2048 for 12500 Hz t_1: 1300 for 6250 Hz
^{1}H-^{1}H COSY 37°C		t_2: 2048 for 12500 Hz t_1: 750 for 6250 Hz
3D NOESY-HSQC 37°C	100 ms	t_2: 512 for 12500 Hz t_1: 40 for 1515 Hz t_3: 256 for 8000 Hz
^{1}H-^{15}N HMQC-J 37°C		t_2: 1024 for 8000 Hz t_1: 320 for 1515 Hz
^{1}H-^{15}N HSQC[a] 37°C		t_2: 1024 for 12500 Hz t_1: 256 for 1515 Hz

[a]the assigned spectrum shown in Fig.3.4

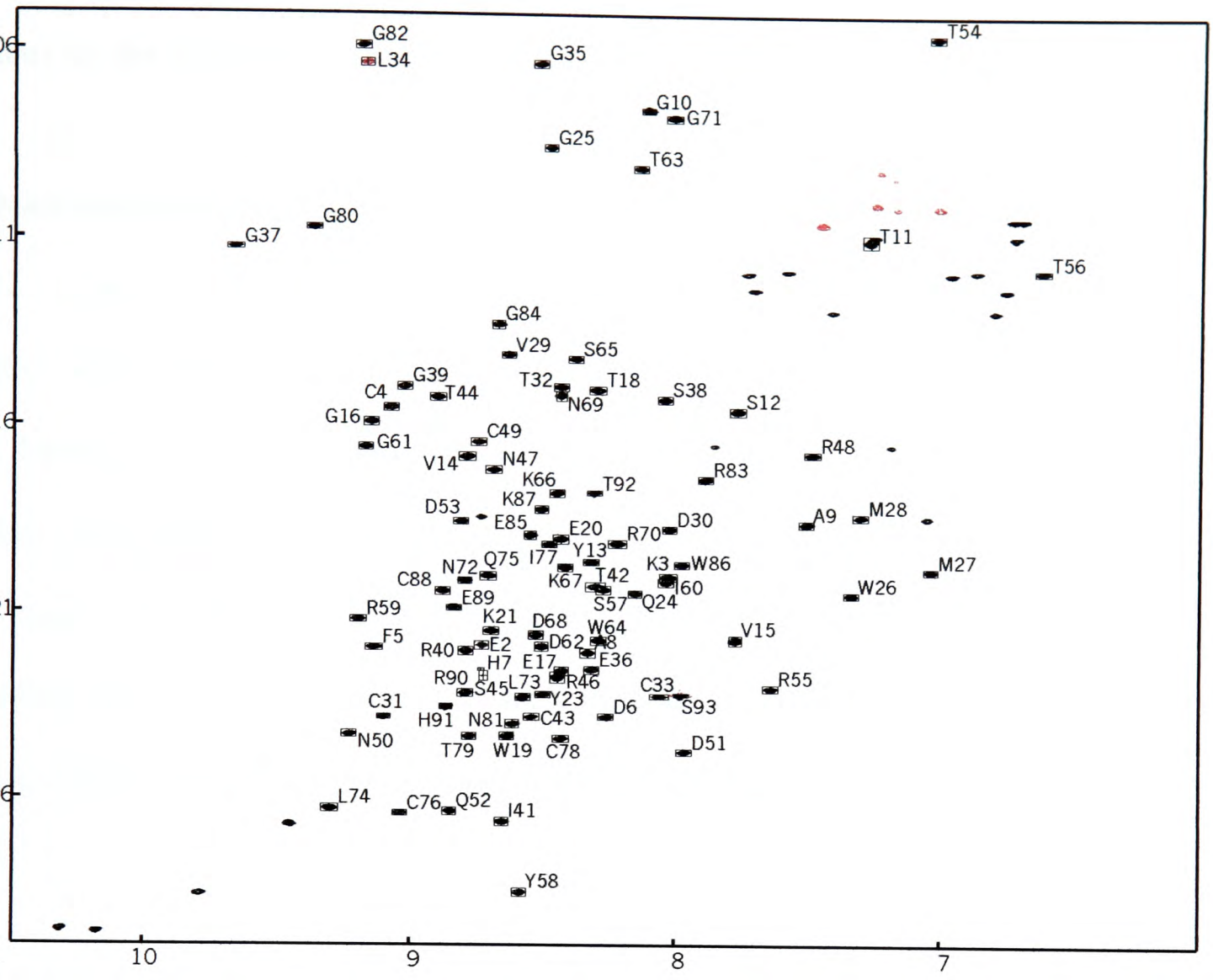


Fig.3.4. The HSQC spectrum of 1 mM ^{15}N-^{4}F1^{5}F1 with labelled backbone NH cross-peaks, performed at 37°C, pH 5.0.

As cross-peaks arising from the same ^{1}H have different intensities in spectra recorded in water and D_2O, separate calibration has to be applied to obtain unified distance restraints for the structure calculations (**Sections 4.1** and **5.1**).

To obtain interproton distance restraints for determination of the structure of unbound $^{4}F1^{5}F1$, intensities were calculated from the NOESY spectra in D_2O and H_2O acquired with mixing times of 100 ms. The value of 100 ms was estimated using 'NOE build-up' curves obtained from a series of 2D NOESY experiments. For the intensity measurement, 8 intraresidue NOEs, which were well-separated from other cross-peaks, were used (Fig.3.5). The value of 100 ms was chosen as the mixing time-dependent peak intensity increase, measured using NMRView 4.0 (Johnson & Blevins, 1994), was still in linear phase but close to the plateau of the 'NOE build-up' curve.

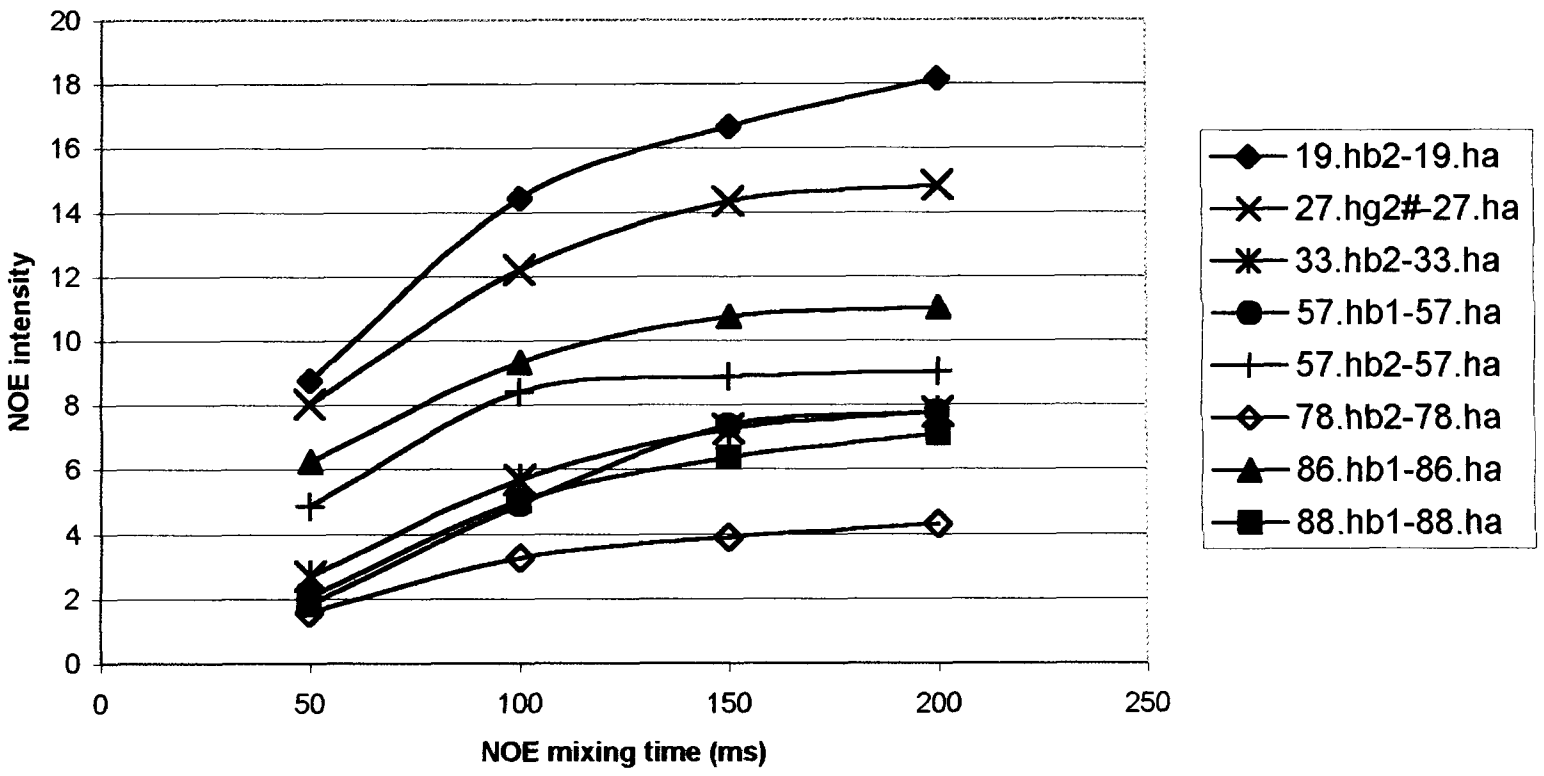


Fig.3.5. NOE intensity 'build-up' curves obtained from experiments acquired with mixing times of 50, 100, 150 and 200 ms.

Table.3.3. ^{4}F1^{5}F1/D3 complex – experiments used for obtaining distance restraints

0.5 mM unlabelled ^{4}F1^{5}F1/1.16 mM unlabelled D3 in 100% D_2O, pH 5.0		
Experiment	TOCSY/NOESY mixing time	Acquisition parameters: total points for sweep width (Hz)
^{1}H-^{1}H TOCSY 37°C	46 ms	t_2: 2048 for 10000 Hz t_1: 800 for 8000 Hz
^{1}H-^{1}H NOESY 37°C	150 ms	t_2: 2048 for 10000 Hz t_1: 1024 for 8000 Hz
0.5 mM ^{15}N-labelled ^{4}F1^{5}F1/1.16 mM unlabelled D3 in 90% H_2O/10% D_2O, pH 5.0		
Experiment	TOCSY/NOESY mixing time	Acquisition parameters: total points for sweep width (Hz)
^{1}H-^{1}H TOCSY 10°C, 25°C, 37°C	46 ms	t_2: 2048 for 10000 Hz t_1: 1200 for 8000 Hz
^{1}H-^{1}H NOESY 10°C, 25°C, 37°C	150 ms	t_2: 2048 for 10000 Hz t_1: 1200 for 8000 Hz
^{1}H-^{1}H COSY 37°C		t_2: 2048 for 10000 Hz t_1: 800 for 8000 Hz
3D NOESY-HSQC 37°C	150 ms	t_2: 1024 for 10000 Hz t_1: 25 for 1515 Hz t_3: 210 for 6250 Hz
^{1}H-^{15}N HMQC-J 37°C		t_2: 1024 for 8000 Hz t_1: 450 for 1263 Hz
^{1}H-^{15}N HSQC[a] 37°C		t_2: 1024 for 10000 Hz t_1: 256 for 1515 Hz

[a]the assigned spectrum shown in Fig.3.6

To obtain interproton distance restraints for determination of the structure of the ^{4}F1^{5}F1/D3 complex, intensities were calculated from the NOESY and NOESY-HSQC spectra in D_2O and H_2O acquired with mixing times of 150 ms. The value of 150 ms was estimated using the 'NOE build-up' curves obtained from a series of 2D NOESY

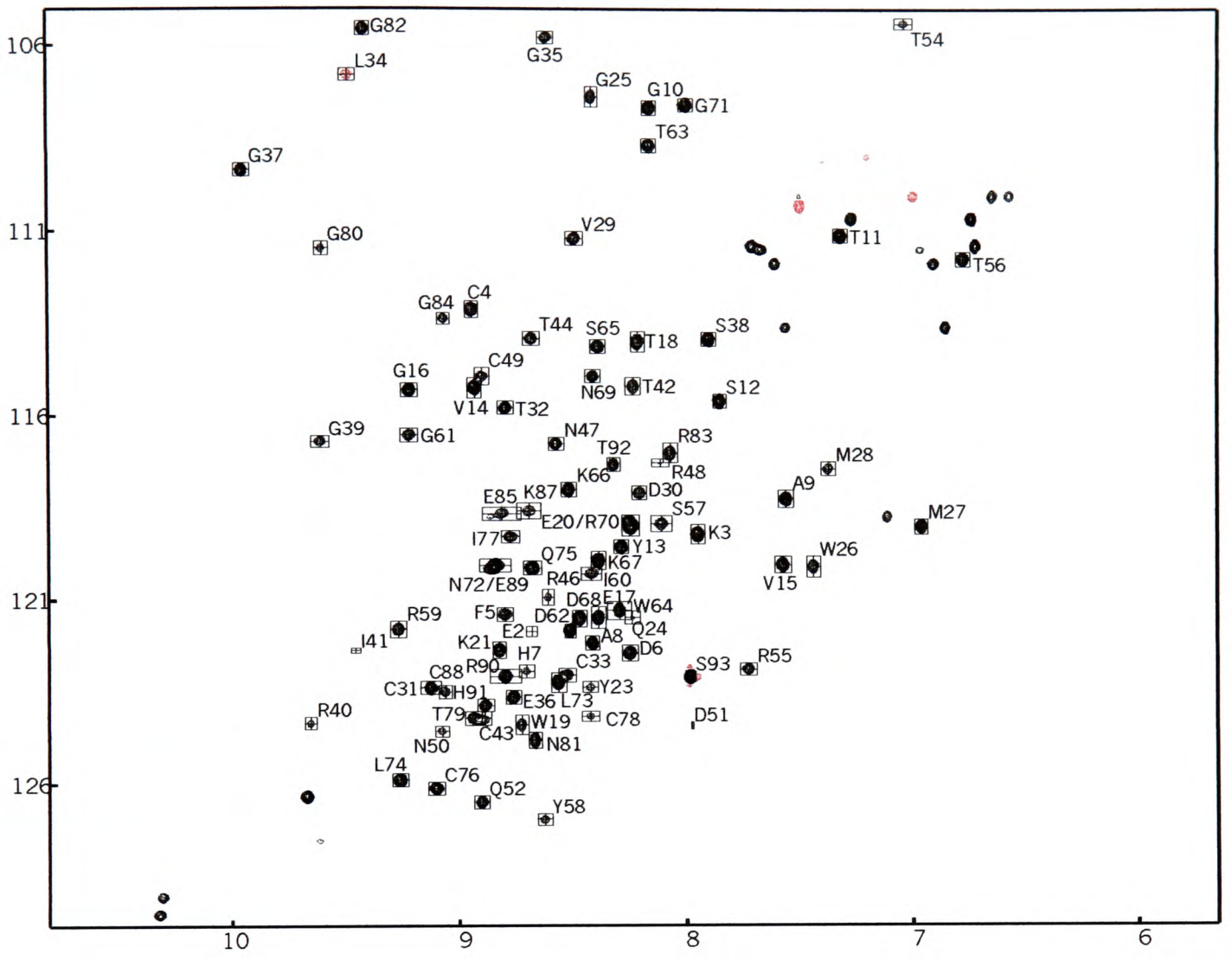


Fig.3.6. The HSQC spectrum of 0.5 mM ^{15}N-$^{4}F1^{5}F1$/ 1.16 mM unlabelled D3 with labelled backbone NH cross-peaks, performed at 37°C, pH 5.0.

experiments in D_2O with mixing times of 50, 100, 150 and 200 ms, using the same intraresidue NOEs as for the unbound $^4F1^5F1$.

The spin-spin $^3J_{HN\text{-}H\alpha}$ couplings are correlated with the backbone dihedral φ angle (Karplus, 1959) and the angle values derived from coupling are used in structure calculations as additional restraints. These J-couplings are easily measured using a ^1H-^{15}N HMQC-J experiment (Heteronuclear Multi-Quantum Correlation – J-coupling – Kay & Bax, 1990). Here, the J-coupling constants were measured by line-shape fitting that of F1 traces from a HMQC-J (C. Redfield, based on Norwood *et al.*, 1992). Exchange experiments were performed to identify slowly exchanging backbone amide protons ($^1H^N$), that might be involved in hydrogen bonds. A ^{15}N-sample dissolved in water was lyophilised and redissolved in 100% D_2O immediately prior to the first acquisition of ^1H-^{15}N HSQC spectrum. A series of experiments was carried out to identify slowly exchanging $^1H^N$. A $^1H^N$ was considered to be slowly exchanging if $^1H^{N15}N$ signal was still present in a HSQC spectrum acquired after 6 h and if $^1H^N$ acceptor (backbone O atom from an adjacent β-strand) could be identified from the secondary structure. The values of φ angles and hydrogen bonds require additional validation before incorporating them into the structure calculations (**Sections 4.1** and **5.1**).

Data processing and analysis

Data were processed with FELIX 2.3 (Biosym Technologies Inc., San Diego, CA, US). For 2D experiments apodization using a Lorentzian-Gaussian function in t_2 and sinebell or sinebell-squared function in t_1 with a phase shift of 70 (or 0 for COSY

experiments) were applied. The 3D experiments were processed using macros that apply a Lorentzian-Gaussian function in t_2 and extending t_1 to twice the acquisition length by linear prediction (Barkhuijsen *et al.*, 1985) prior to applying a sinebell function with phase shift of 70 in t_1 and t_3. Strips were created by the FELIX macro *tease* (A. Pickford). Spectral assignment and the extraction of NOE intensities for structure determination were performed using the program NMRView 4.0 (Johnson & Blevins, 1994).

3.3. ^{15}N relaxation

The function of a protein is often as dependent on its well-defined regions as on the flexible ones. Conventional NMR data, based on relatively short interproton distances, generally provide only averaged information about the ordered regions. Loops are often ill-defined, which in some cases relates to the lack of data obtained for flexible regions in X-ray diffraction. Time-scale information for internal motions can be obtained from ^{15}N relaxation measurements. Fast thermal fluctuations (ps - ns) corresponding to structurally less defined parts and slow conformational changes (μs - ms) between defined conformers can be distinguished. This method can also be helpful in defining the behaviour of domains with respect to each other, which is often difficult due to lack of distance restraints, e.g. Brüschweiler *et al.* (1995).

Relaxation describes the return of a system to thermal equilibrium, mediated by magnetic field fluctuations. The fluctuations are the result of thermal motions of the molecules. The component of the magnetization of the nuclear spins that is aligned

with the external field B_0 can be inverted by applying an rf pulse. The characteristic time constant of the return of this magnetization component to its equilibrium value is called the longitudinal relaxation time constant T_1 (Abragam, 1961).

The magnetization that is oriented perpendicular to the external magnetic field has a characteristic time constant for the return to equilibrium, called the transverse relaxation time constant T_2 (Abragam, 1961). T_2 characterises the loss of phase coherence of the transverse component of magnetization. A third relaxation parameter is the so-called heteronuclear nuclear Overhauser effect (NOE). This is measured by saturating the ^1H signal and observing changes in the ^{15}N signal in comparison to the non-saturated state (review by Roques *et al.*, 1980).

The relaxation time constant T_2 is inversely proportional to the correlation time τ_c. This means that the linewidth increases with increasing of M.W. T_1 increases for short and very long correlation times and goes through a minimum at $1/\omega$ (ω – Larmor frequency). The dependence of the ^1H-^{15}N NOE on the τ_c is low for 'fast' tumbling (10^{-10}s), then rapidly increases around τ_c 10^{-9}s to reach plateau at ~0.85 for 'slow' tumbling (>10^{-8}s). The correlation time dependence of T_1, T_2 and NOE can be used to study molecular motions in proteins on the picosecond to nanosecond time scale (Lipari & Szabo, 1982, Kay *et al.*, 1989).

Another parameter for assessing the motions that influence the data, is the generalized order parameter S. S^2 estimates the spatial restriction that the motion experiences, independent of any model that may be used to describe the motion itself. So that S^2 =

0 indicates no restriction to motion, therefore all possible spin orientations are allowed at the same time. $S^2 = 1$ indicates that the data relates to the motion of a molecule as a whole, without additional components (Lipari & Szabo, 1982).

T_1 and T_2 of ^{15}N nuclei are related to the diffusion tensor of the molecule (Fig.3.7, Woessner, 1962). The quantitative description of the overall diffusion tensor is based on the orientations of the N-H bond vectors within a molecular frame. Apart from the correlation time τ_c another diffusion tensor parameter that can be determined is called D_{ratio}. For the axially symmetric model

$$\mathbf{D_{ratio}} = 2D_{zz}/(D_{xx}+D_{yy})$$

The N-H bond orientations are usually taken from a set of coordinates (PDB).

Details of experiments

A series of ^1H-^{15}N correlation spectra of 0.1 mM ^{15}N-$^4F1^5F1$/0.23 mM unlabelled D3 in 95% H_2O/5% D_2O, pH 5.0 were recorded at 500 MHz for the determination of ^{15}N T_1 and T_2 relaxation time constants. The acquisition parameters were: 1024 complex points covering 8000 Hz in t_2 and 85 complex points for 1263 Hz in t_1. T_2 time constants were determined using a spin-echo sequence with a CPMG delay of 335 μs. Heating introduced to the sample by repeated ^{15}N refocusing pulses during T_2 measurement was assessed by comparing the $^1H^{15}N$ chemical shift in the spectra with the maximum and minimum number of refocusing pulses (Wang & Bax, 1993). To

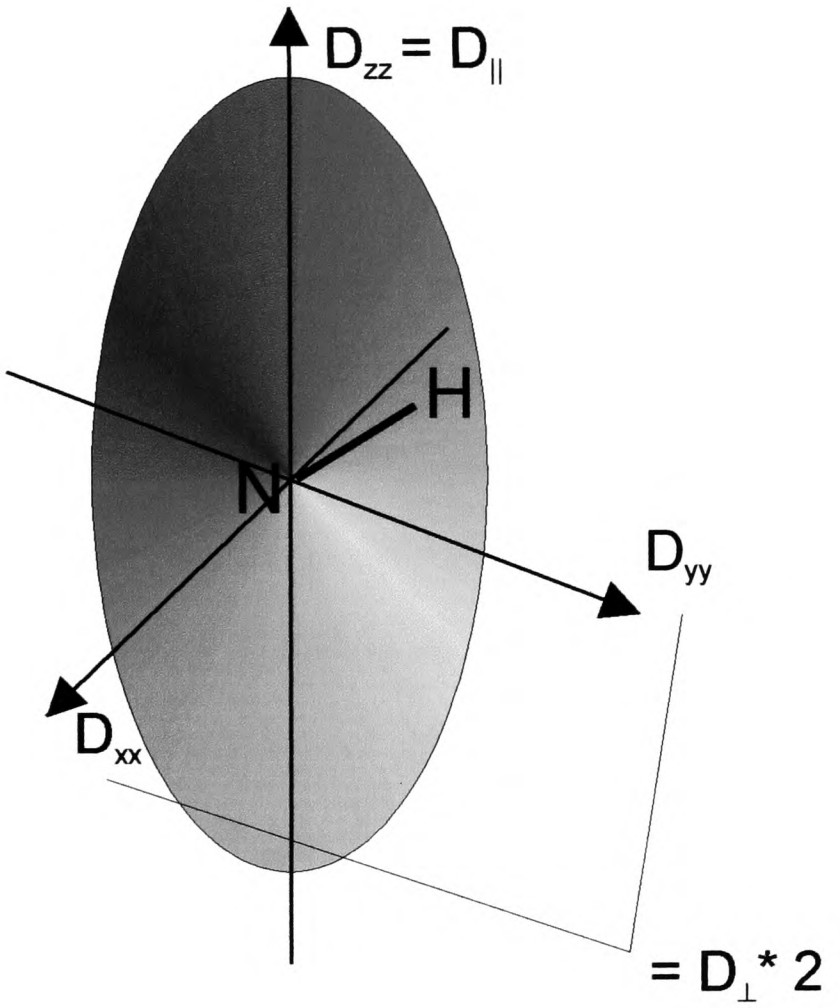


Fig.3.7. Schematic representation of the diffusion tensor for the axially symmetric model. In this model axes D_{xx} and D_{yy} are equivalent and their average is referred to as $D_{\perp}$; the long axis is then called $D_{||}$.

compensate for the solution temperature increase, the sample was cooled down by 2°C. The relaxation delays for T_1 and T_2 experiments were:

T_1: 40 (twice), 120, 200, 320, 440, 600 and 800 ms

T_2: 5.36 (twice), 16.08, 32.16, 53.60, 91.12, 144.72 and 214.40 ms

The diffusion parameters were estimated using Modelfree4 (Mandel *et al.*, 1995) software.

Data processing and analysis

Data were processed with the FELIX 2.3 software package (Biosym Technologies Inc.), using suitable macros (M. Akke & J. Werner). The apodization using a Lorentzian-Gaussian function in t_2 and sinebell-squared function in t_1 with a phase shift of 70 were applied. The data analysis was carried out using series of FELIX macros and scripts kindly provided by A. Palmer & J. Werner.

3.4. Residual dipolar coupling

Dipolar couplings between nuclear spins are usually averaged to zero by the isotropic tumbling of a molecule in solution. If anisotropy can be reintroduced, the dipolar interaction no longer vanishes and residual dipolar couplings (RDCs) are observed. One-bond dipolar couplings can provide a reference to determine the protein alignment tensor (Tjandra & Bax, 1997ab, Prestegard, 1998).

A variety of media is available for alignment of proteins, such as neutral bicelles (Sanders & Prosser, 1998), polyacrylamide gels (Sass *et al.*, 2000), ethylene glycol (Ruckert & Otting, 2000), purple membranes (Sass *et al.*, 1999), and charged phages (Hansen *et al.*, 1998). The medium used here, bicelles (Fig.3.8), represents an intermediate morphology between lipid vesicles and classical mixed micelles, combining some of the putative properties of both. Bicelles are non-compartmentalized and optically transparent, yet maintain some key bilayer properties. Below ~25°C the mixture is in an isotropic, water-like state and NMR spectra obtained should not differ from those recorded in water. Above ~30°C (depending on composition) it undergoes a phase transition becoming anisotropic bicelles, thus aligning the sample molecules through spatial limitation (Sanders & Prosser, 1998). 3-5% aqueous medium of oriented bicelles is widely used to produce moderate degrees of alignment of molecules in a magnetic field while retaining the sensitivity, simplicity and resolution of spectra recorded in an isotropic medium (Tjandra & Bax, 1997a).

Since the first measurement of nuclear dipolar spin-spin couplings in myoglobin caused by partial self-alignment of the protein molecules with respect to the external magnetic field (Tolman *et al.*, 1995), these parameters have become widely used for the determination and the refinement of structures of molecules in solution (Meiler *et al.*, 2001). The simulated annealing approach introduces an RDC restraint energy that is minimized when the dipolar interaction vectors are oriented correctly with the respect to the alignment tensor (N. Tjandra & M. Clore – unpublished results).

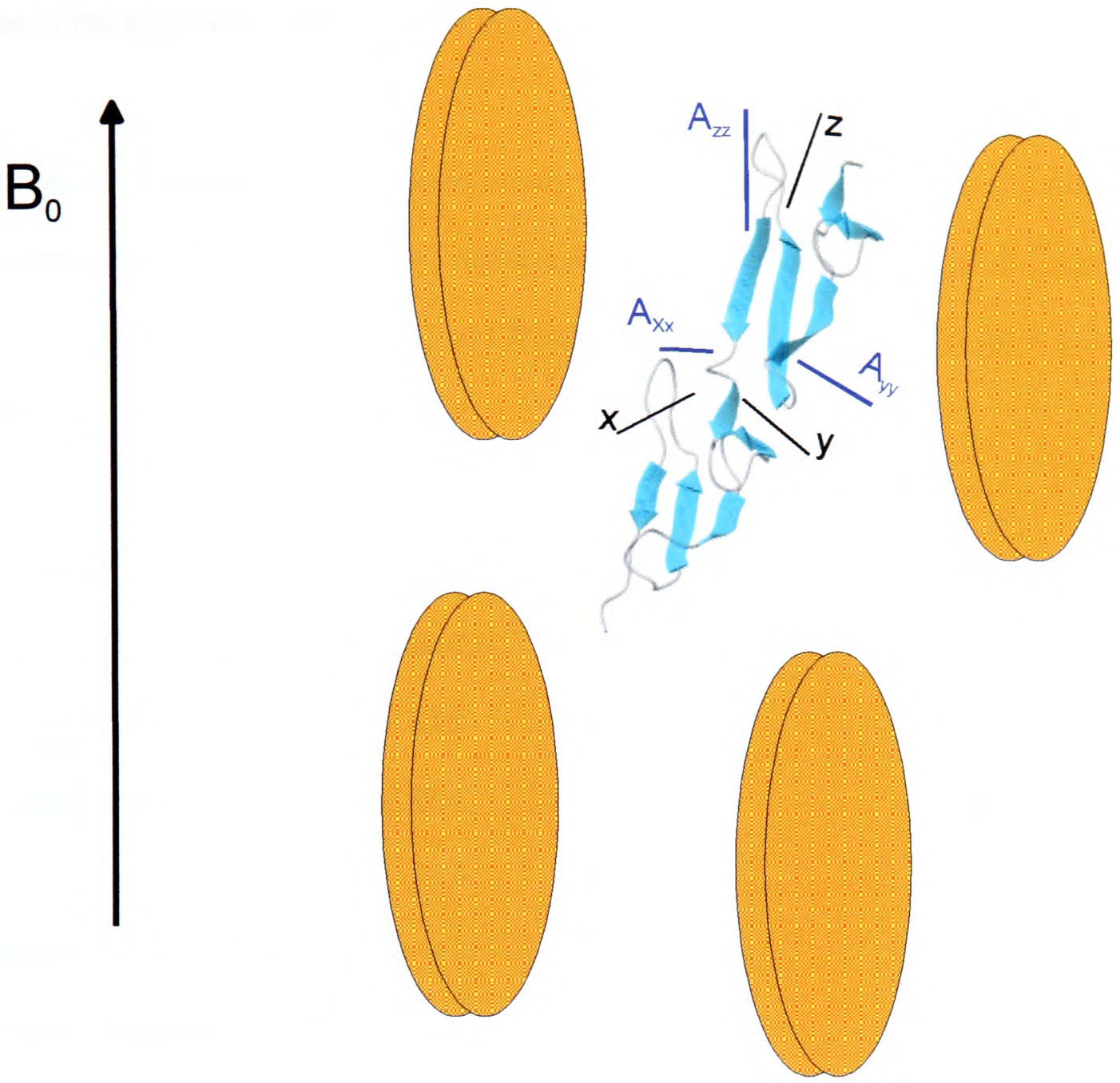


Fig.3.8. Schematic representation of bicelles. The model is presented as proposed in Sanders & Prosser (1998), the model presented recently by A. Bax (named 'Swiss cheese') suggests that the opposite situation occurs. The $^{4}F1^{5}F1$ module pair is presented with the molecule axes (black) and the alignment tensor as modelled in Module (blue).

Assuming an isotropic distribution of N-H bond vectors one can derive the eigen values of the alignment tensor from the distribution of RDCs – 'powder pattern'.

This way of estimating the alignment tensor is rather arbitrary and requires further parameter optimisation after the implementation of restraints in the structure refinement using a grid search of A_a and R values, that agree the best with the rest of experimental data.

'Powder pattern' estimates (Dosset *et al.*, 2001):

A_{xx} – the highest populated value of RDC

A_{yy} – the lowest negative value of RDC

A_{zz} – the highest positive value of RDC

Alignment tensor components:

$\mathbf{A_a}$ – axial component = $\mathbf{1/3\ [A_{zz}-(A_{xx}+A_{yy})/2]}$

$\mathbf{A_r}$ – rhombic component = $\mathbf{1/3\ (A_{xx}-A_{yy})}$

$\mathbf{R=A_r/A_a}$ – rhombicity

Details of experiments

The RDC measurements in bicelles for the backbone N-H vectors were made at 500 MHz at 19°C and 37°C. The modified HSQC pulse sequence (*Grs3e_in*) with disabled decoupling was used, with narrow and broad components of the spectrum recorded as two separate spectra. The lower temperature experiment results in splittings due to scalar coupling alone (J_{HNN} Hz), while the higher temperature one shows splittings due

to the sum of scalar and dipolar couplings ($J_{HN} + D_{HN}$ Hz). Taking the difference between isotropic and aligned state allows the dipolar coupling to be determined.

The sample contained 10% D_2O and 3% (w/v) bicelles composed of 3:1 molar ratio of 1,2-O-ditridecanyl-sn-glycero-3-phosphocholine (DTDPC, Avanti) and 1,2-O-dihexyl-sn-glycero-3-phosphocholine (DHOPC, Avanti) (Sanders & Prosser, 1998) and either 0.5 mM $^{1}H^{15}N$-$^{4}F1^{5}F1$ or 0.5 mM $^{1}H^{15}N$-$^{4}F1^{5}F1$/1.2 mM unlabelled D3 or 0.2 mM $^{2}H^{15}N$-$^{4}F1^{5}F1$/0.47 mM unlabelled D3. All experiments were performed at pH 5.0. The acquisition parameters were: 2048 complex points covering 20000 Hz in t_2 and 160 complex points for 1666 Hz in t_1.

Data processing and analysis

Data were processed with the FELIX 2.3 software package (Biosym Technologies Inc.). Apodization using a Lorentzian-Gaussian function in t_2 and a sinebell-squared function in t_1 with a phase shift of 70 were applied. The sw of 20000 Hz was used in t_2 to improve the baseline in ^{1}H dimension. The t_1 dimension was zero-filled up to 8k to improve the precision of the measurements.

3.5. Structure calculations

Basics

NMR experiments, carried out for the determination of a high-resolution protein structure, in general provide specific, short-range information about the macromolecule. The local nature of the NMR data means that random initial models

can be productively refined, as they contain information about the protein fold (Braun & Go, 1985). In practice the experimental data are complemented by empirical information contained in force fields, which are derived from small molecule and protein structure databases.

The optimisation problem in a high-dimensional parameter space is to find the global minimum of a target function. In the case of macromolecular structure calculation and refinement, one searches for the conformations of the molecule that best fit the experimental data while maintaining reasonable covalent and non-covalent interactions (Fig.3.9 - Braun & Go, 1985). In NMR structure calculation by simulated annealing, an energy target function is used that includes a force field and the experimental information:

$$E=E_{model} + w_{NMR}E_{NMR}$$

E_{model} - describes the covalent and non-bonded interactions

E_{NMR} - represents the experimental data

w_{NMR} - the weight of the NMR energy function

Because of the limitations of the NMR data, force fields have a large impact on the quality and possibly also on the accuracy of NMR structures (Linge & Nilges, 1999). E_{model} consists of terms for covalent bonds, bond angles, chirality, planarity and non-bonded repulsion (Hendrickson, 1985).

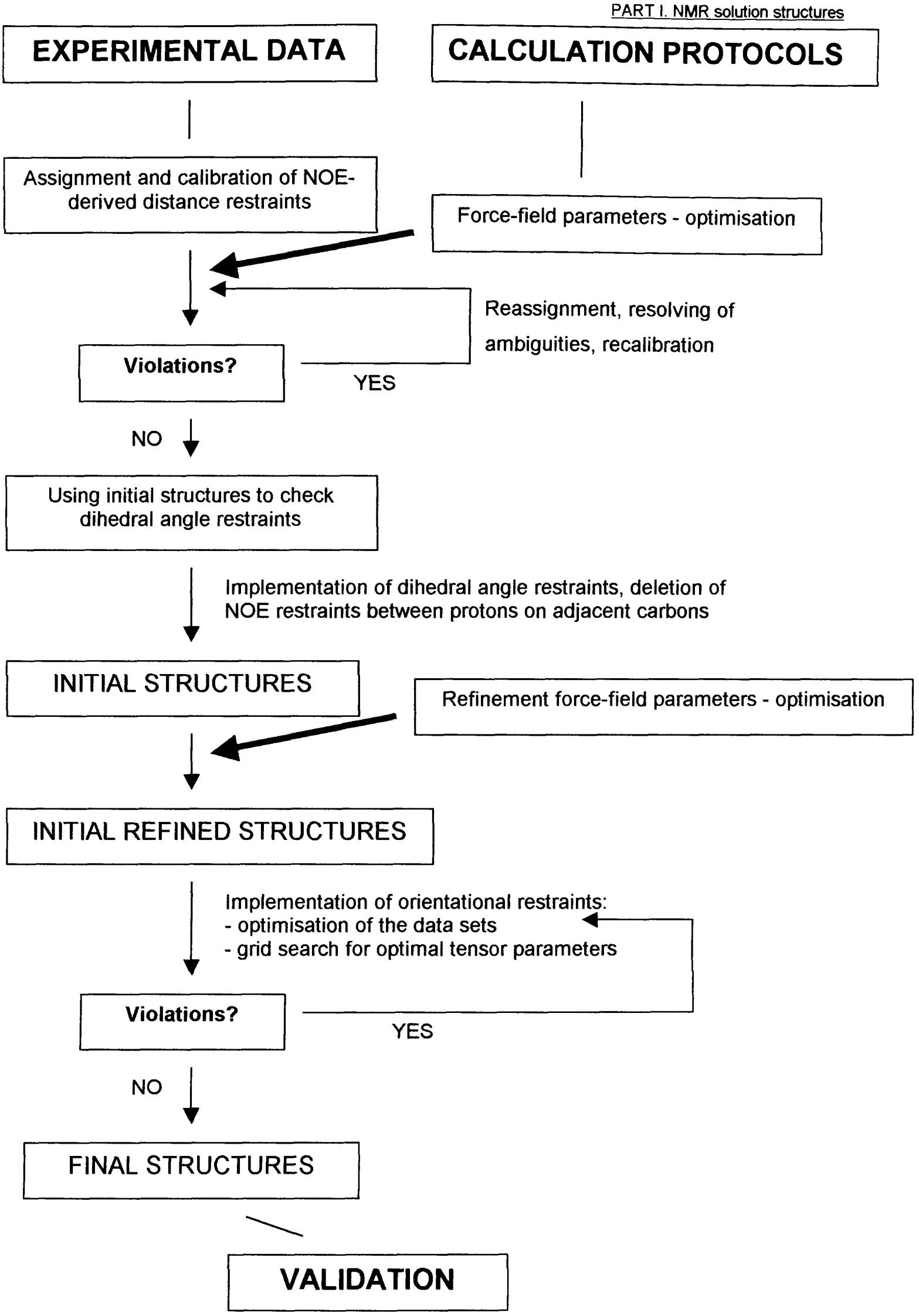


Fig.3.9. Flow diagram of the structure calculation procedures for the $^{4}F1^{5}F1$ module pair

Force fields

The CSDX force-field parameters (Engh & Huber, 1991, based on the CHARMM PARAM19 force field (Brooks *et al.*, 1983)), derived from the data deposited in the Cambridge Structural Database (Allen *et al.*, 1979), are commonly used for X-ray crystal structure refinement, and are the reference parameters for structure validation programs (e.g. WHATIF (Vriend, 1990) and PROCHECK (Laskowski *et al.*, 1996)). They are the basis for the popular PARALLHDG force field.

Recently, the non-bonded interactions from CSDX have been replaced with values taken from the PROLSQ refinement program (Hendrickson, 1985), providing uniformity between X-ray and NMR refinement. The main difference between these two force fields exists in a repulsion energy definition for $r \leq r_{min}$:

r – distance between two atoms

r_{min} – sum of r_{vdW} of these atoms

PARALLHDG

$$E_{repel} = k_{vdW} (s^2 r_{min}^2 - r^2)^2$$

PROLSQ

$$E_{repel} = k_{vdW} (s r_{min} - r)^4$$

k_{vdW} – energy constant

s – scaling factor

Larger van der Waals radii (r_{vdW}) than commonly employed in NMR structure calculation programs significantly improve the definition of secondary structure, if appropriate corrections for vdW interactions of 1-4 atoms (separated by more than three bonds) are applied. As the X-ray refinement normally omits protons, hydrogen radii are included there in extended heavy atoms r_{vdW}. Therefore, unlike in any other force field, in PROLSQ hydrogen r_{vdW} was reduced to avoid clashes between aliphatic protons and extended heavy atoms; also r_{vdW} for 1-4 interactions are smaller than for the others (Linge & Nilges, 1999).

The structures of the unbound ^{4}F1^{5}F1 module pair (**Chapter 4**), were calculated using the PARALLHDG force field; the calculations of the bound state were performed using PROLSQ. Due to the availability of **ISAC** (Implicit Saupe tensor Alignment Constraint) routine and refinement scripts utilizing **DANI** (Diffusion Anisotropy) and **SANI** (Saupe Anisotropy), the XPLOR software (A. Brünger) was chosen for performing the structure calculations.

Calculation protocols

For structure calculations XPLOR v3.851 with the v5.1 parameter and topology files (M. Nilges) and floating chirality file (M. Nilges – modified by K. Downing) were used. All the protocols are included in the **Appendix 5A, 5B** and XPLOR files in the **Appendix 5D**. The simulated annealing protocols used here were written mainly by M. Nilges and J. Linge. They consist of a high-temperature period and two-three cooling stages, normally encompassing a temperature reduction from 2000 K to 25 K.

Usually NOE and CDIH force factors are kept constant during the whole process however, it has been shown that dividing the NOE restraints into intra- and intermodular classes and gradually increasing the force factor for the latter gives much better results in terms of the orientation of intermodular definition (Hashimoto, 2001).

For the DANI routine (kindly provided by M. Clore) diffusion tensor parameters, estimated from T_1/T_2 analysis using coordinates taken from the set of initial structures, are included in the refinement protocol. The orientation of the tensor however, is a function of the coordinates and is allowed to float during the calculation process. The energy term uses T_1/T_2 ratios as input data, and the tensor is represented by four carbon-like atoms: one fixed origin and three representing axes that are allowed to float freely until the correct orientation of the tensor is determined (Tjandra, *et al.*, 1997).

A similar procedure is used to implement RDCs as SANI restraints. The RDC values serve as an input and the additional four carbon-like atoms stand for the fixed origin of the alignment tensor and its coordinate system (Clore *et al.*, 1998). This method requires prior determination of axial and rhombic components of the alignment tensor from the 'powder pattern', as shown above. A recently developed ISAC routine (kindly provided by J. Sass) allows the implementation of RDC-derived restraints without the need for estimation of the alignment tensor parameters. The tensor estimation occurs during the refinement through a least square energy penalty

function for a weak alignment (Sass *et al.*, 2001). In this routine axial and rhombic components of the alignment tensor are defined *de novo* for each refined structure.

In this work all three routines were tested at various stages of the structure refinement. ISAC and DANI were used for the final structure refinement. The validity of final structure ensembles was assessed using PROCHECK-NMR (Laskowski *et al.*, 1996). The structures were displayed using MOLMOL 2.6 (Koradi *et al.*, 1996).

4. THREE-DIMENSIONAL SOLUTION STRUCTURE OF ^{4}F1^{5}F1

4.1. Experimental data

TOCSY spectra of ^{4}F1^{5}F1 were acquired at pH 5.0 and assigned with the assistance of the proton chemical shifts at pH 4.5 (Williams, 1992) (Fig.4.1, **Appendix 2A**). NOESY spectra were assigned independently of the previous chemical shift data. Interproton distances, dihedral φ angle and hydrogen bond restraints obtained from these spectra were used in structure calculations.

Distance and torsion angle restraints

NOE intensities from the NOESY spectra in D_2O and H_2O, acquired with a mixing time of 100 ms, were calibrated using known interproton distances in the aromatic ring of Trp residues, as well as interproton distances in regions of well-defined secondary structure. The peaks were grouped into three main classes according to their intensity: strong (interproton distance $\leq$ 2.8 Å), medium ($\leq$ 3.5 Å) and weak ($\leq$ 5.0 Å). Calibration and creation of restraints tables were carried out using PERL scripts kindly provided by K. Downing. Several of the lowest intensity NOEs were put into an additional (6.0 Å) class. Restraints assigned ambiguously were checked after the refinement – the shorter distance was treated as unambiguous, while

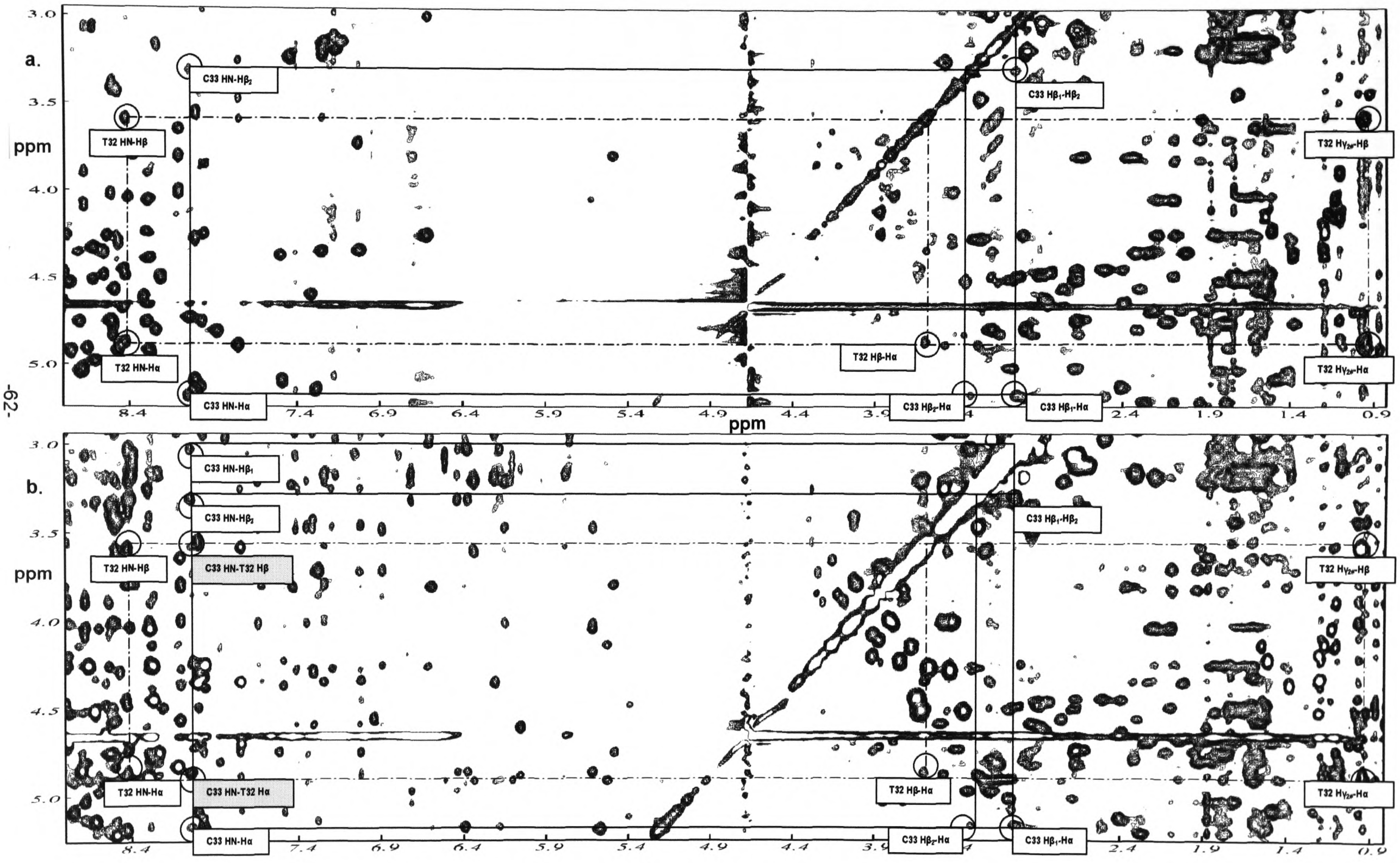


Fig.4.1. Example of assignments of Thr32 and Cys33 of the $^{4}F1^{5}F1$ module pair. **a.** TOCSY in water. **b.** NOESY in water. Apart from the cross-peaks between chemical shifts of intraresidual protons the sequential ones are also seen – indicated in grey.

the longer distance was removed. In case of identical distances for both assignments, they were kept as separate unambiguous restraints.

After the initial structure calculations, hydrogen bond restraints were included if three criteria were met: the presence of the $^{1}H^{15}N$ resonance after 6 h of exchange in D_2O, an N-H···O distance of ≤ 2.3 Å and an N-H···O angle of $\geq 120^{o}$ in more than 70% of the set of 20 structures calculated without these restraints. For each hydrogen bond, two distance restraints were introduced: $d_{HN-O} = 2.3$ Å and $d_{N-O} = 3.2$ Å. Φ backbone torsion angle restraints were added at this stage. The angles were estimated from $^{3}J_{NH-C\alpha H}$ using the Karplus curve (Pardi *et al.*, 1984) and checked within the set of 20 unrestrained structures. The final φ angle restraints were added with an uncertainty ± 40°. The summary of all the restraints used is in **Appendix 3A**.

Long-range orientational restraints

The ^{15}N relaxation data are shown in Fig.4.2. The ^{15}N T_1 and T_2 relaxation time constants in the secondary structure regions (data for 37 residues were available) have averages of 455 ± 24 ms and 121 ± 6 ms, respectively. This yields an estimate for the overall correlation time of 6.3 ns. The data shows only small fluctuations from the average values for the majority of residues, which confirms that the structure is well-ordered. High T_1 and T_2 values indicate motions on a time scale faster than an overall tumbling time of the molecule, such as in the flexible loops or terminal residues. Low T_2 values suggest motions on a μs to ms time scale. Most of the T_1/T_2 data fall into the region of the Lipari-Szabo model consistent with the overall rate of tumbling or faster motion (Lipari & Szabo, 1982). This again indicates a well-defined secondary

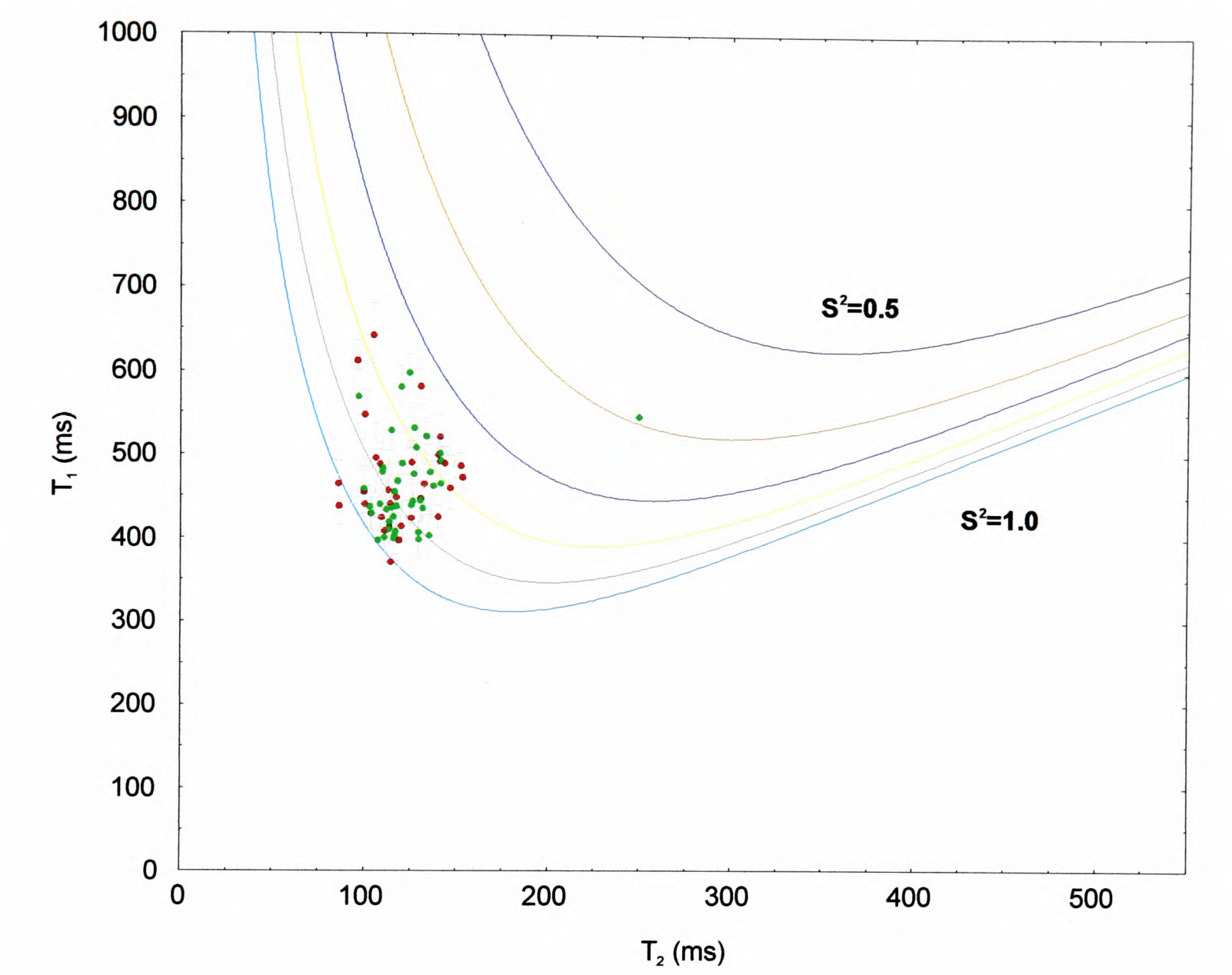


Fig.4.2. Backbone ^{15}N T_1 and T_2 data for the $^4F1^5F1$ module pair (kindly provided by J. Werner). Backbone ^{15}N relaxation time constants for $^4F1$ are indicated by circles. The continuous lines represent T_2vsT_1 values as a function of isotropic correlation time and the order parameter S^2, according to the Lipari-Szabo model. Curves with S^2 values 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 are shown.

structure with some relatively flexible loops. RDC data and its distribution are shown in Fig.4.3. The data were obtained for 62 residues, 40 of which are located in secondary structure regions. This set of RDCs was acquired using a protonated sample, hence the line broadening was observed in spectra, making some of the assignments ambiguous (Fig.4.4).

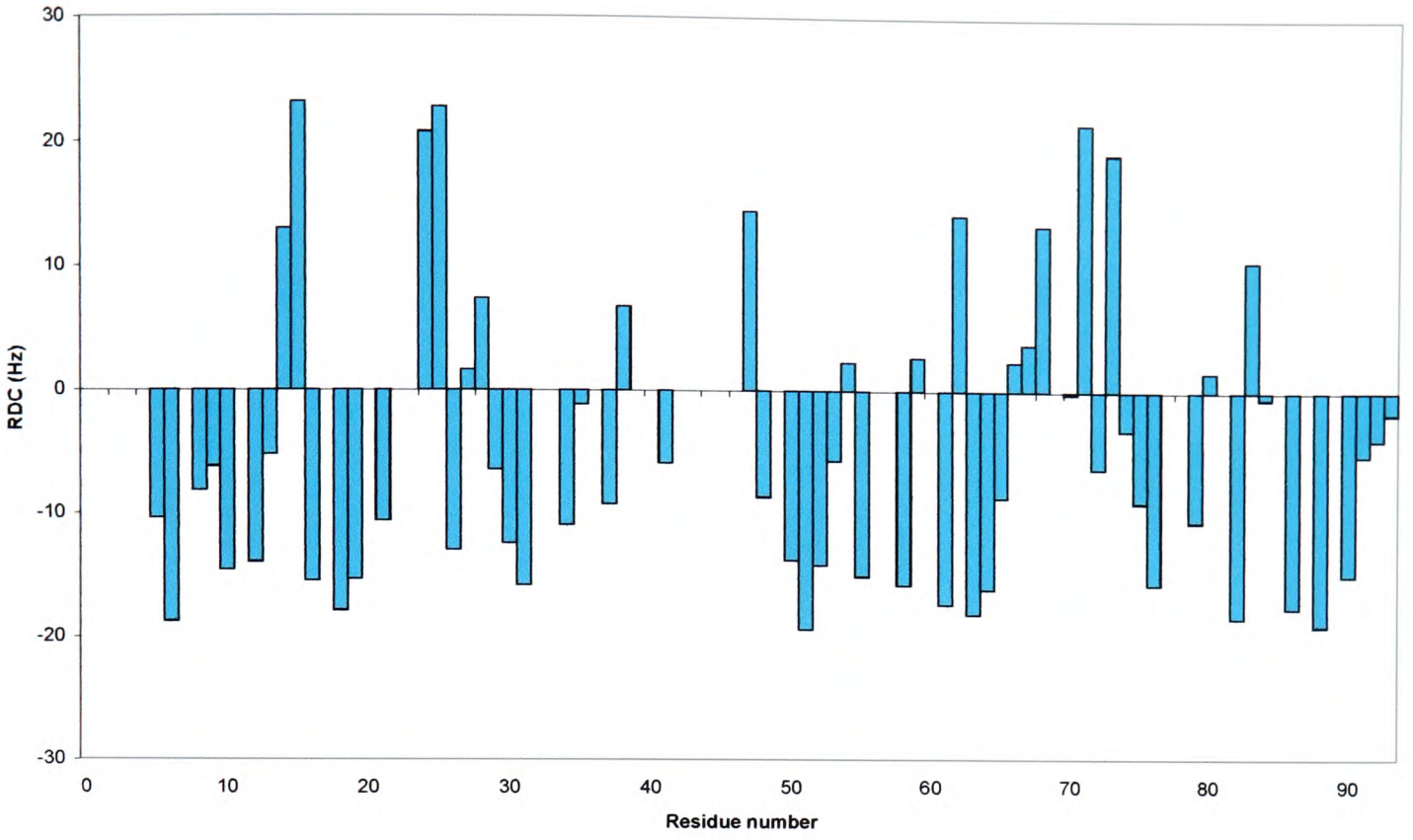
4.2. Structure calculations

It was found previously for structure calculations of a module pair (Wiles *et al.*, 1997), that extended high-temperature and cooling stages improved the quality of calculated structures, especially the convergence of the relative orientations of the modules. For ^{4}F1^{5}F1 the simulated annealing protocol and refinement protocol were optimised in a similar way (**Appendix 5A**).

Initial calculation protocols

A family of structures was calculated using a total of 1311 distance restraints, 10 hydrogen bond restraints (2 for each bond) and 21 φ backbone torsion angle restraints. Intraresidue NOEs between protons on adjacent carbon atoms were not included as restraints. The interproton distance restraints, all assigned unambiguously, included 441 intraresidue, 298 sequential (between adjacent residues), 115 medium-range (between residue *i* and residue $i \pm 4$) and 457 long-range restraints. Among them there were 49 intermodular restraints: 1 medium-range and 48 long-range, as well as 10 long-range restraints between the linker residues (R46 and R47) and the ^{5}F1 module residues. Fig.4.5 presents the secondary structure of the module pair. The

a.



b.

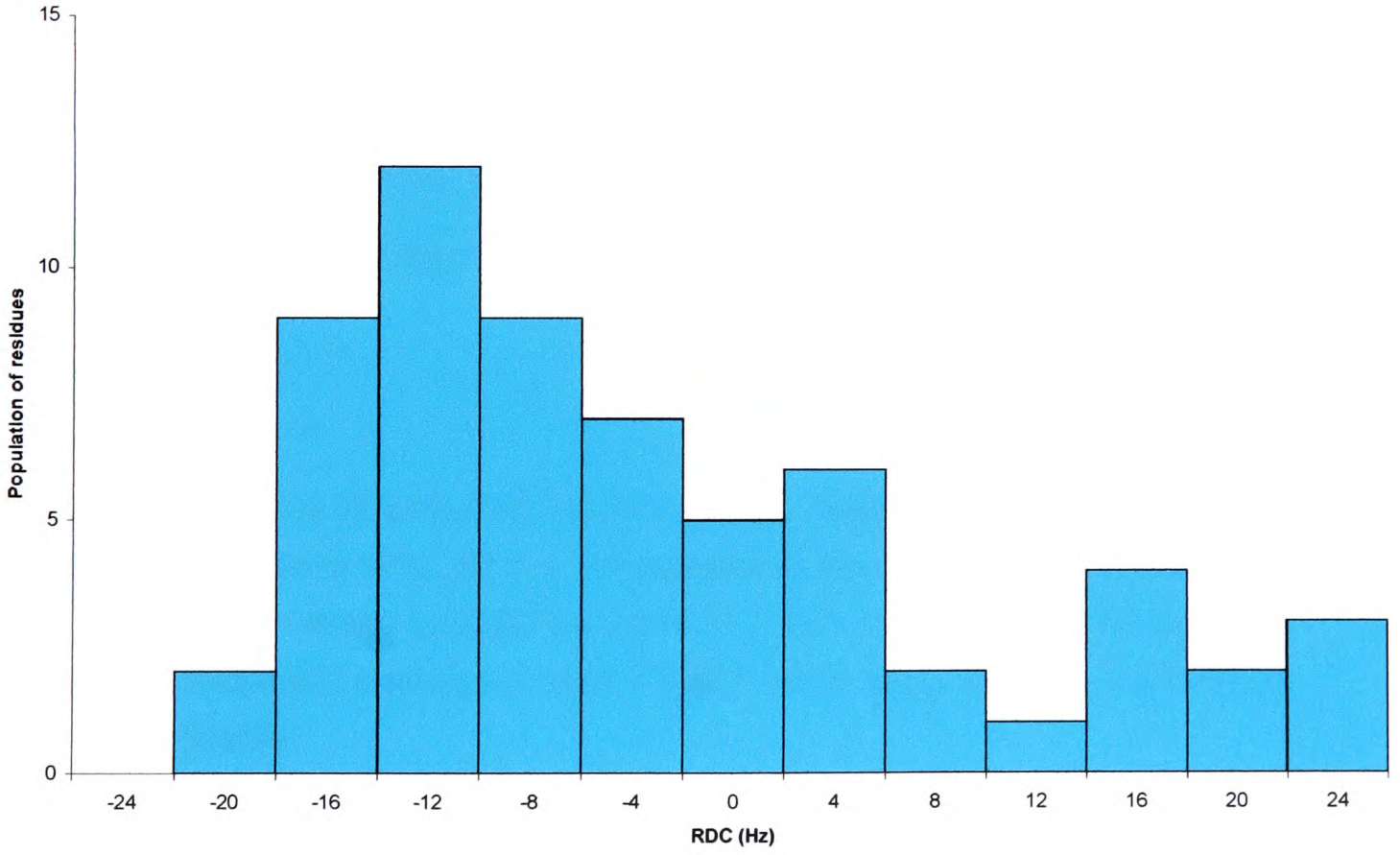


Fig.4.3. RDC data acquired for 0.5 mM ^{15}N-^{4}F1^{5}F1 in the bicelles medium.

a. RDC per residue. Gaps indicate residues for which no data was obtained.

b. RDC distribution - 'powder pattern'.

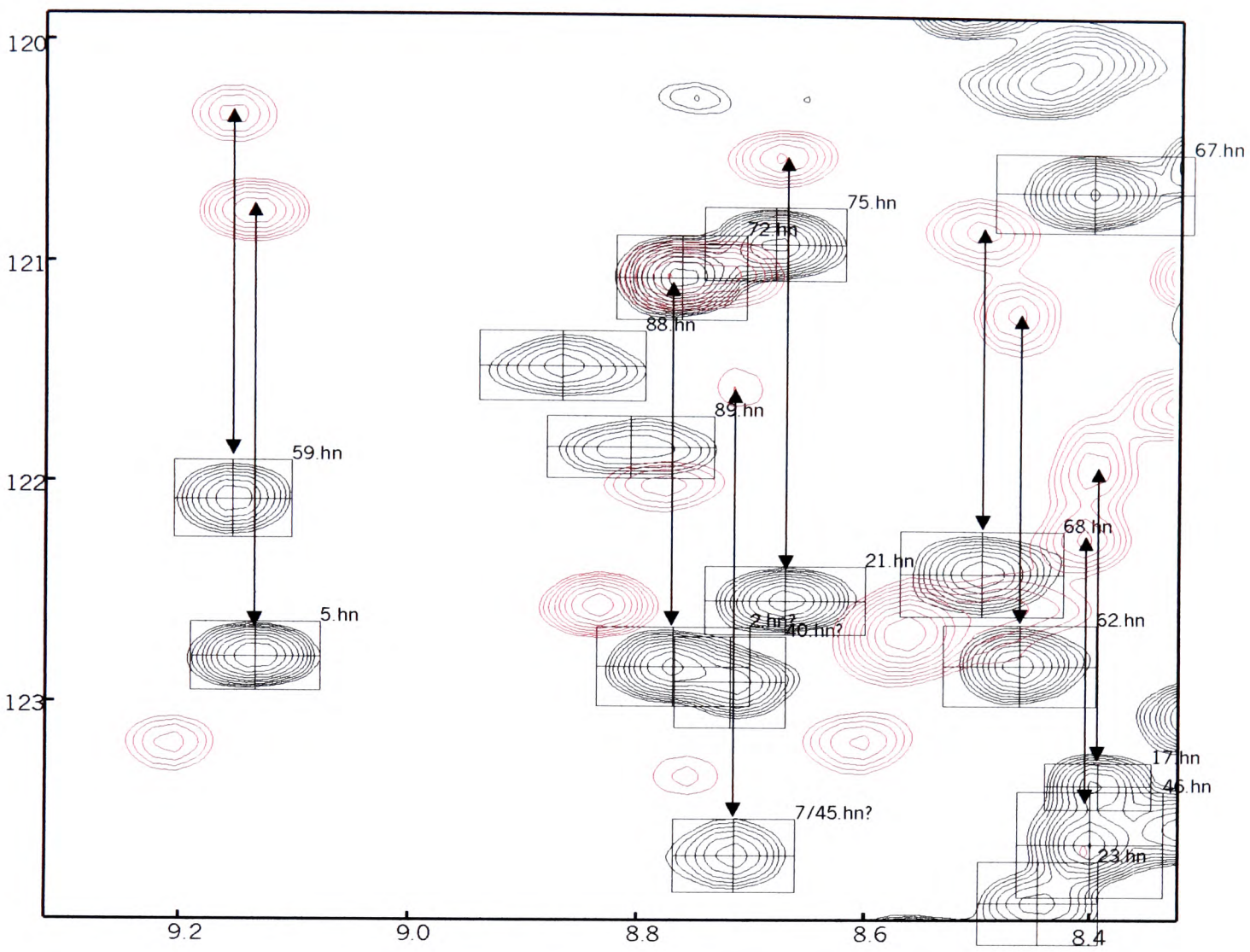


Fig.4.4. The overlay of narrow and broad component spectra of 0.5 mM ^{15}N-^{4}F1^{5}F1 in bicelles, performed at 37°C, pH 5.0. Cross-peaks in the broad component spectrum are labelled, the arrows indicate the splitting. It can bee seen that the signal in the broad component spectrum is much weaker, hence some splittings could not be measured directly.

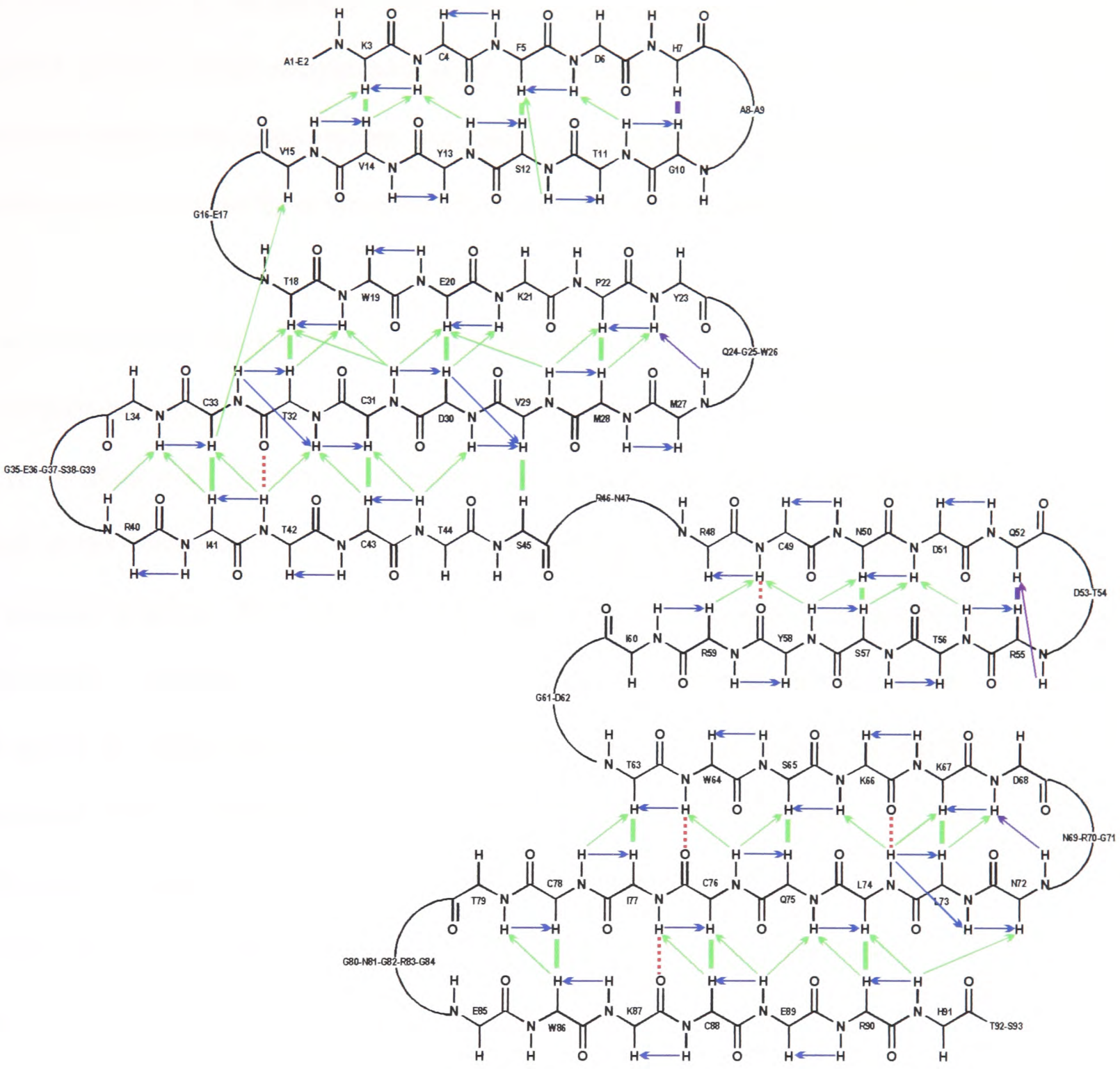


Fig.4.5. Secondary structure of the ^{4}F1^{5}F1 module pair. The backbone NOEs between protons in the ordered regions and hydrogen bonds used as input data for the structure calculations are indicated by solid arrows or lines and dotted lines respectively. Blue - sequential NOEs, purple - medium-range NOEs, green - long-range NOEs, red - hydrogen bonds.

most effective simulated annealing protocol for $^{4}F1^{5}F1$ included a 150 ps high temperature stage at 2000 K. The rmsd of 10 structures obtained using this method was 0.96 ± 0.31 Å, compared to 1.37 ± 0.57 Å for structures calculated with the standard protocol (high temperature stage of 100 ps). The overall energy level remained similar. No improvement was obtained by increasing the length of the cooling stage to two and three times the value from the standard protocol.

From a total of 100 calculated structures, an ensemble of 81 structures was accepted for refinement on the basis that they had no NOE violations > 0.5 Å and no dihedral angle violation > 2°. The choice of refinement protocol (236 ps cooling time) was based on the comparison of 47 structures refined using different cooling times with the average structure. The optimised protocol gave rmsd of 0.9 ± 0.29 Å, compared to 1.05 ± 0.38 Å for the original refinement (118 ps), with the same overall energies. The set of 50 refined structures (characterised by Table.4.2) was chosen for further refinement with an implementation of SANI/ISAC and DANI restraints. For this refinement, a different protocol was used, based on Hashimoto (2001). The cooling is divided into three stages, with differentiated weighting on NOE, RDC and DANI restraints. Distance restraints were divided into intramodular (constant weighting, k = 50) and intermodular (gradual increase of k from 1 to 50).

Selection of sets of long-range orientational restraints

In the implementation the long-range orientational restraints, one has to make sure that the motion of an included residue included reflects the overall tumbling of the molecule. The motion can be characterised by the order parameter S and the ^{1}H-^{15}N

NOE experiment. In this case the ^{1}H-^{15}N NOE measurement at the concentration for which the T_1/T_2 ratios were obtained did not have good enough signal-to-noise ratio. Hence, only residues from well-ordered regions (defined on a basis of the secondary structure – Fig.4.5) were used to estimate the diffusion parameters of the $^{4}F1^{5}F1$. T_1/T_2 ratios from these regions were analysed by Modelfree using 20 structures calculated and refined using NOE and torsion angle restraints. Residues that showed an unusually bad fit (high χ^2), namely F5 and S12, were excluded from further calculations. As a result, 35 out of 37 residues were used for DANI refinement (**Appendix 3A**).

Again, due to a lack of ^{1}H-^{15}N NOE data, only the unambiguously assigned residues from secondary structure regions were considered for the selection of RDCs. For further selection, sets of 20 structures were refined with an implementation of SANI restraints. After ranking the structures according to the overall energy, five of the lowest energy structures were used as models for a back-calculation of RDCs. Residues constantly violated or showing a bad fit (high χ^2), which might be due to the line broadening and therefore large measurement error, were removed from the usable set. This procedure was repeated until no SANI restraints were constantly violated. Finally, for both SANI and ISAC routines the same set of 22 out of 40 residual dipolar couplings was used (**Appendix 3A**).

Determination of the alignment tensor

The alignment tensor parameters A_a (i.e. $\frac{1}{2}A_{zz}$) and R were estimated on the basis of the 'powder pattern' and also modelled using the program Module (Dosset *et al.*,

2001). Modelling was carried out for both modules together as well as separately. Although no significant difference was observed in A_a and R values, the tensor orientation varied strongly between the modules indicating either insufficient data sampling or that the modules align independently. However, the presence of intermodular interface and the relaxation data discussed below, suggest that both modules form a single unit, behaving like one domain. Parameters obtained from the 'powder pattern' were further optimised by refining sets of 20 structures till reaching the stage when the minimal number of NOE violations coincided with a minimal SANI energy. The optimal A_a and R values were used in the structure refinement with implementation of SANI routine.

For the SANI routine the alignment tensor was estimated from the powder pattern and optimised by a grid search. The grid search was carried out from the A_a values of 13 - 16 and for the R values of 0.1 – 0.55. The values of $A_a = 14$ and $R = 0.4$, giving the lowest number of NOE and SANI violations did not result in an uniquely defined alignment tensor orientation, despite efforts to optimise the SANI force constant and use of a simulated annealing procedure.

In general, it appeared impossible to implement SANI restraints into the refinement in without increasing the rmsd of either individual modules or the intermodule orientation. Fig.4.6 shows that the orientation of N-H vectors in initial structures does not fit the experimental RDC data, which is a common case. Back-calculating the couplings for sets of structures before and after the RDC refinement, using Module,

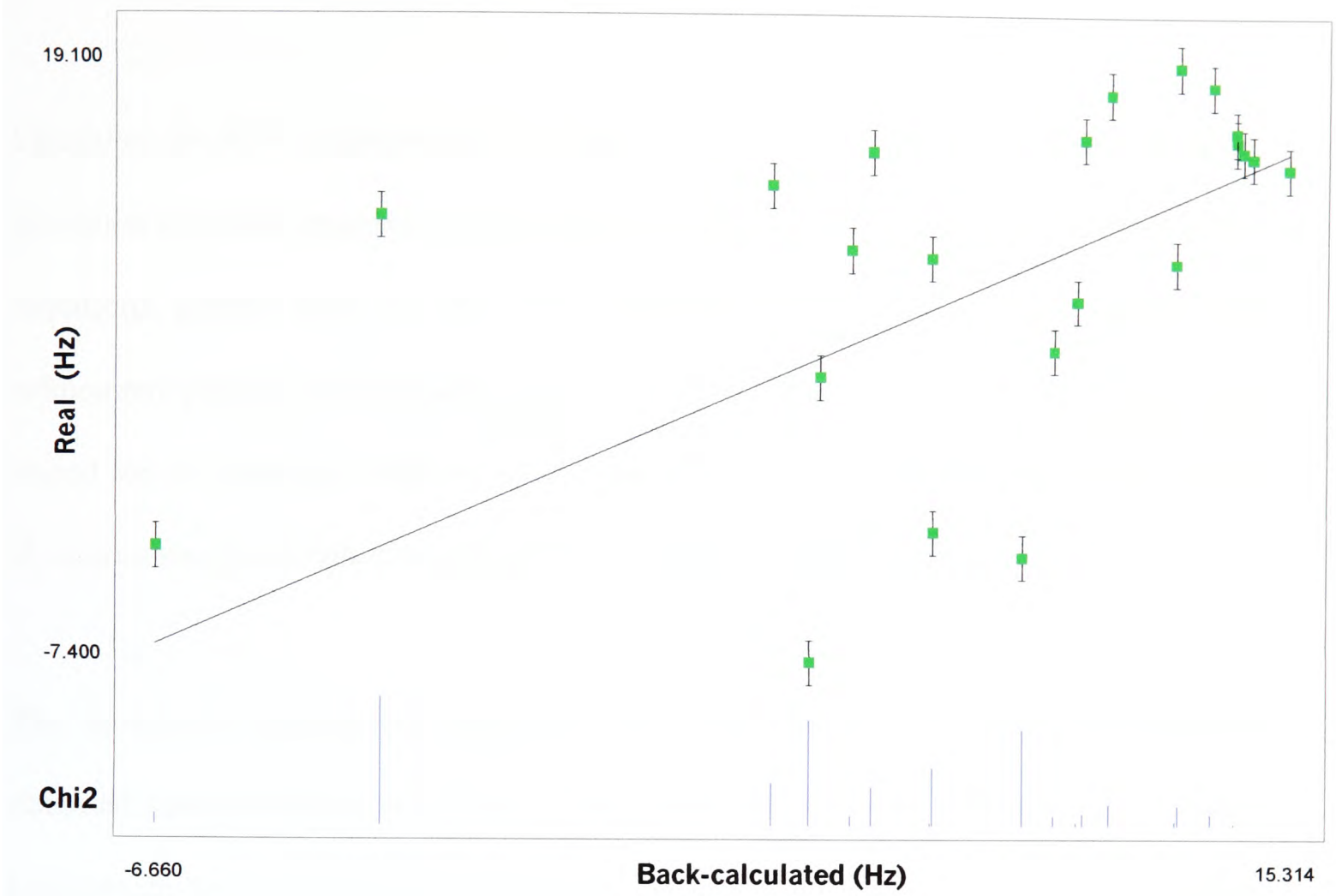


Fig.4.6. Correlation between the experimental and back-calculated RDC values estimated by Module on the lowest energy $^{4}F1^{5}F1$ structures calculated with no RDC restraints.

showed that the positions of N-H vectors were 'corrected' by using either SANI or ISAC routines. However SANI always resulted in an increased rmsd of individual modules and of their relative orientation. Modifications in the refinement protocol did not seem to have a significant effect on the final result.

Changing the RDC implementation routine to ISAC did not reduce the precision of the initial structure ensemble, unlike SANI. In the final structures there were no RDC violations greater than 0.5 Hz. The alignment tensor is determined during the refinement process in this subroutine. The tensor parameters from the output files varied for A_{zz} between values of 34 - 40 and R of 0.1 – 0.2, and the orientations of N-H vectors were well fitted to each of individual alignment tensors.

The rhombicity determined using the ISAC routine was very similar to estimates obtained using Module (R = 0.1 – 0.2). However A_{zz} from Module would range between 26 and 30, which is smaller than values determined using ISAC, but similar to the estimate from the 'powder pattern' and the grid search described above, used in the SANI refinement: A_{zz} = 28. However, the rhombicity determined for SANI disagreed completely: R = 0.4. The difficulty in obtaining a precise estimate of the alignment tensor can arise due to the difficulty of measuring large RDCs. In such cases the intensity of the broad component of the spectrum is often indistinguishable from the noise. Also in highly anisotropic molecules, such as ^{4}F1^{5}F1, the sampling of vectors almost parallel to A_{zz} is poor (Boyd *et al.*, 2001). Therefore using only the measured RDC values usually results in underestimation of the axial component of

the alignment tensor and is insufficient for a correct determination of the alignment tensor *a priori*.

Determination of diffusion tensor

It was shown previously that the relaxation data for the ^{4}F1^{5}F1 module pair are consistent with an axially symmetric model and the diffusion tensor was modelled with $D_{\parallel}/D_{\perp}$ ratio of 1.9 (Phan *et al.*, 1996). The $D_{\parallel}/D_{\perp}$ ratio from the fit of the ^{15}N relaxation data to previously calculated structures is 1.6 (J. Werner, pers. com.), indicating a cigar-like shape for the molecule.

Prior to DANI refinement of newly calculated structures, the diffusion tensor parameters τ_c and D_{ratio} were estimated using an ensemble of 20 structures determined without using the relaxation data. From this analysis the τ_c and D_{ratio} values were 6.2 ns and 1.2 respectively, and although lower than determined from previously calculated structures (Phan *et al.*, 1996, Marsden, 2000), they were considered reliable and used for DANI refinement. Neither the precision of the structures nor the T_1/T_2 fit seemed to be affected by this procedure, suggesting that the relaxation data agree well with the results from RDC-refinement.

4.3. Comparison with the previous calculations

The overlay of 47 structures refined using only NOE and torsion angle data (characterised by Table.4.1) shows well-defined positions of the core hydrophobic residues in both modules. All 10 β-strands are well-defined within the typical F1

module fold (Fig.4.7d). In each module a short double-stranded antiparallel β-sheet folds over the triple-stranded antiparallel β-sheet, enclosing a hydrophobic core. The major intermodule interface, that stabilises the relative orientation of the modules (Fig.4.7a&b&c), is formed between the C-D loop in ^{4}F1 and the A-B β-sheet in ^{5}F1 (mainly between W26 and R55). In addition, the linker residues are constrained by NOEs to the D-E loop of ^{5}F1 (mainly R46 and R83) - consistent with Marsden, 2000.

The previous structures of ^{4}F1^{5}F1 were determined at pH 4.5, which is not optimal for binding studies with the D3 peptide. D3 and the ^{4}F1^{5}F1/D3 complex tend to aggregate at pH < 5.0, which precludes the preparation of samples sufficiently concentrated for NMR experiments (J. Potts, pers. com.). Assignments and preferably the ensemble of structures at pH 5.0 were required for the direct comparison of the module pair in a free and a bound state. In addition, since the assignments for the structure determination were obtained in 1992 (Williams *et al.*, 1994), it seemed likely that use of higher magnetic field and improved probes would improve the quality of input data. Also, previous recalculation of structures, using a new force-field (PARALLHDG, the same as used here), and T_1/T_2 data in the structure refinement had already improved the quality of structures and the definition of intermodule orientation (Marsden, 2000).

The individual modules in newly calculated structures do not differ much from the original calculations (rmsd values are within the range for the family of structures), however the intermodule orientation varies between the average structures (Table.4.1 – the rmsd for superimposing the backbone of residues 3-91 is higher than estimated

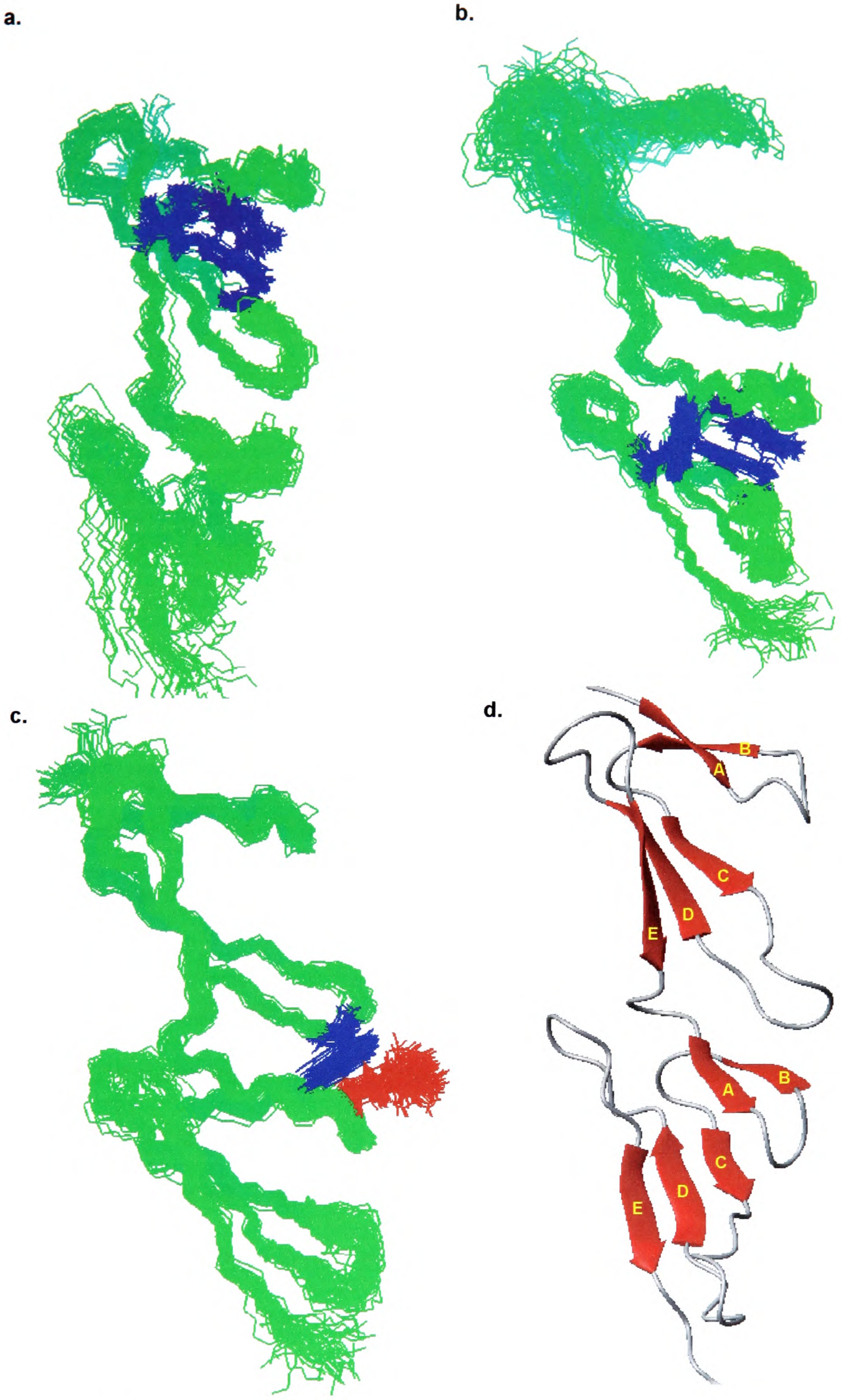


Fig.4.7. Overlays of the 47 final ^{4}F1^{5}F1 structures. **a.** superposition on backbone atoms of ^{4}F1 (residues 3-45), **b.** superposition on backbone atoms of ^{5}F1 (residues 48-91); the sidechains of three conserved hydrophobic core residues are shown. **c.** superposition on backbone atoms of ^{4}F1^{5}F1 (residues 3-91); sidechains of two residues involved in the intermodule interactions (W26 and R55) are shown **d.** Average structure calculated from 47 structures - β-strands are labelled.

for a family of 25 present structures). Further analysis was carried out with the set of structures calculated by Marsden (2000), as they showed an improvement in comparison with the original ones.

Table.4.1. Comparison of average structure from Williams, 1992 and present one.

superposition of backbone atoms	rmsd (Å)
residues 3-45	1.21
residues 48-91	0.78
residues 3-91	1.62

The final structures calculated here have higher precision (Table.4.2) than previous calculations. The main difference is that the intermodule orientation is much better defined, which results in lower overall rmsd when overlaying the whole molecule. This improvement has been achieved both by changes in input restraints and optimisation of calculation protocols, described above.

In the previous work mixing times of 150 ms and 200 ms were used for NOESY experiments (Williams *et al.*, 1993). Many NOEs used in the original structure calculations did not appear in the 100 ms spectra in the present work and these are listed in the **Appendix 3B**. The NOEs inconsistent with the new structure, but which appear in the present work with an increase in mixing time (indicated by * in the **Appendix 3B**), could be the result of spin diffusion. Some additional restraints were also obtained from the spectra at pH 5.0 and some previously misassigned restraints were corrected. These new restraints were included only after checking they were consistent with preliminary structures (**Appendix 3C**).

Table 4.2. Comparison of three sets of calculated structures of $^{4}F1^{5}F1$ module pair (the minimised average structures are shown in Fig.4.8)

	Marsden, 2000 (49 structures)	New calculation (25 structures)
rmsd (±sd) from the experimental restraints		
all NOE restraints (Å)	0.027 ± 0.001	0.006 ± 0.002
dihedral angle (deg)	0.059 ± 0.039	0.018 ± 0.024
rmsd (±sd) from the idealized covalent geometry		
bond length (Å)	0.0025 ± 0.0001	0.0007 ± 0.0001
angles (deg)	0.368 ± 0.0236	0.220 ± 0.0035
rmsd (±sd) of Cartesian coordinates (Å)[a]		
backbone atoms: residues 3-91	1.1 ± 0.3	0.9 ± 0.3
backbone atoms: residues 3-45	0.7 ± 0.2	0.5 ± 0.3
backbone atoms: residues 48-91	0.6 ± 0.2	0.5 ± 0.3
all heavy atoms: residues 3-91	1.6 ± 0.2	1.2 ± 0.4
all heavy atoms: residues 3-45	1.2 ± 0.2	0.8 ± 0.4
all heavy atoms: residues 48-91	1.4 ± 0.2	1.0 ± 0.5

[a] rmsd for the new structures was calculated by comparison of each structure with the average structure

The secondary structure in both calculations is very similar. However, there is a difference between the orientation of side-chains of residues from the hydrophobic cores. Some restraints involving aromatic ring protons of W19 and W64 were misassigned and have been corrected (**Appendix 3B, 3C**). Also four weak restraints between W26 and residues 52-57, together with one weak restraint between R46 and R83, have been identified only in NOESY spectra acquired with mixing times larger than 100 ms and therefore were not included in the present structure calculations. The only significant difference in core residues arising from different assignments is in the position of Y13 in relation to two other core residues in $^{4}F1$. The previous misassignments consistently resulted in violated NOE restraints during previous

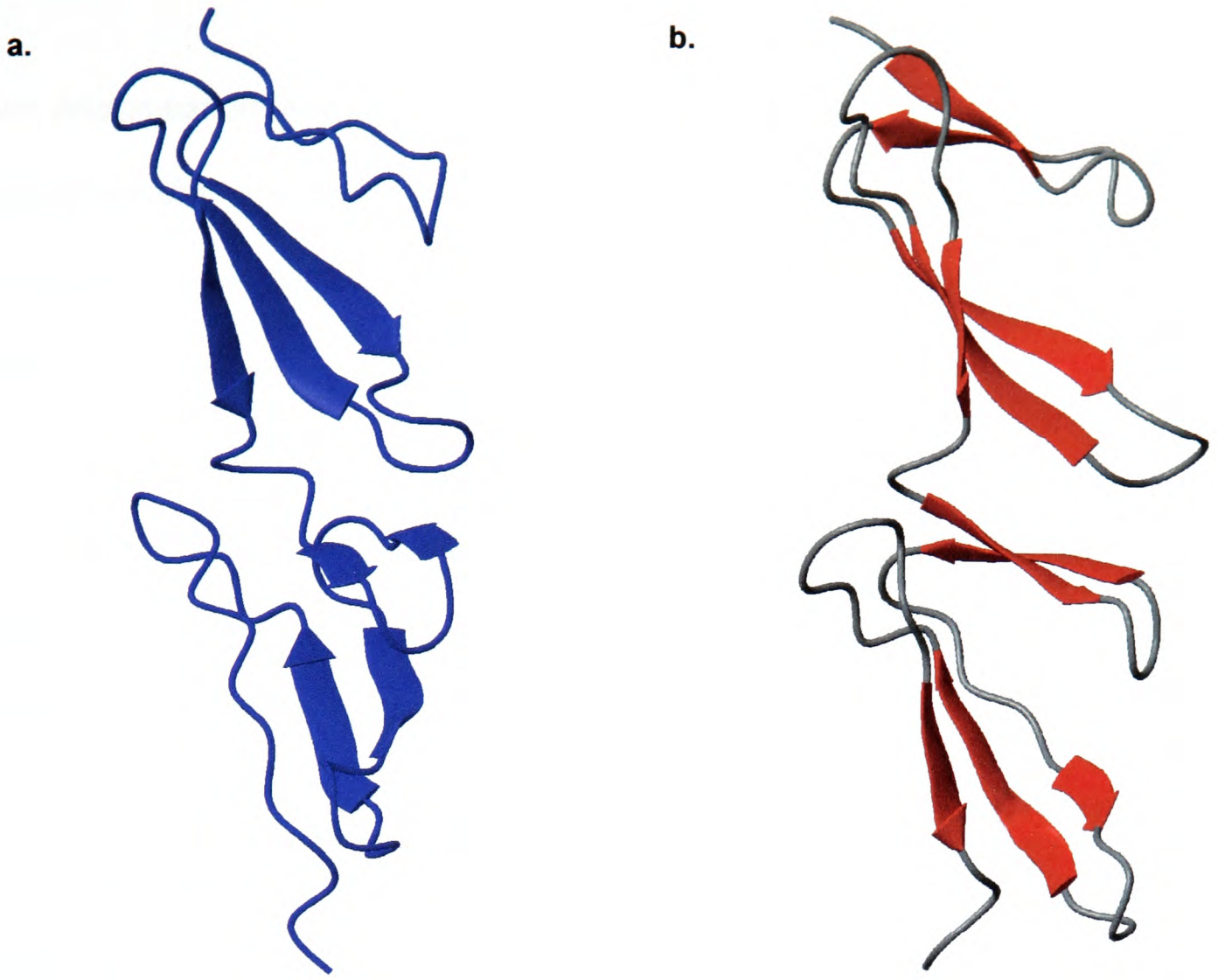


Fig.4.8. The comparison of the minimised average structures.

a. Williams, 1992

b. Marsden, 2000 (the lowest energy structure after DANI refinement)

c. Present calculations with ISAC/DANI implementation (stereoview)

structure determination (Marsden, 2000). The comparison of structures from all three calculations are shown in Fig.4.8. The present structure exhibits very well defined β-sheets already after NOE/CDIH refinement, which is not affected by the implementation of long-range orientational restraints (Fig.4.8c).

Some of the recorded T_1/T_2 data points appeared inconsistent with the structures calculated on a basis of NOE- and RDC-derived restraints. This may indicate that additional, faster motions occur. To observe their presence, molecular dynamics (MD) simulations on a 5 ns timescale were carried out for ^{4}F1^{5}F1 structures (Williams *et al.*, 1994) in water (Marsden, 2000). During the simulations several fast-motion events were observed. The transient unfolding occurred in the A-B and D-E β-sheets of ^{4}F1 and ^{5}F1 respectively. Probably in physiological conditions both these regions would be stabilised by adjacent modules. In addition to the transient unfolding, the opening angles between A-B and C-D-E β-sheets (Fig.4.9a) appeared to change on a ps timescale. Also the sheer angles (Fig.4.9b) between these sheets varied up to 30°. Each atypical conformation usually returned to the average state consistent with calculated structures within 100-200 ps (Marsden, 2000).

On the timescale of the MD simulations both modules were shown to behave as independent bodies with the linker residues in the centre of changes of the orientation. The intermodule orientation changes can be described as hinge-like (changes in a tilt angle, Fig.4.9c) with the linker and interface itself acting as the pivot points, and shear-like (changes of a twist angle, Fig.4.9d) (Gerstein *et al.*, 1994) when the interface reorganises slightly without significantly increasing the distance between

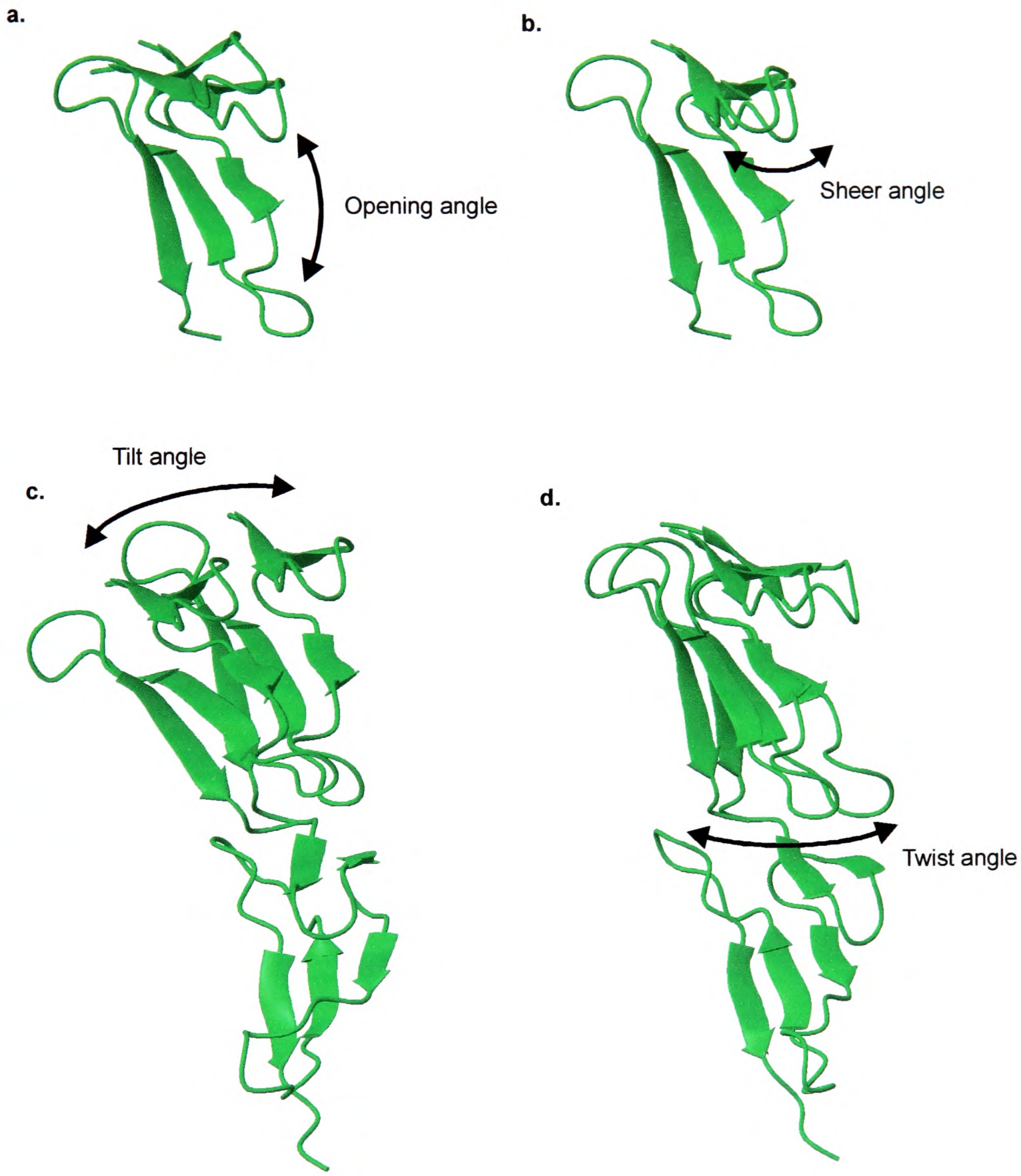


Fig.4.9. Schematic representation of possible conformational changes in β-sheet orientation in ^{4}F1 (**a**&**b**) and intermodule orientation in ^{4}F1^{5}F1 (**c**&**d**) resulting in alteration of respective angles.

stabilising residues (W26, R46, R48, N50, R55, S57 and R83). Since the intermodule NOE restraints (Williams, 1992) are reproduced by the simulations, it can be concluded that the interface is stable thorough the simulations (Marsden, 2000).

The D_{ratio} estimated from the relaxation data and calculated structures is significantly lower than the values consistent with structure-based models, which indicates that the $^{4}F1^{5}F1$ is exploring far wider ranges of tilt/twist space than the structures calculated from the experimental data suggest. Alternative intermodule orientations might then lead to a more compact structure i.e. a less extended overall shape (larger tilt angles - Marsden, 2000).

The observed correlation between changes in module conformation induced by changes in the other module is small (Marsden, 2000). This suggests that fast internal motions on ps timescale can still be treated independently. The events measured by the relaxation experiments occur on ns scale. Therefore from the motions listed above mainly averages would be recorded.

4.5. Summary

A set of 50 NOE/CDIH-refined structures was refined either with an implementation of ISAC or ISAC/DANI restraints. Both refinements resulted in a very good fit of the experimental data to the existing structures. 25 of the lowest energy structures were chosen for further analysis, which is summarised in Table.4.3.

Table 4.3. Comparison of three sets of 25 calculated structures of $^{4}F1^{5}F1$ module pair

	NOE/CDIH restraints	+ ISAC restraints	+ ISAC/DANI restraints
rmsd (±sd) from the experimental restraints			
NOE (Å)	0.007 ± 0.001	0.005 ± 0.001	0.006 ± 0.002
hydrogen bonds (Å)	0.0001 ± 0.0005	0.001 ± 0.0004	0
CDIH (deg)	0.020 ± 0.037	0.017 ± 0.025	0.018 ± 0.024
RDC (Hz)	na	0.066 ± 0.030	0.240 ± 0.007
T_1/T_2 fit (χ^2 p/residue)	1.23	1.18	1.18
rmsd (±sd) from the idealized covalent geometry			
bond length (Å)	0.0008 ± 0.0001	0.0006 ± 0.0001	0.0007 ± 0.0001
angles (deg)	0.240 ± 0.005	0.220 ± 0.003	0.220 ± 0.0035
impropers (deg)	0.380 ± 0.020	0.390 ± 0.018	0.390 ± 0.016
No of violations			
NOE > 0.2 Å	0	0	0
CDIH > 2 deg	0	0	0
RDC > 0.5 Hz	na	0	0
PROCHECK statistics			
allowed	94.7%	94.7%	93.6%
generous	3.2%	3.2%	3.2%
disallowed	2.1%	2.1%	3.2%
rmsd (±sd) of Cartesian coordinates (Å)[a]			
backbone sec. structure atoms: $^{4}F1^{5}F1$[b]	0.7 ± 0.2	0.7 ± 0.2	0.7 ± 0.2
backbone sec. structure atoms: $^{4}F1$[b]	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1
backbone sec. structure atoms: $^{5}F1$[b]	0.3 ± 0.1	0.4 ± 0.1	0.4 ± 0.1
backbone atoms: residues 3-91	0.9 ± 0.3	0.9 ± 0.3	0.9 ± 0.3
backbone atoms: residues 3-45	0.5 ± 0.3	0.5 ± 0.3	0.5 ± 0.3
backbone atoms: residues 48-91	0.5 ± 0.3	0.5 ± 0.3	0.5 ± 0.3
all heavy atoms: residues 3-91	1.2 ± 0.5	1.2 ± 0.5	1.2 ± 0.4
all heavy atoms: residues 3-45	0.8 ± 0.4	0.8 ± 0.4	0.8 ± 0.4
all heavy atoms: residues 48-91	1.0 ± 0.5	1.0 ± 0.5	1.0 ± 0.5

[a]rmsd for the new structures was calculated by comparison of each of 25 structures with the average structure

[b]secondary structure residues were used: from $^{4}F1$: 3-7, 10-15, 18-23, 27-33, 41-45, and from $^{5}F1$: 48-52, 55-60, 63-68, 72-78, 86-91.

The use of PARALLHDG force-field resulted as previously (Marsden, 2000), in good quality structures, exhibiting good geometry and agreement with the experimental data (the final structures are presented in Fig.4.10). Although the use of long-range orientational restraints did not further improve the convergence, these restraints were

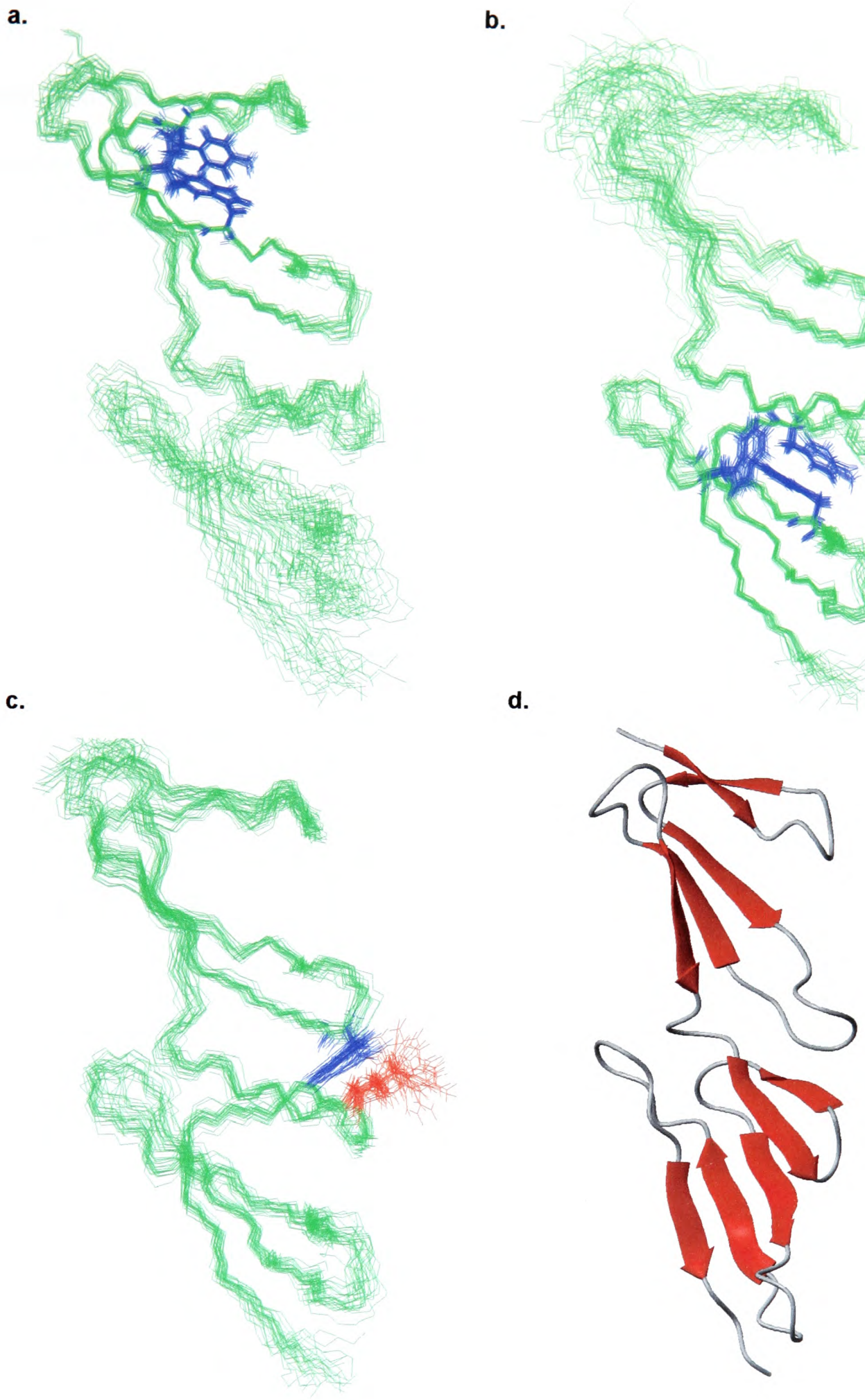


Fig.4.10. Overlays of the 25 final ^{4}F1^{5}F1 structures after tenso/DANI refinement. **a.** superposition on backbone atoms of ^{4}F1 (residues 3-45), **b**. superposition on backbone atoms of ^{5}F1 (residues 48-91); the side-chains of three conserved hydrophobic core residues are shown. **c.** superposition on backbone atoms of ^{4}F1^{5}F1 (residues 3-91); side-chains of two residues involved in the intermodule interactions (W26 and R55) are shown. **d.** average structure calculated from 25 structures.

consistent with the other experimental data. The intermodular orientation is well-defined due to strong side-chain interactions between the modules and between the linker and ^{5}F1. The intermodule orientation is much better defined than in the previous cases, which allows for more precise comparison of the unbound ^{4}F1^{5}F1 structure with its structure in complex with bacterial peptides.

5. THREE-DIMENSIONAL STRUCTURE OF THE ^{4}F1^{5}F1 MODULE PAIR BOUND TO THE D3 REPEAT OF FNBPA OF *S. aureus*

5.1. Experimental data

Assignment of ^{4}F1^{5}F1 bound to D3

TOCSY and NOESY spectra of ^{4}F1^{5}F1 in the complex with D3 were acquired at pH 5.0 and the ^{4}F1^{5}F1 resonances were assigned on the basis of the proton chemical shifts for the unbound module pair, with the help of backbone proton assignment data for the bound ^{4}F1^{5}F1 module pair from Penkett, 1997 (**Appendix 2A**). The linewidths in the spectra were generally broader than for the unbound module pair, which increased the overlap and, as a result, the ambiguity of assignments. The intermodule NOEs between residues W26 and R55 were still observed, but there were only two weak NOEs between the linker and the D-E loop of ^{5}F1 (six NOE restraints in the unbound form, one of ~3.5 Å), and therefore a strong interaction between these regions, that stabilises the intermodule orientation in the unbound state, does not appear to be present in the complex.

Assignment of D3

The unbound D3 peptide was assigned at pH 5.0 (**Appendix 2B**). The chemical shifts indicate a random coil conformation, which is consistent with Penkett *et al.*, 1998. In

the complex, the resonances of the first fourteen D3 residues were assigned on the basis of the chemical shifts for the unbound form (Fig.5.1.). Since the chemical shifts are identical to those of an unbound D3, these residues do not appear to be involved in binding to $^{4}F1^{5}F1$. This is also consistent with the previous data (Penkett *et al.*, 2000).

To perform ligand-selective experiments, a fully deuterated ^{15}N-labelled $^{4}F1^{5}F1$ module pair was expressed and purified. The experiments were carried out mainly with fully bound D3 (0.5 mM unlabelled D3 and 1.2 mM $^{2}H^{15}N$-$^{4}F1^{5}F1$, pH 5.0). In this situation only ^{1}H belonging to the D3 peptide and the exchangeable $^{1}H^{N}$ from the $^{4}F1^{5}F1$ should be visible.

Unfortunately, the assignment of the bound region of D3 is difficult due to a drastic drop in the intensity of cross-peaks arising from protons interacting with the module pair. The 37°C TOCSY fingerprint region shows only H^{N}-H^{α} cross-peaks of the unbound D3 residues (Fig.5.2a). NOESY spectra also show very weak or no NOE transfer between protons (Fig.5.2b). Performing NMR experiments at 10°C and 40°C to alter the proton solvent-exchange rate and the exchange rate of the complex did not improve the quality of the spectra. As a protein:peptide ratio of 1:2 is required for the saturation of the module pair, the problem might be caused by chemical exchange between the bound and free states of D3. A less likely explanations might be that either D3 binds in several conformations or it cross-links forming larger aggregates. In conclusion, the signal from the D3 residues remained undetectable, despite the presence of NOEs originating from protons belonging to the bound $^{4}F1^{5}F1$ module pair.

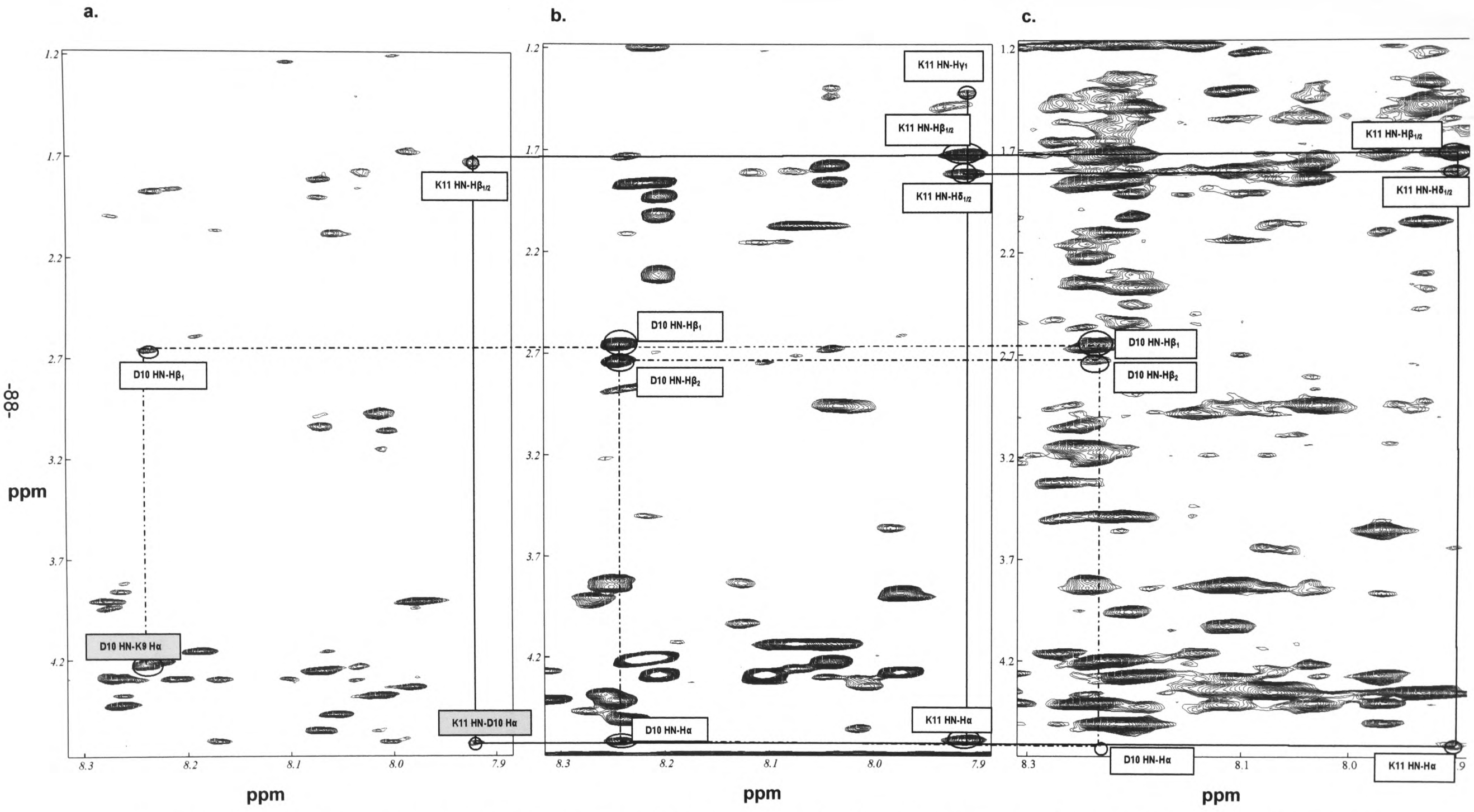


Fig.5.1. An example of assignments of D3 in complex with $^{4}F1^{5}F1$. The Asp10 and Lys11 residues do not bind and the positions of their proton chemical shifts remain unchanged. The fingerprint regions of the following spectra are shown:
a. NOESY in water of the unbound D3
b. TOCSY in water of the $^{4}F1^{5}F1$/D3 complex
c. NOESY in water of the $^{4}F1^{5}F1$/D3 complex. Sequential NOEs are indicated in grey

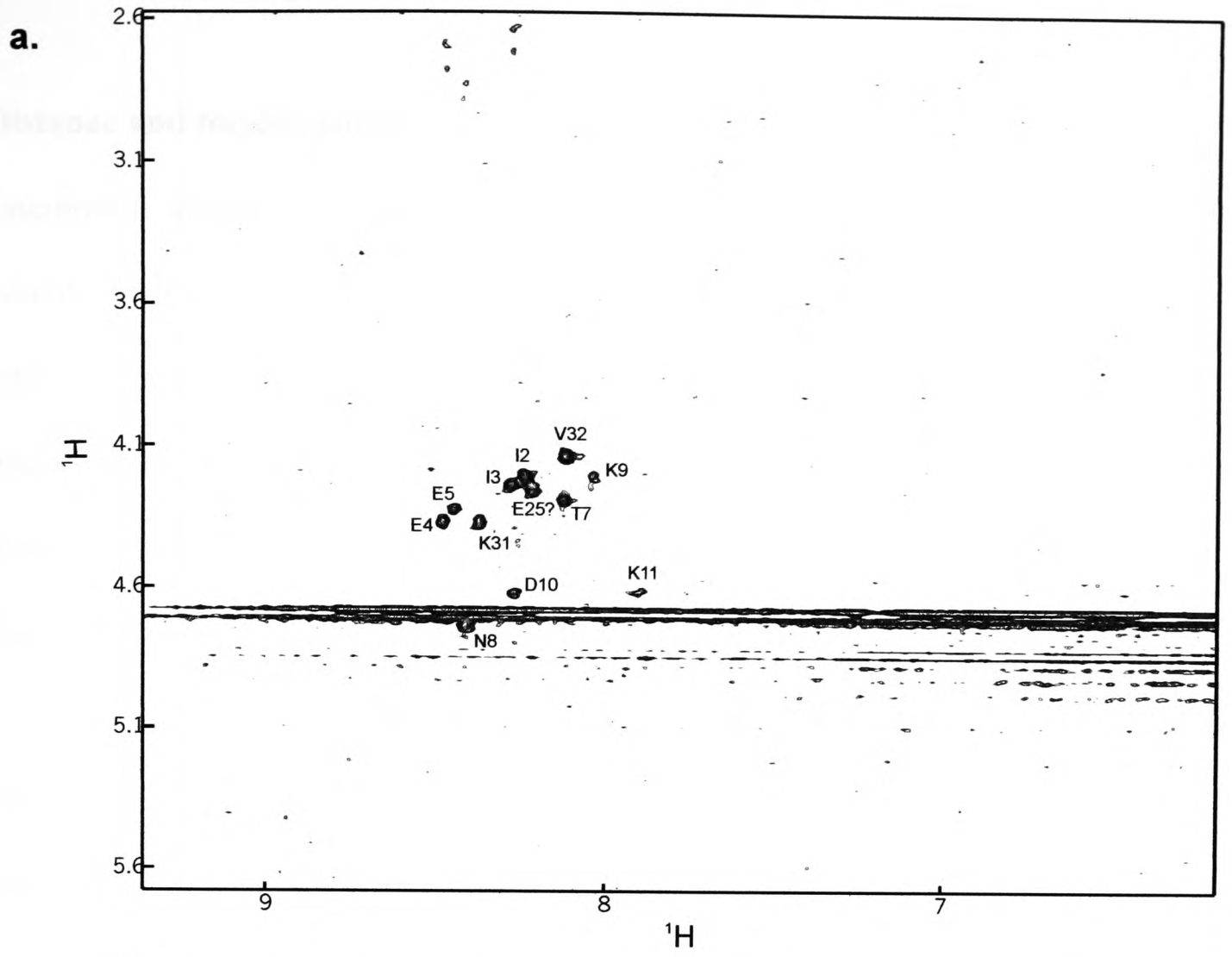


Fig.5.2. 2D spectra of $^2H^{15}N$-$^4F1^5F1$/ unlabelled D3 acquired at 37°C. **a.** TOCSY (46 ms mixing time), the assignments of H^N-H^α cross-peaks are shown, **b.** NOESY (150 ms mixing time). The only visible cross-peaks belong to the unbound part of D3, no module pair-peptide NOEs could be identified.

Distance and torsion angle restraints

Interproton distances, dihedral φ angle and hydrogen bond restraints obtained for bound ^{4}F1^{5}F1 were used in structure calculations. For the distance restraints, intensities were obtained from the NOESY spectra in D_2O and H_2O (150 ms mixing time). The intensities were calibrated using known interproton distances in the aromatic ring of Trp residues, as well as interproton distances in regions of well-defined secondary structure. The peaks were grouped into three main classes according to their intensity: strong (interproton distance ≤ 2.8 Å), medium (≤ 3.5 Å) and weak (≤ 5.0 Å). Calibration and creation of restraints tables were carried out using PERL scripts kindly provided by K. Downing. Several of the lowest NOEs were put into an additional (5.5 Å) class. Ambiguous restraints were checked after refinement – the shorter distance was treated as unambiguous, while the longer distance was removed. In the case of identical distances for both assignments, they were kept as separate unambiguous restraints. As the PROLSQ force field is characterized by a slightly larger atom radius, the tables created for PARALLHDG have been recalibrated i.e. restraints on the lower boundary of each class have been reassigned to a weaker category. After initial structure calculations, hydrogen bond restraints and φ backbone torsion angle restraints were included as described in **Section 4.1**. The summary of all the restraints used is in the **Appendix 4**.

Long-range orientational restraints

The ^{15}N relaxation data are shown in Fig.5.3. The ^{15}N T_1 and T_2 relaxation time constants in the secondary structure regions (data for 48 residues were available) have averages of 494 ± 113 ms and 107 ± 9 ms, respectively. This yields an estimate for

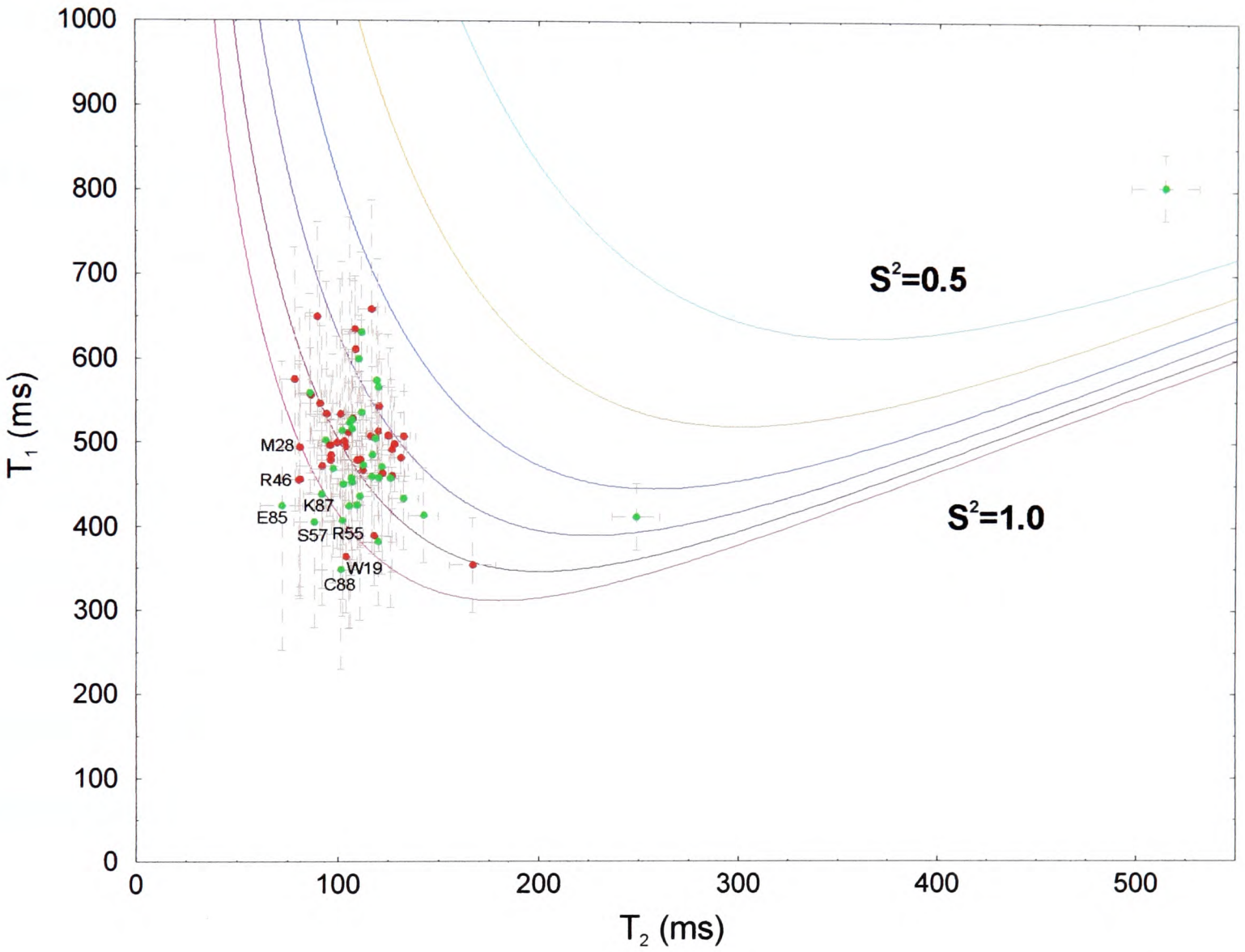


Fig.5.3. Backbone ^{15}N T_1 and T_2 data for $^4F1^5F1$ in complex with D3. ^{15}N relaxation time constants for $^4F1^5F1$ are indicated by circles. The continuous lines represent T_2vsT_1 values as a function of isotropic correlation time and the order parameter S^2, according to the Lipari-Szabo model. Curves with S^2 values 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 are shown. Residues with chemical exchange component are indicated.

the overall correlation time of 7.5 ns. The data show small fluctuations from the average values for the majority of residues, which confirms that the structure is well-ordered, similar to the unbound state. Most of the T_1/T_2 data fall into the region of the Lipari-Szabo model consistent with the overall rate of tumbling or faster motion (Lipari & Szabo, 1982). This again indicates a well-defined secondary structure with some relatively flexible loops.

Two separate sets of RDC data were acquired for a protonated and a uniformly deuterated module pair in the complex with a protonated D3 peptide. The signal-to-noise ratio from the deuterated sample is much better and lines narrower, which resulted in better quality spectra and more RDCs could be measured (Fig.5.4). The RDC data and its distribution are shown in Fig.5.5 ($^{2}H^{15}N$-$^{4}F1^{5}F1$ - 68 residues, 44 in secondary structure regions) and Fig.5.6 (^{15}N-$^{4}F1^{5}F1$ - 48 residues, 32 in secondary structure regions).

5.2. Structure calculations

Initial calculation protocols

The simulated annealing and refinement protocols utilizing the PROLSQ force-field were used with heating and cooling times identical to the unbound $^{4}F1^{5}F1$ structure calculations (**Appendix 5B**). Changes in the number of heating/cooling steps did not result in any significant improvement of the rmsd of the structure ensemble.

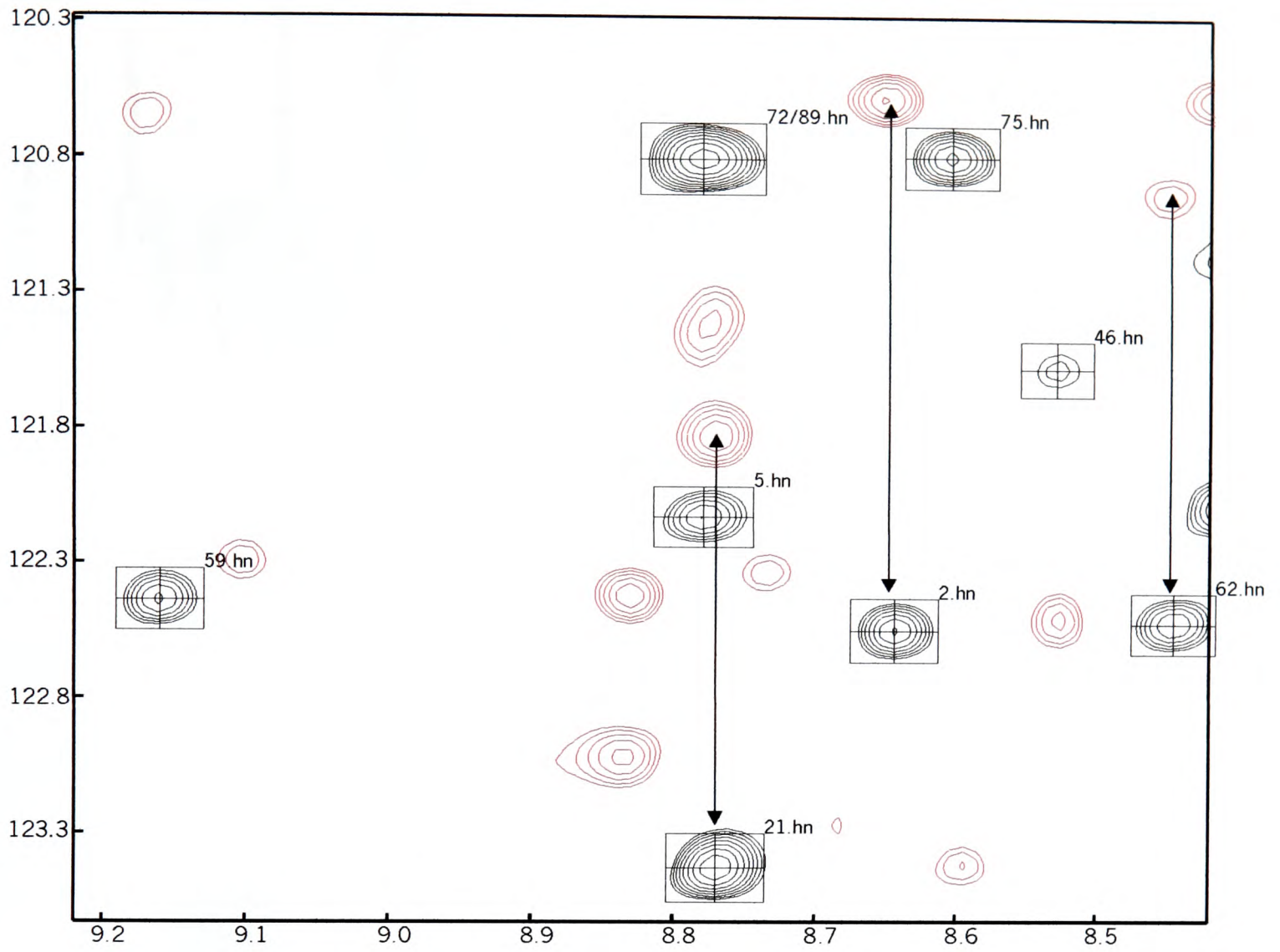
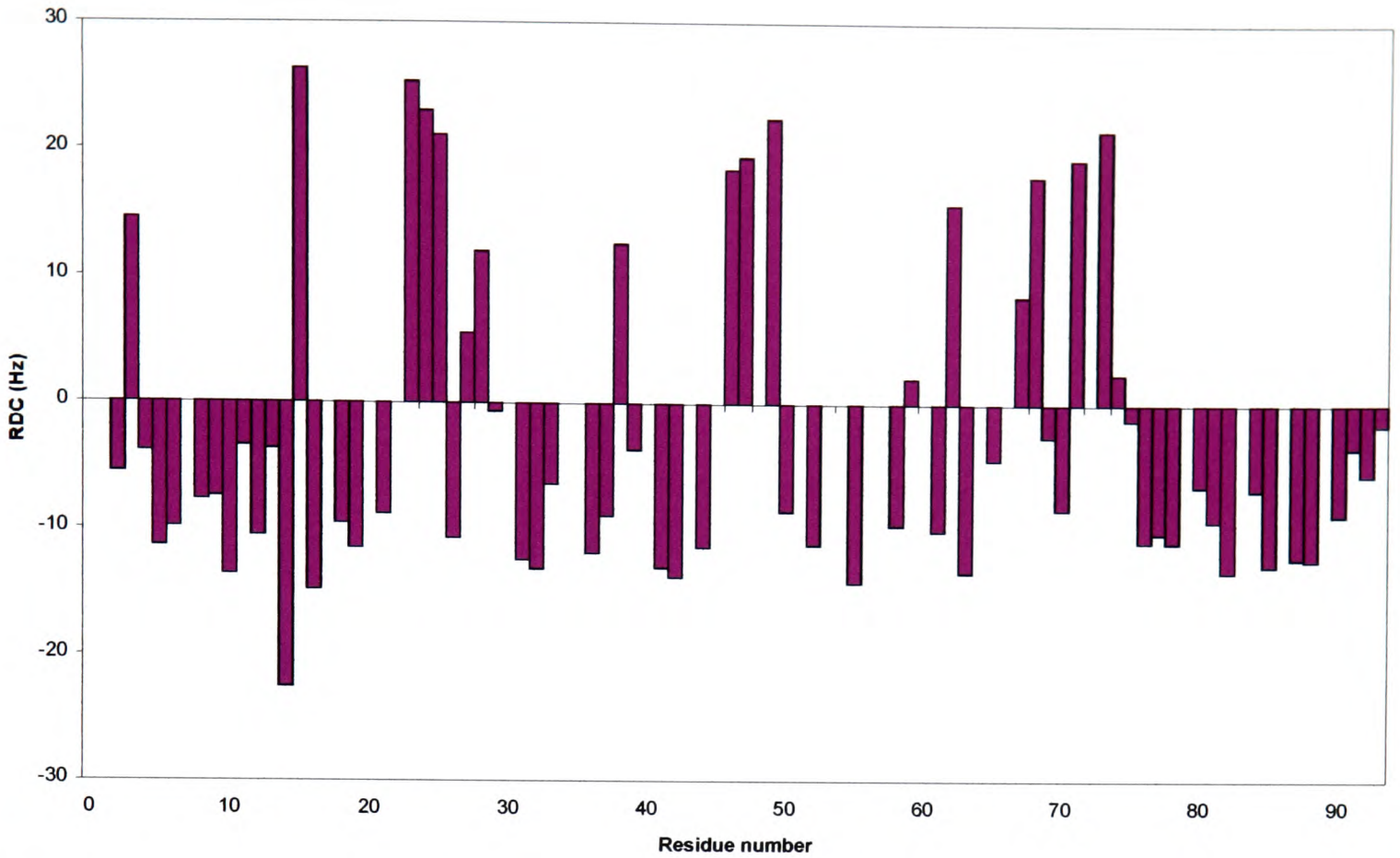


Fig.5.4. The overlay of narrow and broad component spectra of 0.5 mM $^{2}H^{15}N$-$^{4}F1^{5}F1$/ 1.2 mM D3 in bicelles, performed at 37°C, pH 5.0. Cross-peaks in the broad component spectrum are labelled, the arrows indicate the splitting. It can bee seen that the signal in the broad component spectrum is much weaker, hence some splittings could not be measured directly. Lines in the spectra recorded with deuterated $^{4}F1^{5}F1$ are much narrower than in the case of fully protonated protein (see Fig.4.4), allowing for more accurate assignments.

a.



b.

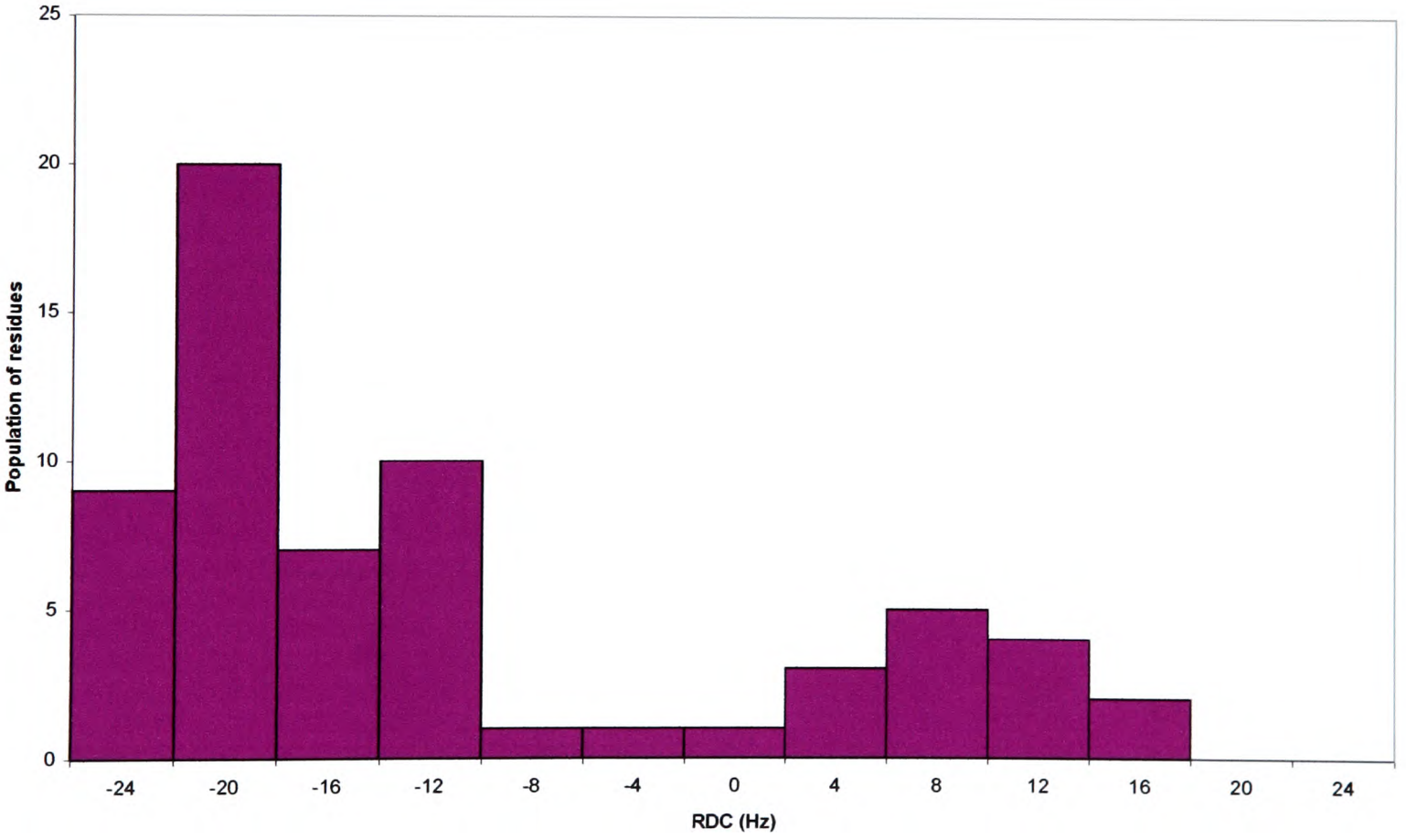
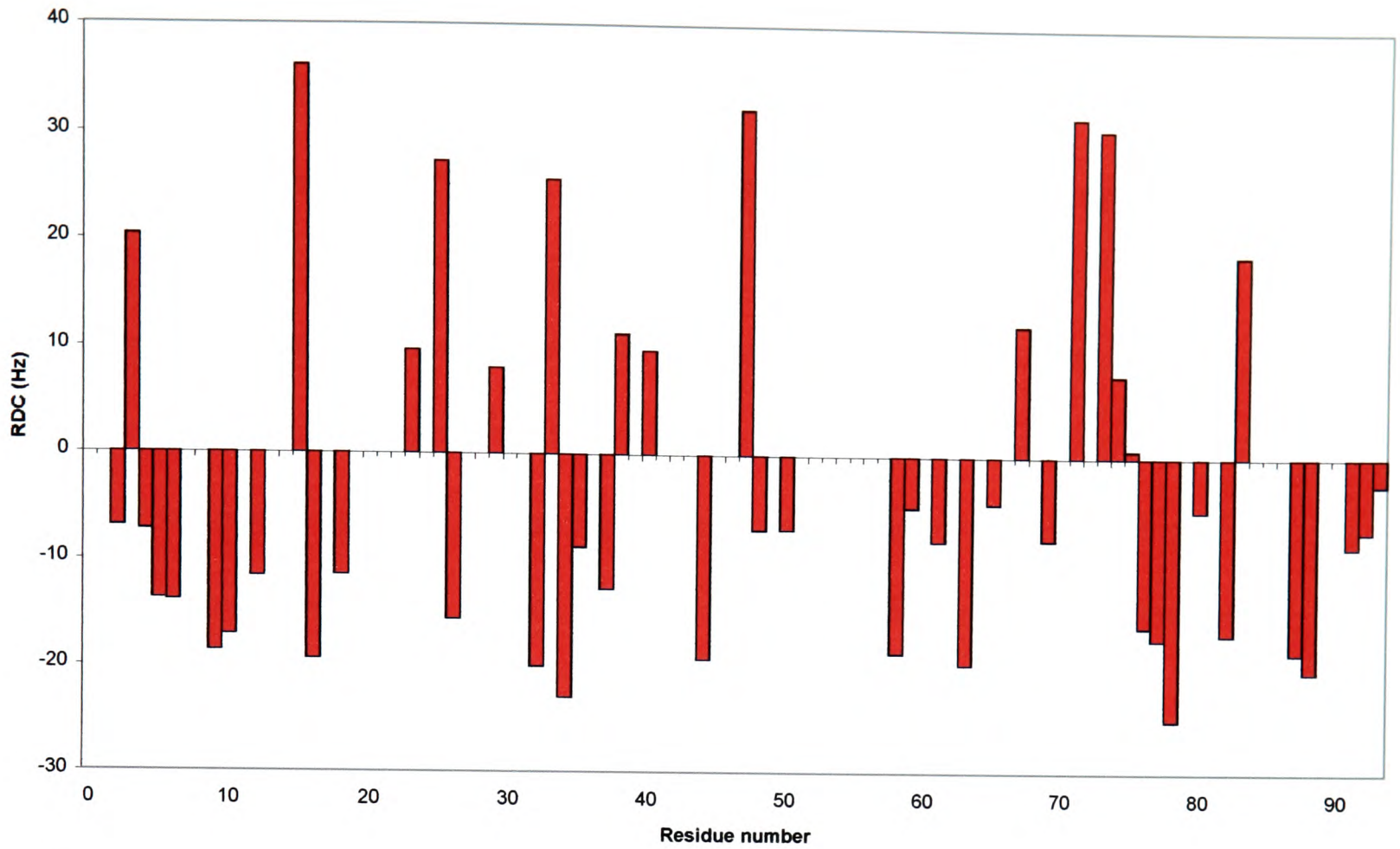


Fig.5.5. RDC data acquired for 0.2 mM $^{2}H^{15}N$-$^{4}F1^{5}F1$ in complex with 0.47 mM D3 in the bicelles medium.

a. RDC per residue. Gaps indicate residues for which no data was obtained.

b. RDC distribution - 'powder pattern'.

a.



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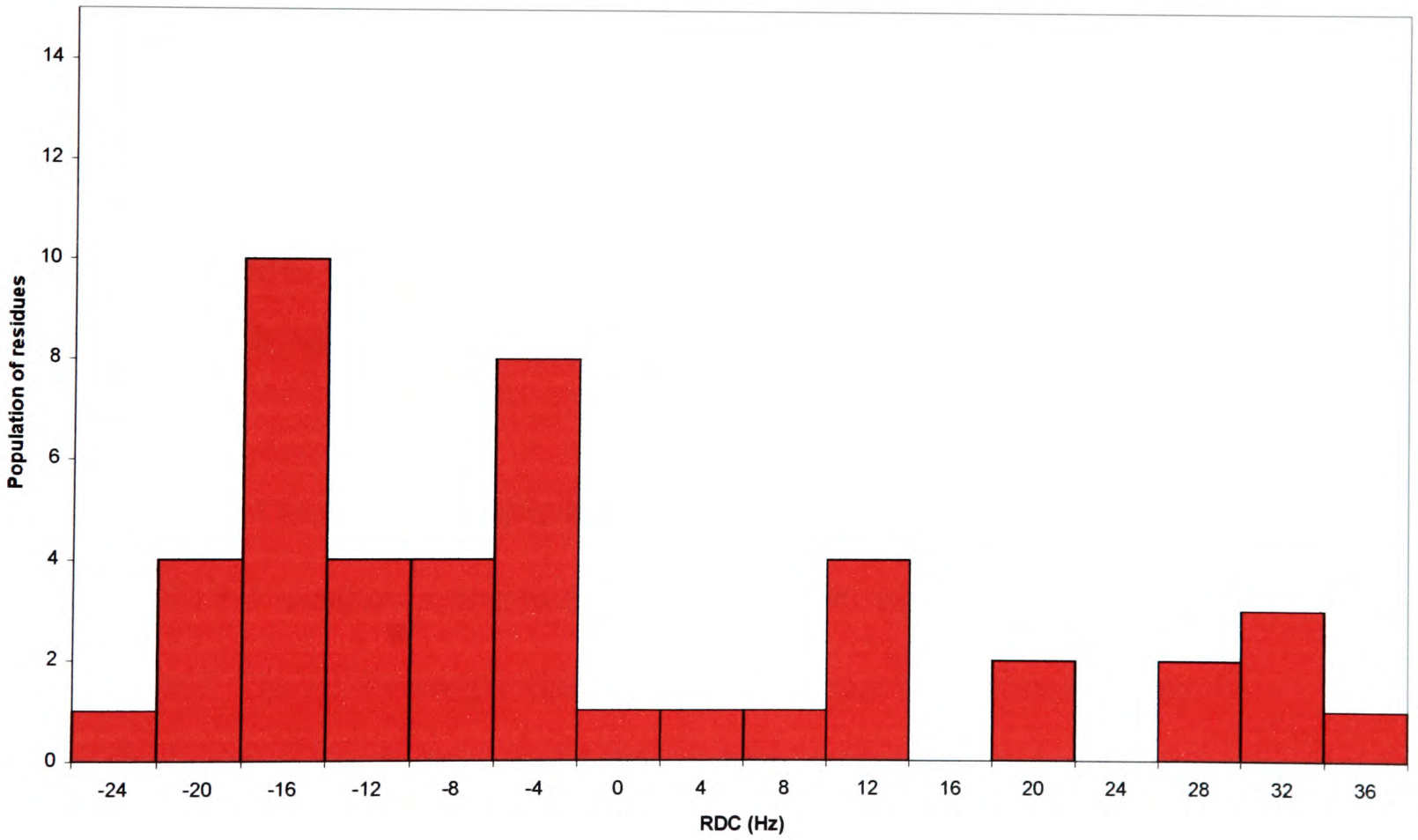


Fig.5.6. RDC data acquired for 0.5 mM ^{15}N-^{4}F1^{5}F1 in complex with 1.2 mM D3 in the bicelles medium.

a. RDC per residue. Gaps indicate residues for which no data was obtained.

b. RDC distribution - 'powder pattern'.

A family of 100 structures was calculated using a total of 1098 distance restraints, 10 hydrogen bond restraints (2 for each bond) and 9 φ backbone torsion angle restraints. Intraresidue NOEs between protons on adjacent carbon atoms were not included as restraints. The interproton distance restraints, all assigned unambiguously, included 397 intraresidue, 300 sequential (between adjacent residues), 81 medium-range (between residue *i* and residue $i \pm 4$) and 320 long-range restraints. Among them there were 44 intermodular restraints: 3 medium-range and 41 long-range, as well as 5 long-range restraints between the linker residues (R46 and N47) and the ^{5}F1 module residues. Fig.5.7 presents the secondary structure of the module pair.

From a total of 100 calculated structures, an ensemble of 72 structures was accepted for the refinement on the basis that they had no NOE violations > 0.5 Å and no dihedral angle violation > 3°. The set of 50 refined structures (characterised by Table.5.4) was chosen for further refinement with the implementation of SANI/ISAC and DANI restraints. The several cooling-stages protocol described in **Section 4.2**, were also used here, but using the PROLSQ force-field.

Selection of sets of long-range orientational restraints

For the ^{4}F1^{5}F1/D3 complex a heteronuclear ^{1}H-^{15}N NOE experiment was acquired using a 0.2 mM ^{15}N-^{4}F1^{5}F1 sample (Fig.5.8). The data were then analysed using the minimised average structure of bound ^{4}F1^{5}F1, and Modelfree with the application of five dynamical models to fit the data points (Mandel *et al.,* 1995). Model 1 is consistent with the Lipari-Szabo model, models 2-5 assume more complex dynamics of the residue. Thus, the following possibilities were considered for model: S^2 (1), S^2

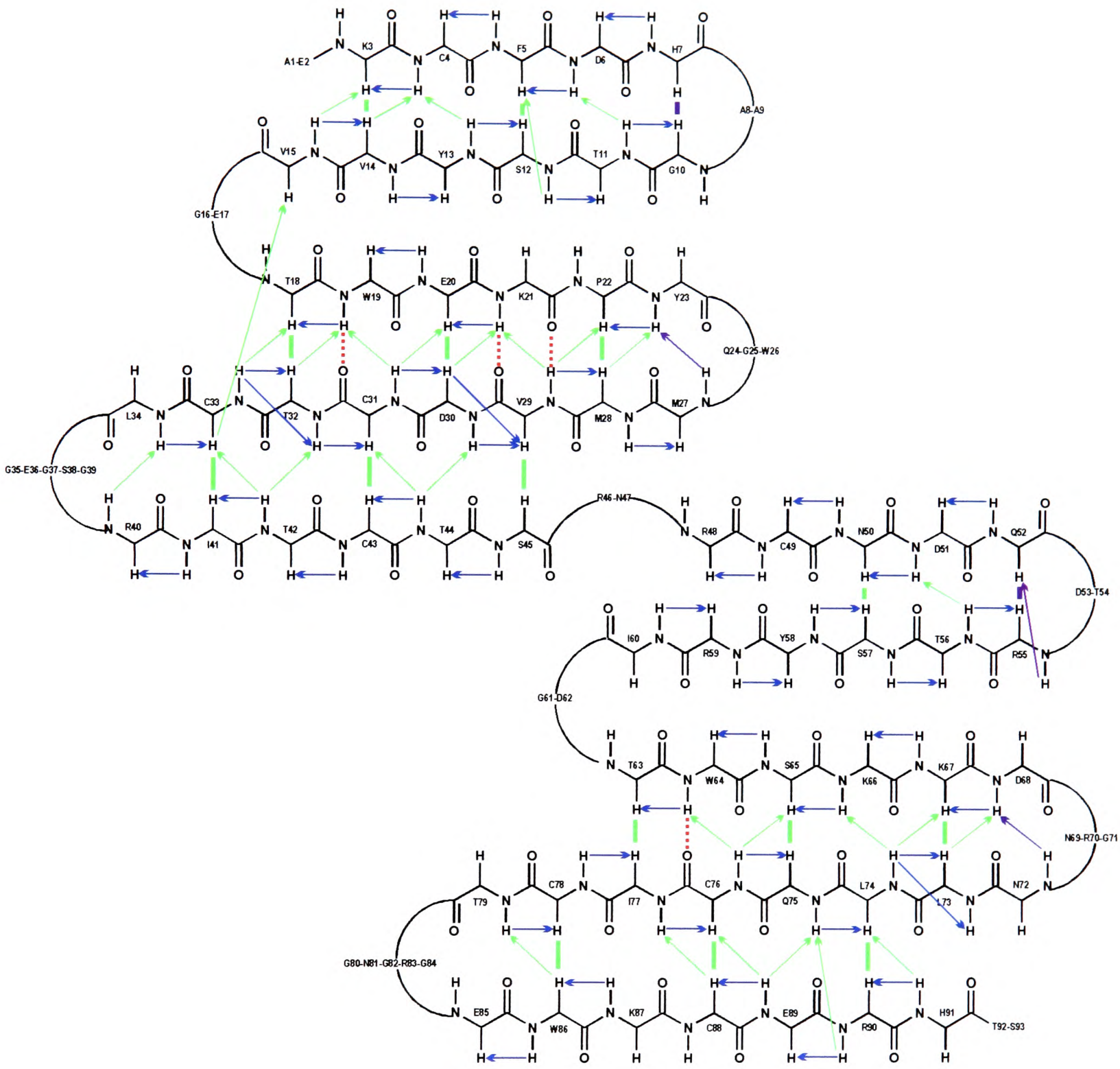


Fig.5.7. Secondary structure of the ^{4}F1^{5}F1 module pair bound to D3. The backbone NOEs between protons in the ordered regions and hydrogen bonds used as input data for the structure calculations are indicated by solid arrows or lines and dotted lines respectively. Blue - sequential NOEs, purple - medium-range NOEs, green - long-range NOEs, red - hydrogen bonds.

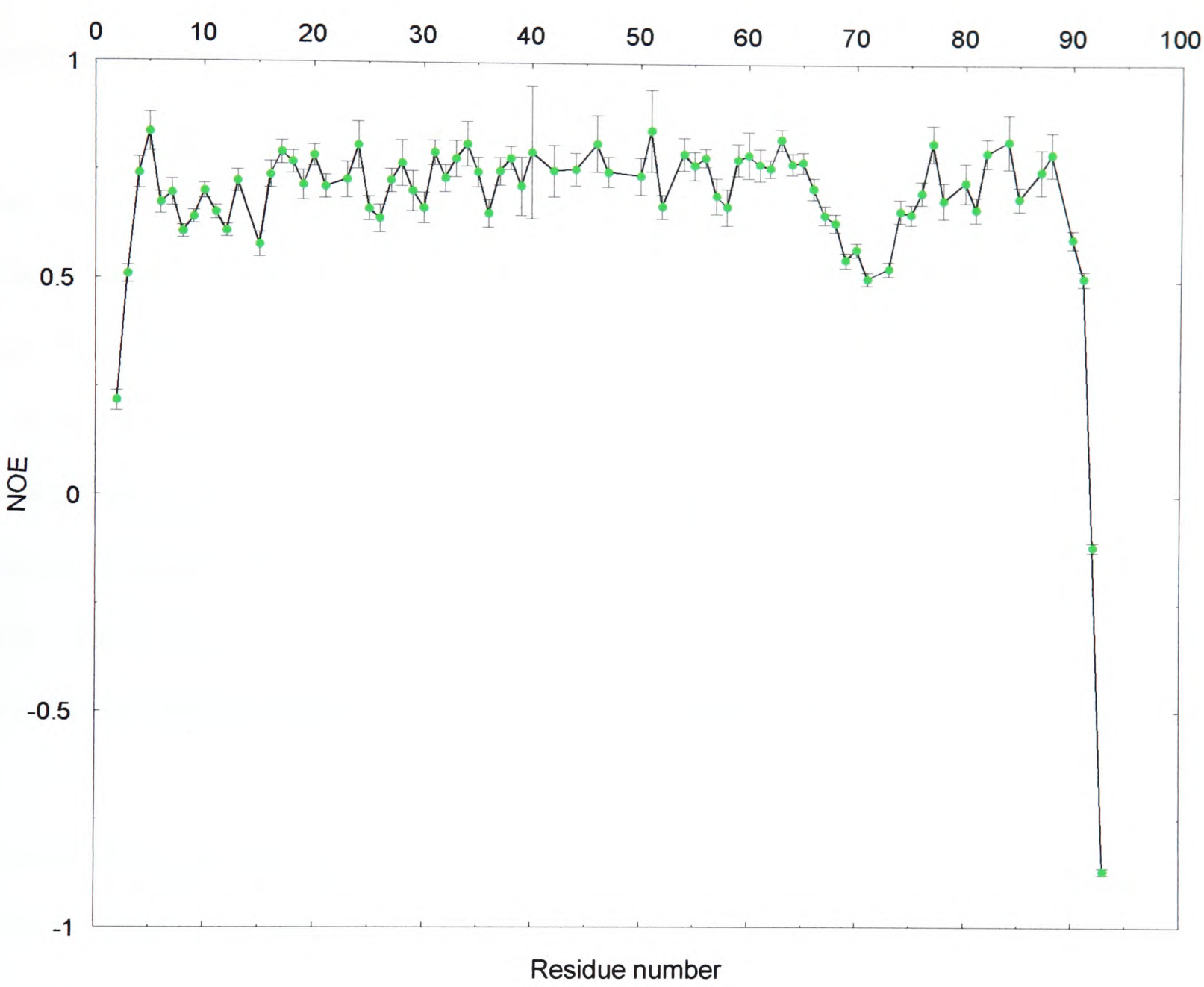


Fig.5.8. ^{1}H-^{15}N NOE experiment for 0.2 mM $^{4}F1^{5}F1$ in complex with 0.47 mM D3. NOE was calculated as a ratio of HSQC peak intensity from the NOE experiment to the intensity without NOE.

and τ_e (2), S^2 and R_{ex} (3), S^2, τ_e and R_{ex} (4), S^2_f, S^2_s and τ_s (5). S is a general order parameter, S_f and S_s are order parameters relating to fast and slow motions respectively, τ_e is a correlation time for internal motions and R_{ex} is a chemical exchange component. The results of the analysis are shown in Table.5.1 and summarized in Fig.5.9.

The residues that could be fitted into model 1 or model 2 with $\tau_e < 45$ ps were selected for further Modelfree analysis using 20 structures calculated and refined using NOE and torsion angle restraints. Residues that fitted to the model 2 but with $\tau_e > 45$ ps were excluded from further studies, as well as the residues that fitted to models 1 and 2 but showed high χ^2 when a prolate model was assumed in Modelfree analysis (Table.5.3). The excluded residues were: D6, A8, A9, T11, S12, E17, W26, M28, V29, L34, E36, R40, Q52, T54, K67, L74, Q75, G84, C88 and R90. As a result, 38 out of 58 residues were used for DANI refinement (**Appendix 4**).

A set of RDC data was again selected on the same basis, as T_1/T_2 (the residues that could be fitted into models 1 and 2 with $\tau_e < 45$ ps in Modelfree analysis). These residues were assumed to be unaffected by the additional effects due to a presence of the D3 peptide. For further selection, sets of 20 structures were refined with an implementation of SANI restraints. After ranking the structures according to the overall energy five of the lowest energy ones were used as models for back-calculation of the couplings. Residues constantly violated or showing a bad fit (high χ^2) were removed from the usable set. A possible explanation for the bad fit is given in a similar subchapter in **Section 4.2**. The procedure was repeated until no residues

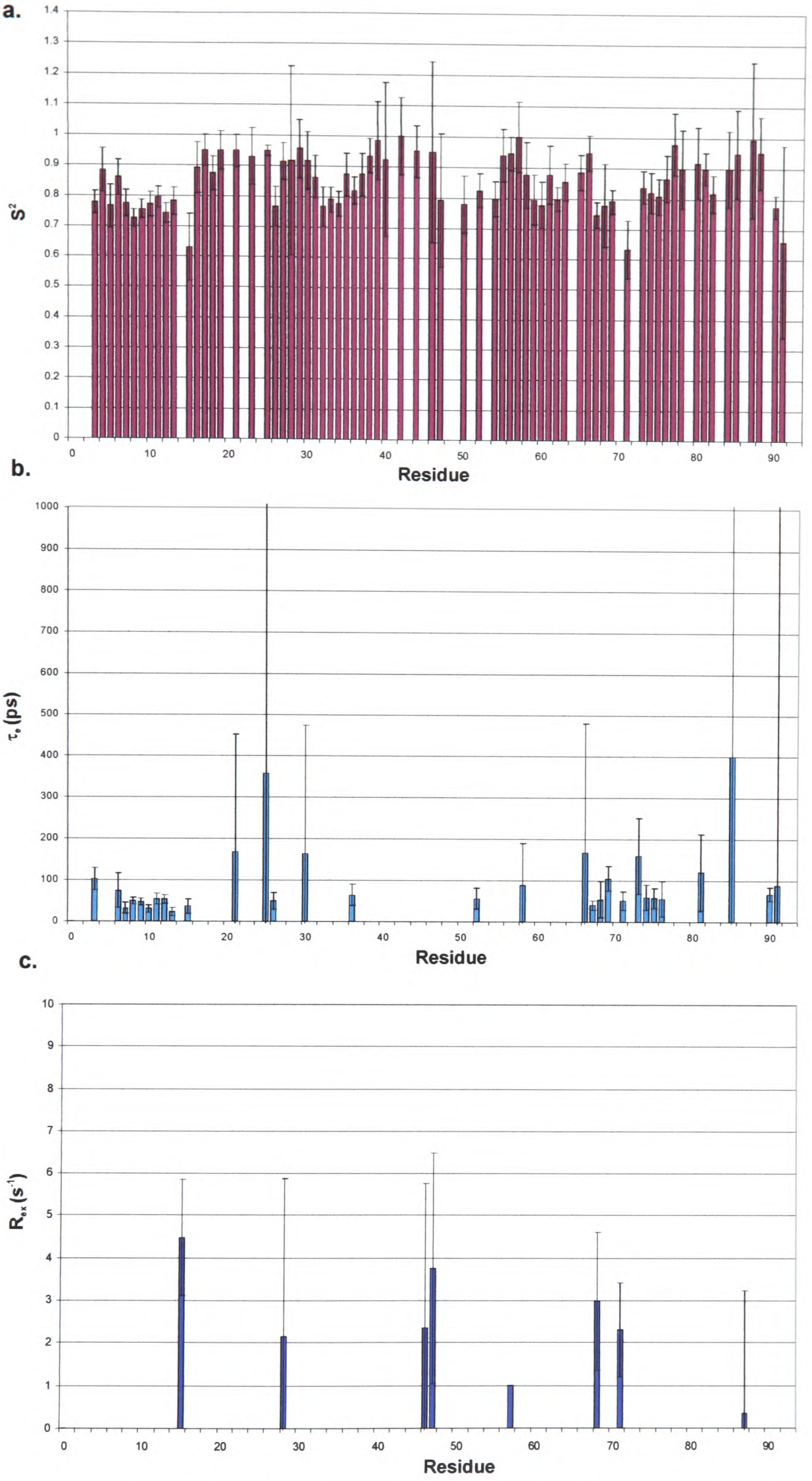


Fig.5.9. Modelfree parameters for ^{4}F1^{5}F1 bound to D3 (Table.5.1)

a. Order parameter S^2

b. Effective correlation time for internal motions (τ_e)

c. Chemical exchange contribution (R_{ex})

showed a consistently bad fit. The data for protonated and deuterated $^{4}F1^{5}F1$ were treated in the same way. Finally, for both SANI and ISAC routines sets of 38 out of 38 ($^{2}H^{15}N$-$^{4}F1^{5}F1$) and 30 out of 43 (^{15}N-$^{4}F1^{5}F1$) residual couplings were used (**Appendix 4**).

Table.5.1. Modelfree analysis of ^{15}N-relaxation data for ^{15}N-$^{4}F1^{5}F1$ bound to D3

Residue	Secondary[a]	Model[b]	S^2	S_f^2	τ_e (ps)	R_{ex} (s^{-1})	χ^2
k3	β	2	0.777 ± 0.037		102 ± 27		9.78
c4	β	1	0.881 ± 0.071				2.46
p5	β	1	0.763 ± 0.069				1.63
d6	β	2	0.858 ± 0.059		74 ± 42		0.03
h7	β	2	0.773 ± 0.044		31 ± 13		0.91
a8		2	0.724 ± 0.029		49 ± 8		1.21
a9		2	0.754 ± 0.031		47 ± 9		0.72
g10	β	2	0.77 ± 0.041		30 ± 9		0.48
t11	β	2	0.793 ± 0.034		54 ± 13		0.03
s12	β	2	0.742 ± 0.031		53 ± 10		1.38
y13	β	2	0.781 ± 0.044		23 ± 10		0.01
v15	β	4	0.629 ± 0.109		36 ± 18	4.48 ± 1.37	0
g16		1	0.889 ± 0.083				3.41
e17		1	0.947 ± 0.051				4.52
t18	β	1	0.872 ± 0.055				0.93
w19	β	1	0.948 ± 0.062				5.98
k21	β	2	0.947 ± 0.053		168 ± 283		0.13
y23	β	1	0.929 ± 0.094				2.87
g25		2	0.947 ± 0.017		358 ± 3460		3.14
w26		2	0.765 ± 0.064		49 ± 21		0.08
m27	β	1	0.913 ± 0.061				6.83
m28	β	3 (1)[c]	0.916 ± 0.308			2.14 ± 3.74	0.28
v29	β	1	0.955 ± 0.096				4.37
d30	β	2	0.916 ± 0.094		164 ± 311		0.45
c31	β	1	0.863 ± 0.071				0.06
t32	β	1	0.766 ± 0.065				4.87
c33	β	1	0.789 ± 0.041				6.35
l34	β	1	0.777 ± 0.04				0.62
g35		1	0.872 ± 0.07				1.89
e36		2	0.82 ± 0.044		64 ± 26		0.002
g37		1	0.872 ± 0.07				1.69
s38		1	0.934 ± 0.056				0.34
g39		1	0.984 ± 0.127				1.41
r40	β	1	0.922 ± 0.252				0.58
t42	β	1	1 ± 0.126				1.99

Residue	Secondary[a]	Model[b]	S^2	S_f^2	τ_e (ps)	R_{ex} (s^{-1})	χ^2
t44	β	1	0.95 ± 0.084				1.11
r46		3 (1)[c]	0.948 ± 0.294			2.35 ± 3.40	0.14
n47		3 (1)[c]	0.791 ± 0.218			3.75 ± 2.72	1.81
n50	β	1	0.778 ± 0.093				1.61
q52	β	2	0.823 ± 0.056		56 ± 26		0.26
t54		1	0.795 ± 0.057				2.67
r55	β	1	0.938 ± 0.086				0.83
t56	β	1	0.944 ± 0.054				0.09
s57	β	3 (1)[c]	1 ± 0.115			0.99 ± 0.00	4.96
y58	β	2	0.875 ± 0.107		88 ± 103		0.06
r59	β	1	0.794 ± 0.081				0.38
i60	β	1	0.777 ± 0.075				0.004
g61		1	0.876 ± 0.094				0.47
d62		1	0.797 ± 0.038				3.74
t63	β	1	0.853 ± 0.06				2.28
s65	β	1	0.885 ± 0.057				0.6
k66	β	2	0.947 ± 0.058		168 ± 312		1.12
k67	β	2	0.745 ± 0.042		40 ± 11		0.06
d68	β	4	0.776 ± 0.136		54 ± 44	2.98 ± 1.63	0
n69		2	0.789 ± 0.038		105 ± 30		4.41
g71		4	0.632 ± 0.095		51 ± 22	2.3 ± 1.1	0
l73	β	2	0.837 ± 0.052		160 ± 92		0.79
l74	β	2	0.819 ± 0.066		59 ± 31		0.05
t75	β	2	0.806 ± 0.059		57 ± 24		0.004
c76	β	2	0.866 ± 0.077		56 ± 43		0.16
i77	β	1	0.977 ± 0.101				0.54
c78	β	1	0.895 ± 0.129				6.32
g80		1	0.916 ± 0.117				2.01
n81		2	0.897 ± 0.053		120 ± 93		0.01
g82		1	0.815 ± 0.06				0.51
g84		1	0.897 ± 0.125				0.22
e85	β	2	0.947 ± 0.145		400 ± 13233		2.72
k87	β	3 (1)[c]	0.993 ± 0.253			0.34 ± 2.88	0.56
c88	β	1	0.95 ± 0.117				0.55
r90	β	2	0.774 ± 0.036		67 ± 16		2.41
h91	β	5	0.658 ± 31.5	0.885 ± 30	88 ± 13107[d]		6.74

[a] secondary structure as defined in Fig.5.6.

[b] dynamical models applied to fit the relaxation data

[c] residues could be fitted to model 1 but Fig.5.3 indicates the presence of the slow motions, therefore model 3 was applied

[d] $\tau_e = \tau_s$ for model 5

Determination of the alignment tensor

The theoretical alignment tensor parameters A_a (i.e. $\frac{1}{2}A_{zz}$) and R were estimated from the 'powder pattern' and modelled using Module (Dosset *et al.*, 2001). Modelling was carried out for both modules together as well as separately, and no significant difference was observed between an individual and a collective alignment tensor (Fig.5.10).

The grid search of the optimal alignment tensor parameters, obtained from the 'powder pattern', was carried out on 10 structures and for A_a values of 13 - 21 and for R values of 0.1 – 0.55. The values giving the lowest number of NOE and SANI violations in 5 of the lowest energy structures (A_a = 15 and R = 0.5 for $^{2}H^{15}N$-$^{4}F1^{5}F1$, and A_a = 20 and R = 0.45 for ^{15}N-$^{4}F1^{5}F1$) were used in the structure refinement with implementation of SANI routine. Using lower value for rhombicity resulted in numerous SANI violations after the refinement. Similar to the free module pair, use of the SANI routine in the refinement did not improve the structures. Modelling the alignment tensor in Module has shown that although the positions of N-H vectors with RDC present in the refinement were readily 'corrected', as in case of the unbound $^{4}F1^{5}F1$ (**Section 4.2**), the SANI refinement with both data sets either increased the rmsds of the module pair and the individual modules or did not affect the initial rmsd values. Changes made to the refinement protocol did not seem to have a significant effect on the final result.

The effectiveness of the ISAC routine has been checked by calculating and refining the structures of the unbound $^{4}F1^{5}F1$ module pair, without using intermodular and

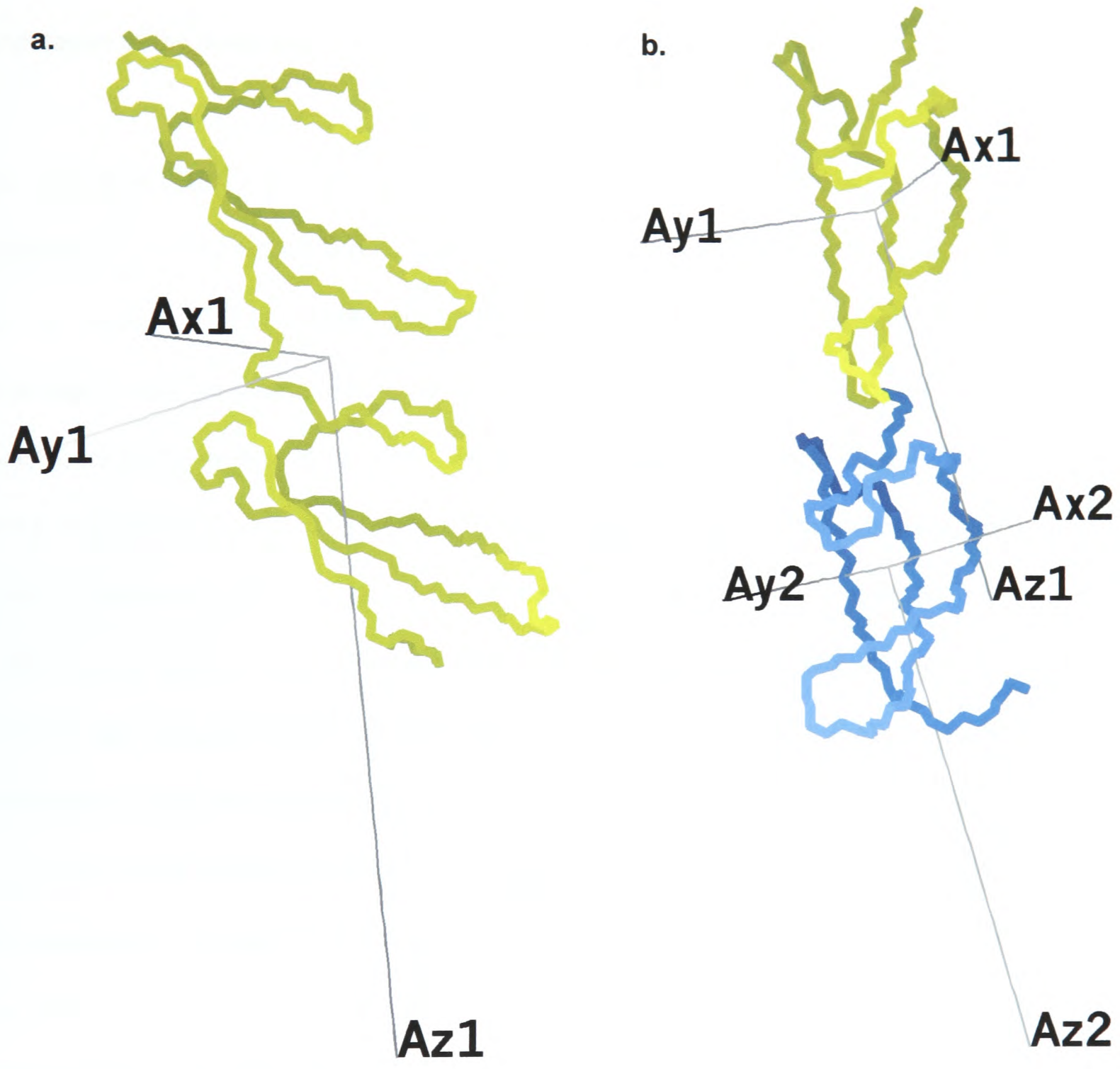


Fig.5.10. The alignment tensors modelled using Module: **a.** For both modules in the lowest energy structure of ^{4}F1^{5}F1 bound to D3. **b.** For the individual modules in this structure.

linker-^{5}F1 NOEs. The respective rmsds for the backbone of residues 3-91 for the sets of 10 structures refined, without and with RDC-derived restraints, were: 2.2 ± 0.7 Å and 1.5 ± 0.5 Å (Fig.5.11). This shows that RDCs can be used to define the relative orientation of the domains.

As the set of RDCs recorded for ^{2}H^{15}N-^{4}F1^{5}F1 in complex with D3 was more complete, it was chosen as the one to focus on. Using an ISAC routine for refinement of the bound ^{4}F1^{5}F1 module pair improved the definition of the intermodule orientation, with no constant RDC violations greater than 1 Hz. The summary of the best structure ensembles after NOE/CDIH, NOE/CDIH + SANI and NOE/CDIH + ISAC refinements are shown in Table.5.2. The alignment tensor parameters from the ISAC refinement output files varied for A_{zz} between values of 36 - 29 and R of 0.1 – 0.25, slightly greater than the values estimated and optimised using the 'powder pattern' and the grid search described above (A_{zz} = 30 and R = 0.5) for SANI refinement. Using the alignment tensor parameters defined by ISAC (A_{zz} = 34 and R= = 0.15) in SANI refinement resulted in rmsd values similar to obtained, when using the parameters estimated by grid search. For an explanation of the underestimation of A_{zz} and the inability to determine the alignment tensor *a priori* see **Section 4.2**. The underestimation in this case was not as significant as for the free module pair, probably due to narrower lines and larger signal-to-noise ratio obtained by using fully deuterated protein. This makes the coupling measurement more precise and allows for more data points to be obtained from overlapped regions of the spectra.

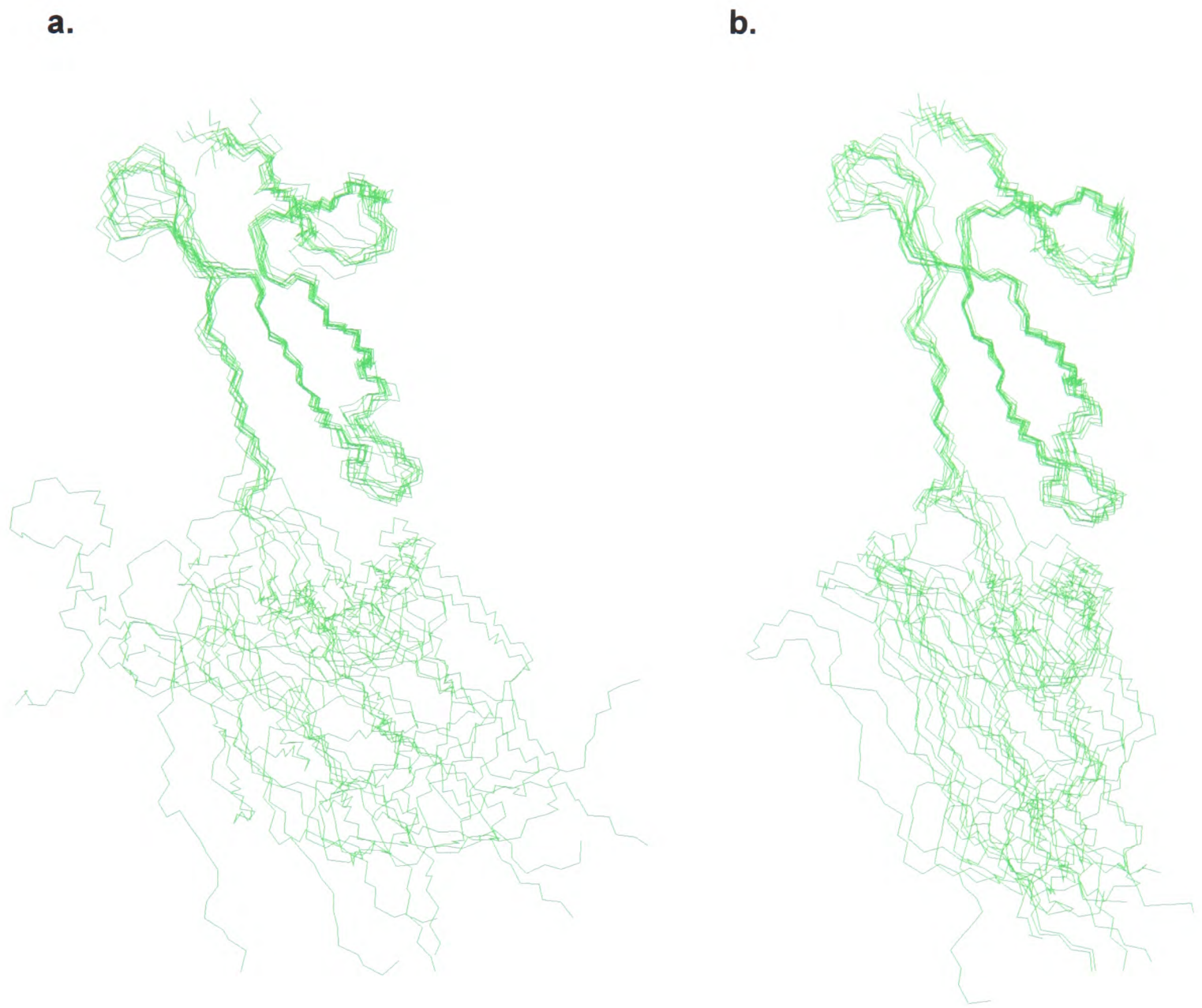


Fig.5.11. Overlays of 10 ^{4}F1^{5}F1 structures in an unbound state, calculated and refined with the exclusion of intermodular NOE restraints. Superposition on the ^{4}F1 backbone (residues 3-45) shows the spread of allowed intermodule orientations. **a.** NOE/CDIH refinement resulted in the backbone rmsd for the residues 3-91 of 2.2 ± 0.7 Å. **b.** NOE/CDIH refinement with the implementation of ISAC restraints improved this rmsd to 1.5 ± 0.5 Å.

Table 5.2. Comparison of the best sets of 20 calculated structures of ^{4}F1^{5}F1 module pair in the complex with D3 after SANI and ISAC refinement.

rmsd (±sd) of Cartesian coordinates (Å)[a]	No RDC	SANI	ISAC
backbone atoms: residues 3-91	1.2 ± 0.5	1.4 ± 0.5	1.0 ± 0.5
backbone atoms: residues 3-45	0.6 ± 0.5	0.8 ± 0.6	0.7 ± 0.5
backbone atoms: residues 48-91	0.9 ± 0.6	1.0 ± 0.5	0.8 ± 0.6
all heavy atoms: residues 3-91	1.5 ± 0.7	1.8 ± 0.7	1.4 ± 0.7
all heavy atoms: residues 3-45	1.0 ± 0.6	1.2 ± 0.7	1.0 ± 0.6
all heavy atoms: residues 48-91	1.4 ± 0.7	1.5 ± 0.8	1.3 ± 0.8

[a]rmsd for the new structures was calculated by comparison of each of 20 structures with the average structure

Determination of the diffusion tensor

Prior to the DANI refinement, the diffusion tensor parameters τ_c and D_{ratio} were estimated using Modelfree analysis of an ensemble of 20 structures refined first using NOE/CDIH restraints, and then RDCs (Table.5.3). As more T_1/T_2 data could be used due to the availability of an ^{1}H-^{15}N NOE experiment, the N-H angle sampling was much better for individual modules than in the case of the free module pair (Fig.5.12). The isotropic τ_c for bound ^{4}F1^{5}F1 is very similar for both modules together and separately: 7.3 – 7.4 ns, suggesting that, as found for unbound ^{4}F1^{5}F1, ^{4}F1 and ^{5}F1 tumble together, when bound to D3.

No significant difference in the anisotropy (D_{ratio}) was observed between the module pair and the ^{5}F1 module, although the diffusion tensor for ^{4}F1 appeared inconsistent with them. This is probably due to limited sampling of N-H bond angles that can be measured. The ^{4}F1^{5}F1 data still give the best fit with the prolate model. The D_{ratio} indicates that the module pair is more elongated than in the unbound state. The τ_c and D_{ratio} values of 7.4 ns and 1.4 respectively, were used for DANI refinement.

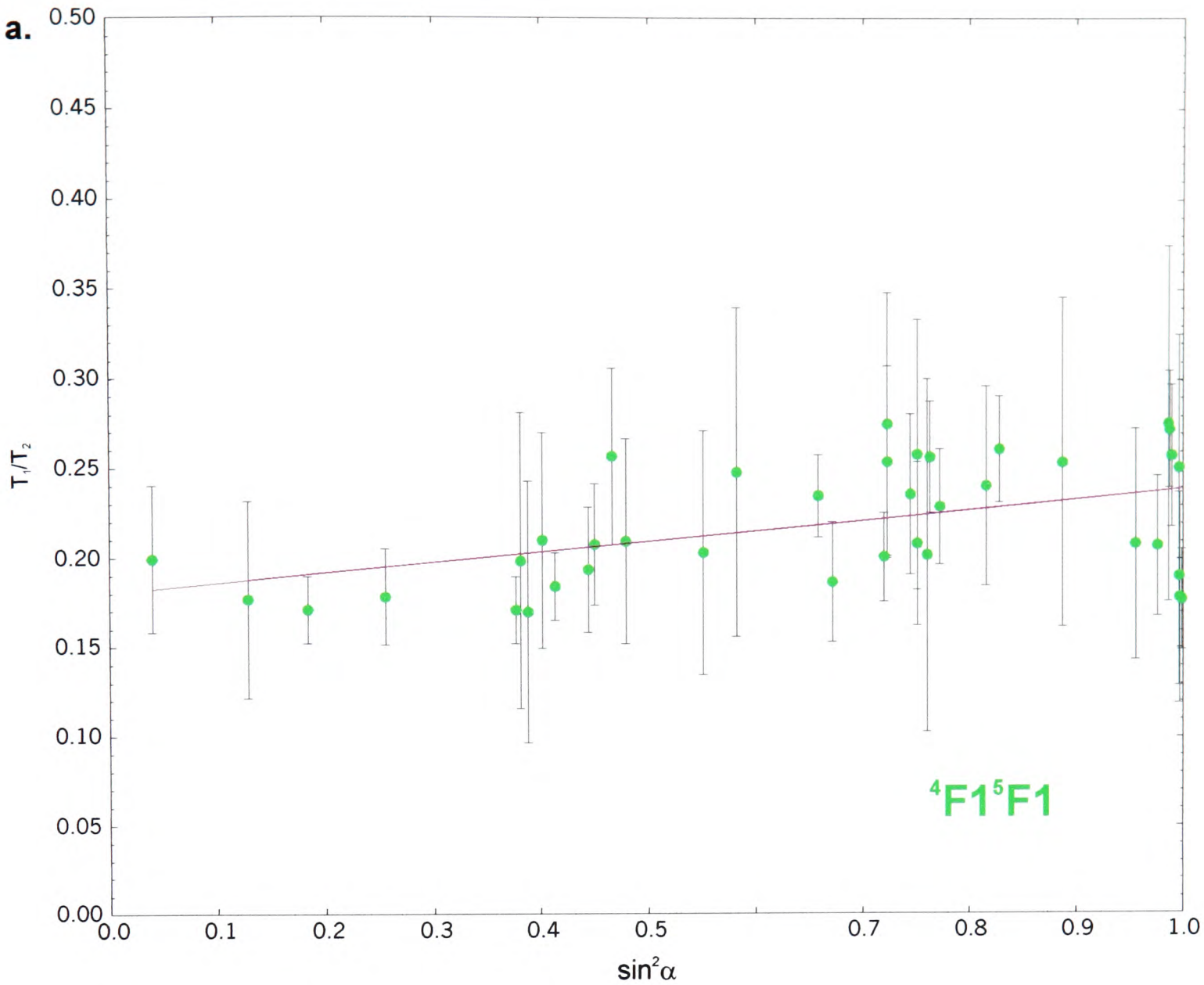


Fig.5.12. Distribution of N-H bond angles in $^4F1^5F1$ bound to D3 (the lowest energy structure) and in the individual modules according to T_1/T_2 ratio.

Table.5.3. Diffusion parameters for 90% of 20 ^{4}F1^{5}F1 structures from ^{4}F1^{5}F1/D3 complex (10% giving the highest χ^2 were removed)

	τ_c	D_{ratio}	θ	φ	χ^2 per residue	Q^a
^{4}F1^{5}F1 pair – NOE (38 res.)						
Isotropic	7.4	1	-	-	1.03	-
Axially symmetric						
prolate	7.4 ± 0.11	1.4 ± 0.19	40 ± 9	62 ± 13	0.92	$5.69 \cdot 10^{-6}$
oblate	7.4 ± 0.07	0.8 ± 0.07	96 ± 12	-14 ± 2	0.95	$4.54 \cdot 10^{-4}$
^{4}F1 in ^{4}F1^{5}F1 pair – NOE (17 res.)						
Isotropic	7.3	1	-	-	0.73	-
Axially symmetric						
prolate	7.1 ± 0.11	1.6 ± 0.14	114 ± 8	77 ± 6	0.42	$1.21 \cdot 10^{-6}$
oblate	7.5 ± 0.08	0.6 ± 0.04	105 ± 11	-11 ± 13	0.41	$7.94 \cdot 10^{-7}$
^{5}F1 in ^{4}F1^{5}F1 pair – NOE (21 res.)						
Isotropic	7.3	1	-	-	0.45	-
Axially symmetric						
prolate	7.2 ± 0.08	1.5 ± 0.22	59 ± 6	153 ± 18	0.37	$1.38 \cdot 10^{-2}$
oblate	7.3 ± 0.13	0.8 ± 0.08	52 ± 13	202 ± 19	0.41	$3.31 \cdot 10^{-1}$
^{4}F1^{5}F1 pair – NOE + ISAC						
Isotropic	7.4	1	-	-	1.03	-
Axially symmetric						
prolate	7.4 ± 0.07	1.4 ± 0.17	-40 ± 8	56 ± 6	0.91	$9.14 \cdot 10^{-7}$
oblate	7.4 ± 0.09	0.8 ± 0.06	92 ± 12	129 ± 17	0.94	$6.95 \cdot 10^{-4}$
^{4}F1 in ^{4}F1^{5}F1 pair – NOE + ISAC						
Isotropic	7.3	1	-	-	0.73	-
Axially symmetric						
prolate	7.2 ± 0.04	1.7 ± 0.06	61 ± 7	65 ± 7	0.41	$7.94 \cdot 10^{-7}$
oblate	7.5 ± 0.08	0.5 ± 0.04	63 ± 6	178 ± 6	0.38	$6.93 \cdot 10^{-8}$
^{5}F1 in ^{4}F1^{5}F1 pair – NOE + ISAC						
Isotropic	7.3	1	-	-	0.45	-
Axially symmetric						
prolate	7.3 ± 0.11	1.4 ± 0.18	46 ± 12	88 ± 7	0.38	$1.94 \cdot 10^{-2}$
oblate	7.3 ± 0.08	0.8 ± 0.05	48 ± 12	193 ± 11	0.41	$3.31 \cdot 10^{-1}$

[a]Q factor for the axially symmetric model was calculated by its χ^2 comparison to the isotropic χ^2 (for the prolate the highest value from the ensemble was used, for the oblate the lowest one). The asymmetric model was compared to the prolate, as the real representation of the diffusion tensor of the molecule.

5.3. Structure analysis

The set of 50 NOE/CDIH-refined structures was further refined either with implementation of ISAC or ISAC/DANI restraints. Both refinements resulted in a very good fit of the experimental data to the structures. 20 of the lowest energy structures were chosen for further analysis, which is summarised in Table.5.4. The overlay of 20 final structures refined only with using NOE and torsion angle restraints shows well-defined positions of the core hydrophobic residues in both modules, but the intermodule orientation is less well-defined then in the previous case (Fig.5.13a&b&c). After the ISAC refinement however, the definition of intermodule orientation improved to a level almost identical with the family of unbound structures. The slightly lower convergence of the ^{5}F1 module structure in comparison with the unbound form (Table.5.5) is probably due to the lower number of restraints caused by a larger number of overlapped NOEs in the spectra of ^{4}F1^{5}F1/D3 complex. Using PROLSQ resulted in slightly higher energies due to the tighter atom packing but also resulted in better-defined secondary structure (Fig.5.14).

The final structures of ^{4}F1^{5}F1 in a bound state were used molecular replacement trials to solve the crystal structure of the ^{4}F1^{5}F1/D3 complex (**Chapter 6**). Comparison of unbound and bound ^{4}F1^{5}F1 (Table.5.5) shows that long-range orientational restraints can efficiently compensate for the limited distance constraints and give a result very close to an implementation of a large number of NOE-derived distances. In future experiments, the extraction of additional RDCs involving C and C$^{\alpha}$ from ^{13}C^{15}N-

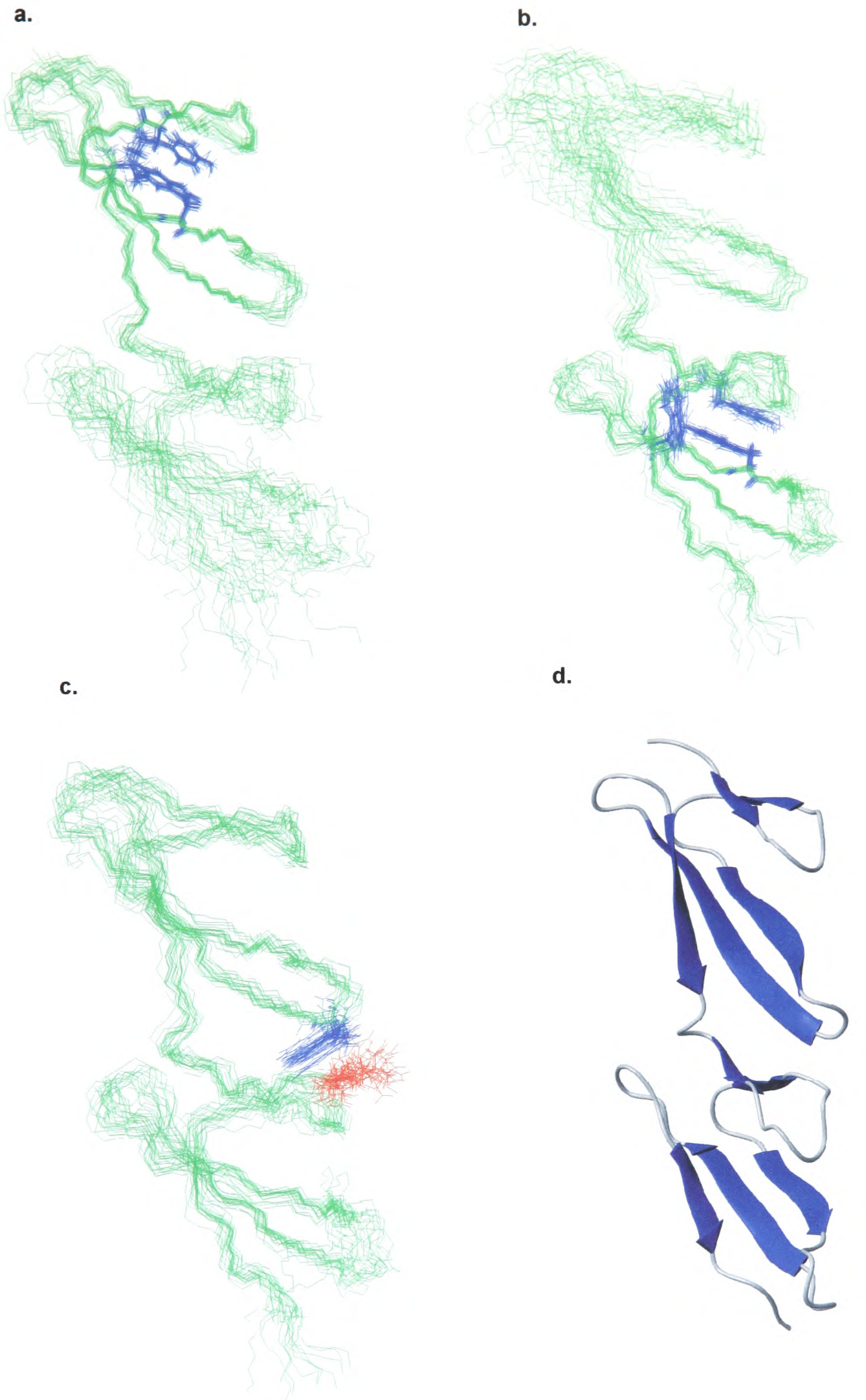


Fig.5.13. Overlays of the 20 final ^{4}F1^{5}F1 structures bound to D3. **a.** superposition on backbone atoms of residues 3-45, **b.** superposition on backbone atoms of residues 48-91; the side-chains of three conserved hydrophobic core residues are shown, **c.** superposition on backbone atoms of residues 3-91; side-chains of two residues involved in the intermodule interactions (W26 and R55) are shown, **d.** Average structure calculated from 20 structures.

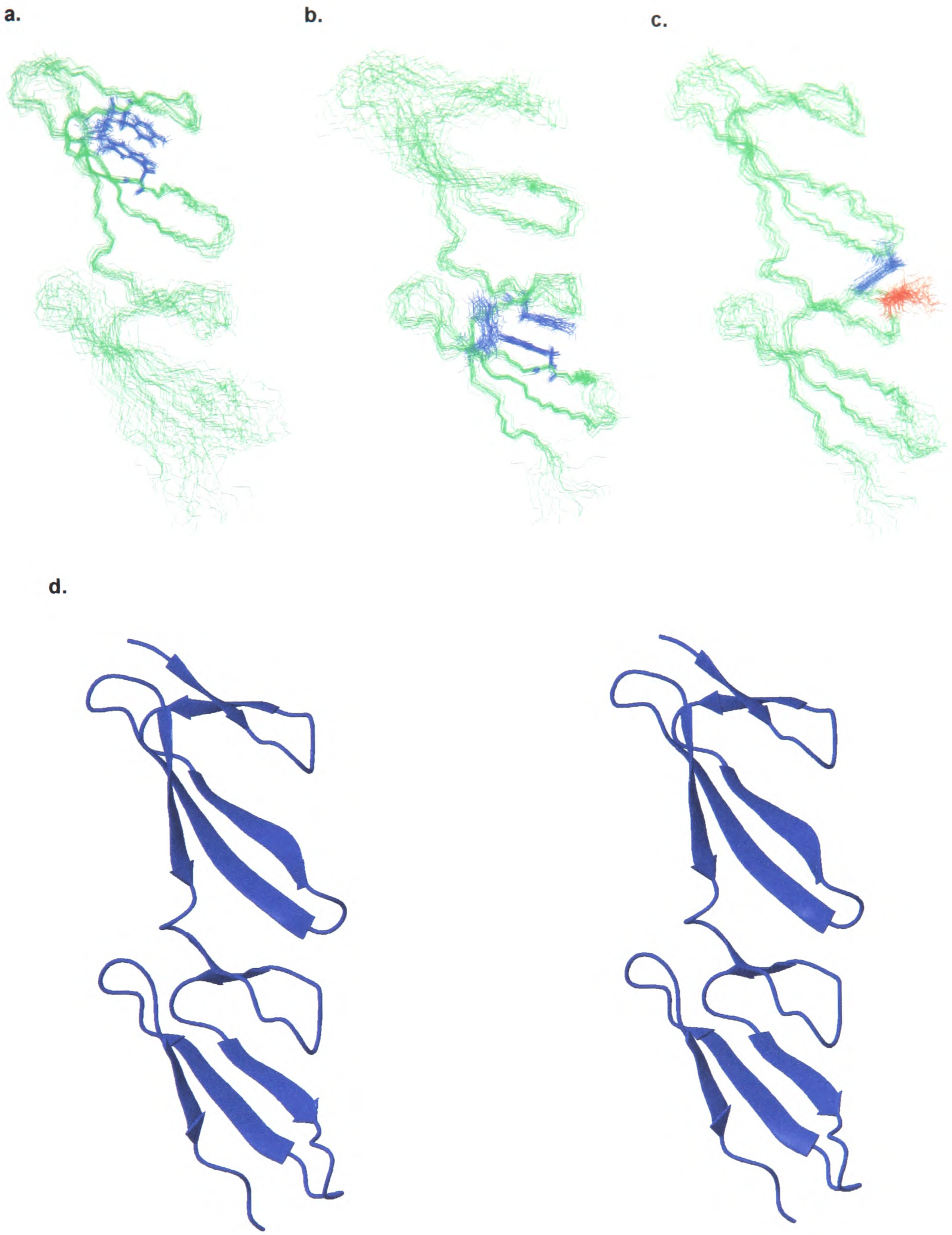


Fig.5.14. Overlays of the 20 final ^{4}F1^{5}F1 structures bound to D3 after ISAC/DANI refinement. **a.** superposition on backbone atoms of residues 3-45, **b.** superposition on backbone atoms of residues 48-91; the side-chains of three conserved hydrophobic core residues are shown. **c.** superposition on backbone atoms of residues 3-91; side-chains of two residues involved in the intermodule interactions (W26 and R55) are shown. **d.** Stereoview of an average structure calculated from 20 structures.

labelled samples might define the relative domain orientation more precisely even in the absence of the NOE distance restraints (Mueller *et al.*, 2000).

Table 5.4. Comparison of three sets of 20 calculated structures of ^{4}F1^{5}F1 module pair in a complex with D3

		NOE/CDIH restraints	+ ISAC restraints	+ ISAC/DANI restraints
rmsd (±sd) from the experimental restraints				
	NOE (Å)	0.013 ± 0.002	0.015 ± 0.002	0.015 ± 0.001
	hydrogen bonds (Å)	0.003 ± 0.002	0.004 ± 0.004	0.004 ± 0.003
	CDIH (deg)	0.016 ± 0.033	0.004 ± 0.018	0.020 ± 0.064
	RDC (Hz)	na	0.220 ± 0.065	0.550 ± 0.018[d]
T_1/T_2 fit (χ^2 p/residue)		0.93	0.91	0.90
rmsd (±sd) from the idealized covalent geometry				
	bond length (Å)	0.0014 ± 0.0001	0.0015 ± 0.0001	0.0015 ± 0.0001
	angles (deg)	0.270 ± 0.014	0.290 ± 0.012	0.286 ± 0.008
	impropers (deg)	0.350 ± 0.017	0.350 ± 0.015	0.350 ± 0.014
No of violated residues				
	NOE > 0.2 Å	2 (11, 19, 23)[c]	1 (11)[c]	1 (12, 19)[c]
	CDIH > 2 deg	0	0	0
	RDC > 0.5 Hz	na	3 (no 2, 13, 20)[c]	3 (no 16, 20)[c]
PROCHECK statistics				
	allowed	94.7%	93.6%	94%
	generous	3.2%	4.3%	6%
	disallowed	2.1%	2.1%	0%
rmsd (±sd) of Cartesian coordinates (Å)[a]				
backbone sec. structure atoms: ^{4}F1^{5}F1[b]		1.0 ± 0.3	0.8 ± 0.3	0.8 ± 0.3
backbone sec. structure atoms: ^{4}F1[b]		0.4 ± 0.2	0.4 ± 0.2	0.4 ± 0.2
backbone sec. structure atoms: ^{5}F1[b]		0.6 ± 0.3	0.6 ± 0.2	0.6 ± 0.2
backbone atoms: residues 3-91		1.2 ± 0.5	1.0 ± 0.5	1.0 ± 0.5
backbone atoms: residues 3-45		0.6 ± 0.5	0.7 ± 0.5	0.7 ± 0.5
backbone atoms: residues 48-91		0.9 ± 0.6	0.8 ± 0.6	0.8 ± 0.5
all heavy atoms: residues 3-91		1.5 ± 0.7	1.4 ± 0.7	1.4 ± 0.7
all heavy atoms: residues 3-45		1.0 ± 0.6	1.0 ± 0.6	1.0 ± 0.6
all heavy atoms: residues 48-91		1.4 ± 0.7	1.3 ± 0.8	1.3 ± 0.7

[a]rmsd for the new structures was calculated by comparison of each of 20 structures with the average structure

[b]secondary structure residues were used: from ^{4}F1: 3-7, 10-15, 18-23, 27-33, 41-45, and from ^{5}F1: 48-52, 55-60, 63-68, 72-78, 86-91

[c] in brackets the number of structure in an energy ranking

[d] the increase in RDC rmsd is probably due to differences in N-H vectors orientations defined by RDC and T_1/T_2 data

ISAC/DANI-refined structures (Fig.5.14) retain a good internal geometry and low rmsd, confirming the consistency of all the experimental restraints implemented. The RDCs recorded for $^{2}H^{15}N$-$^{4}F1^{5}F1$ in the complex with D3 have shown quality superior to the set acquired using the protonated sample, though that might be simply due to higher completeness of the data (the result of narrower lines and higher signal-to-noise ratio).

Table.5.5. Comparison of final sets of structures of an unbound and a bound $^{4}F1^{5}F1$

	Unbound structures (25 structures)	Bound structures (20 structures)
rmsd (±sd) from the experimental restraints		
all NOE restraints (Å)	0.006 ± 0.002	0.015 ± 0.001
hydrogen bonds (Å)	0	0.004 ± 0.003
CDIH (deg)	0.018 ± 0.024	0.020 ± 0.064
RDC (Hz)	0.240 ± 0.007	0.550 ± 0.018
T_1/T_2 fit (χ^2 p/residue)	1.19	0.90
rmsd (±sd) from the idealized covalent geometry		
bond length (Å)	0.0007 ± 0.0001	0.0015 ± 0.0001
angles (deg)	0.220 ± 0.0035	0.286 ± 0.008
impropers (deg)	0.390 ± 0.016	0.350 ± 0.014
rmsd (±sd) of Cartesian coordinates (Å)[a]		
backbone atoms: residues of sec. structure[b]	0.7 ± 0.2	0.8 ± 0.3
backbone atoms: residues 3-91	0.9 ± 0.3	1.0 ± 0.5
backbone atoms: residues 3-45	0.5 ± 0.3	0.7 ± 0.5
backbone atoms: residues 48-91	0.5 ± 0.3	0.8 ± 0.5
all heavy atoms: residues 3-91	1.2 ± 0.4	1.4 ± 0.7
all heavy atoms: residues 3-45	0.8 ± 0.4	1.0 ± 0.6
all heavy atoms: residues 48-91	1.0 ± 0.5	1.3 ± 0.7

[a]rmsd for the new structures was calculated by comparison of each of 20 structures with the average structure

[b]secondary structure residues were used: from $^{4}F1$: 3-7, 10-15, 18-23, 27-33, 41-45, and from $^{5}F1$: 48-52, 55-60, 63-68, 72-78, 86-91.

An ISAC refinement with RDCs from ^{15}N-$^{4}F1^{5}F1$ also resulted in correct orientations of N-H vectors but without any improvement in rmsd values. It is possible that after

incorporation of RDCs, acquired using the unbound $^{2}H^{15}N$-$^{4}F1^{5}F1$, into the refinement, the convergence of the final structures would improve further. Both sets of structures were used for comparison studies of the D3 binding, presented in **Chapter 7**.

6. PRELIMINARY X-RAY CRYSTALLOGRAPHY OF THE $^{4}F1^{5}F1/D3$ COMPLEX

6.1. Introduction to crystallography

Under certain circumstances, many molecular substances, including proteins, form crystals - a highly ordered three-dimensional arrangement of molecules, held together by non-covalent interactions. The basic building block of the crystal is the unit cell, which is defined as the minimum volume, which can be replicated within the crystal simply by translation (Fig.6.1a). The number and kind of symmetry operations that need to be applied to a single protein molecule to obtain the full content of a unit cell is described as a symmetry (space) group (65 different space-groups exist for proteins).

From the X-ray diffraction experiment (Fig.6.1b) a diffraction pattern is obtained as a result of X-rays scattering from the electron clouds that surround atoms in the crystallized molecule. The aim is to use this information to reconstruct the atom positions in the unit cell. This process requires working in two systems of coordinates: the real one of the unit cell (Fig.6.1a - direct space and lattice) and the imaginary one of the diffraction pattern (reciprocal space and lattice). The relation between the edges of real and reciprocal unit cell is $\mathbf{a^* = 1/a}$, $\mathbf{b^* = 1/b}$ and $\mathbf{c^* = 1/c}$ (**a**, **b** and **c** are the lengths of the edges of a real unit cell). Crystallographers usually use x, y and z to specify the atom position in the unit

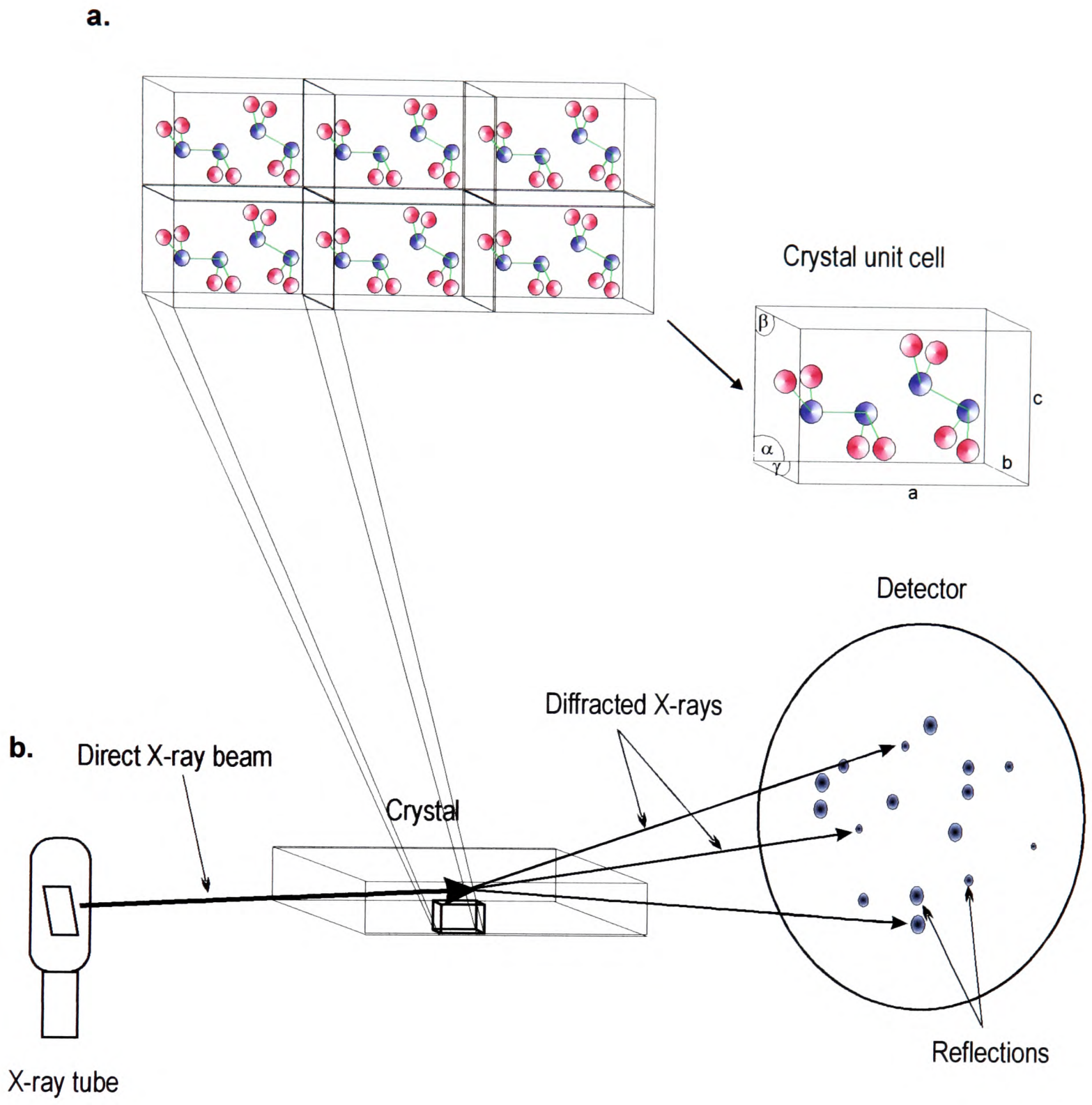


Fig.6.1.

a. An example of the arrangement of asymmetric unit cells in a crystal, each containing two molecules in different orientations. The unit cell edges are denoted a, b and c, and the angles α, β and γ.

b. A schematic representation of X-ray diffraction. A crystal is mounted between an X-ray source and an X-ray detector. It then diffracts the source beam into many discrete beams, each of which produce a distinct spot on the detector plate. The positions and intensities of these reflections contain part of the information needed to determine the molecular structure (from Rhodes, 2000).

cell (with associated temperature factor B, describing the motion of the atom about its mean position), and h, k and l to designate the positions of individual reflections in reciprocal space (Rhodes, 2000).

In order to compute the electron density from the diffraction pattern, the amplitude and phase of each diffracting ray have to be known. The amplitude can be determined directly from the intensity of the diffraction spot, but the phases have to be derived indirectly. The widely used techniques for phase determination are multiple isomorphous replacement (MIR) or multiwavelength anomalous dispersion (MAD) using heavy-atom derivatives, and molecular replacement (MR) using previously determined structures.

6.2. Crystallization techniques

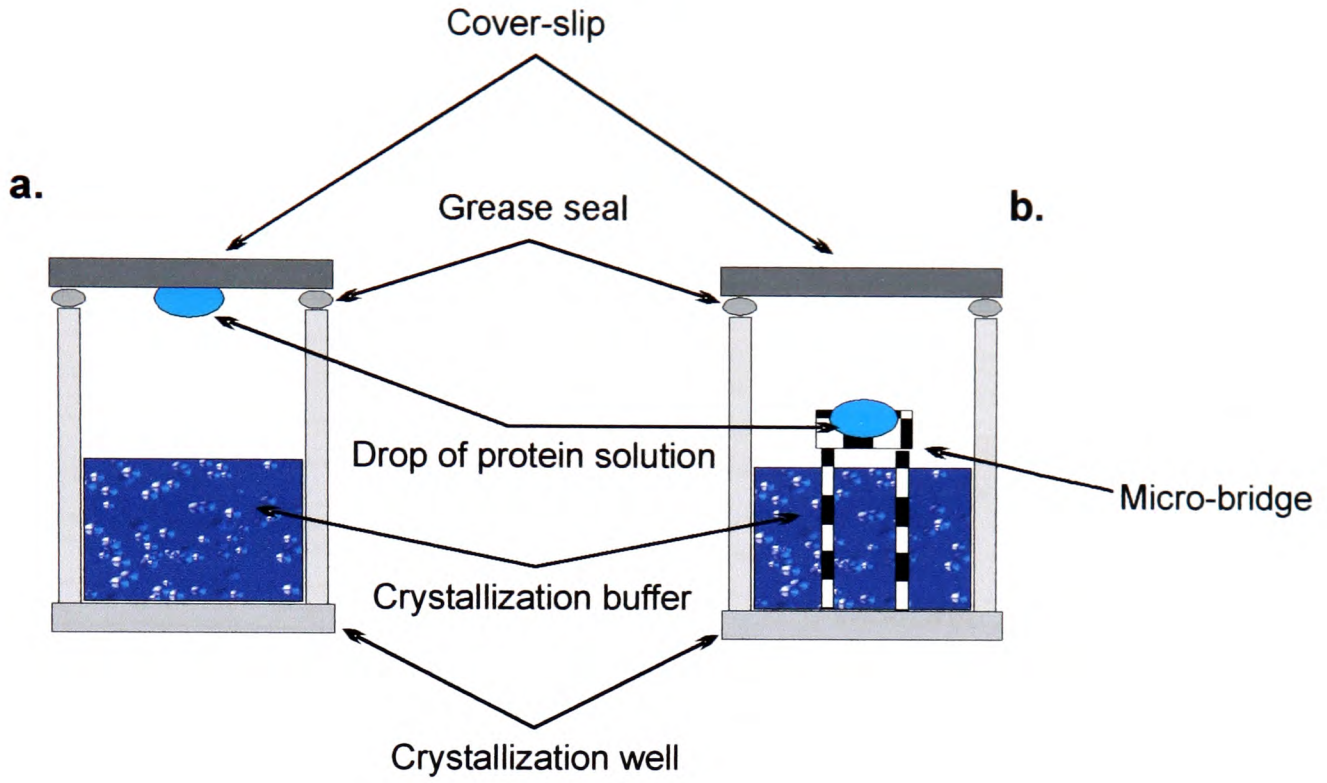
Crystals of proteins are usually grown by slow, controlled precipitation from aqueous solution under conditions that do not denature the protein. One simple means of causing slow precipitation is to add a precipitant to an aqueous protein solution until its concentration is just below that required to precipitate the protein. Then water is allowed to evaporate slowly (McPherson, 1982), which gently increases the concentration of both protein and precipitant until precipitation occurs. Whether the protein forms crystals or instead forms a useless amorphous solid depends on many properties of the solution, including protein purity and concentration, temperature, pH and ionic strength. Crystallization is still the most unpredictable process during the crystal structure determination, as well as the hardest one to reproduce.

Desalting

To remove trifluoroacetic acid (TFA) remaining after the HPLC purification step, unlabelled $^{4}F1^{5}F1$ and D3 were desalted on an ÄCTA FPLC HiPrep26/10 column using 50 mM acetate pH 5.0 as loading buffer and 50 mM acetate pH 5.0, 100 mM NaCl solution for an elution. A typical chromatograph is shown in Fig.6.2c. After desalting, the protein solutions underwent buffer to water exchange and concentration *via* centrifugal ultrafiltration in Centricons with M.W. cutoff 3 kDa (Millipore, Bedford, MA, US).

Crystallization

Crystallization was performed at 21°C using the vapour-diffusion technique (McPherson, 1982) for hanging and sitting drops (Fig.6.2a&b). Initial trials were set up with Standard Protein Screen I (50 solutions from Molecular Dimensions – Table.6.1). The hanging drops were prepared by mixing 1 µl each of 8 mg/ml $^{4}F1^{5}F1$/D3 (in a ratio of 1:2 in water) and reservoir solutions (400 µl in each well). The screen resulted in precipitation in ~30% of drops, indicated in Table.6.1 by '+'. Further tests were carried out with the protein solution at a concentration of 16 mg/ml and led to the identification of polyethylene glycol (PEG - powerful precipitant and weak denaturant) and $(NH_4)_2SO_4$ (strong salt, buffering the solution to pH 5.5) as the agents causing phase separation and/or precipitation. Optimization of conditions was carried out by varying the PEG 8000 concentration between 6 and 16% and the $(NH_4)_2SO_4$ concentration between 0.2 and 0.6 M (pH 5.5). Identical trays were set up at 4°C, 21°C and 25°C.



c.

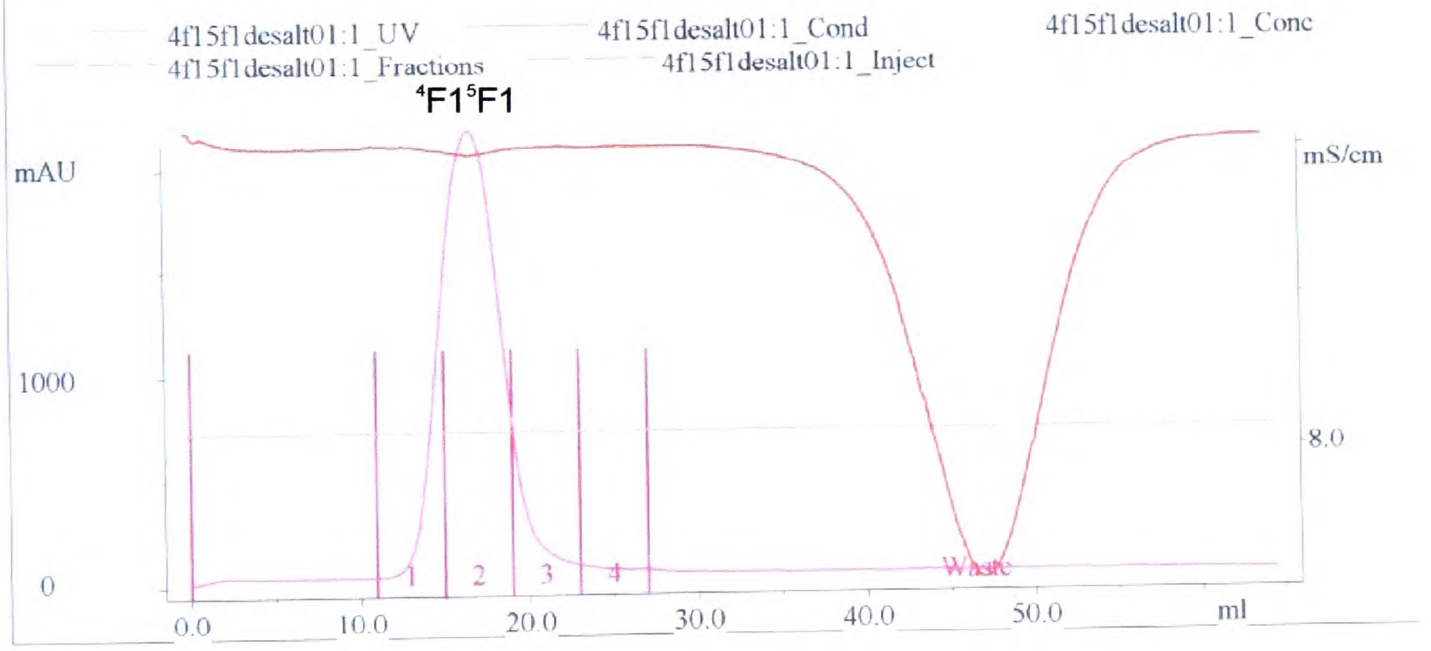


Fig.6.2.

a. The hanging-drop vapour diffusion setup in a crystallization well

b. The sitting-drop vapour diffusion setup in a crystallization well

c. Chromatograph of $^{4}F1^{5}F1$ desalting as described in the text (**6.2**)

Table.6.1. Results of the first ^{4}F1^{5}F1/D3 crystallization screen. '+' indicates precipitation in a drop

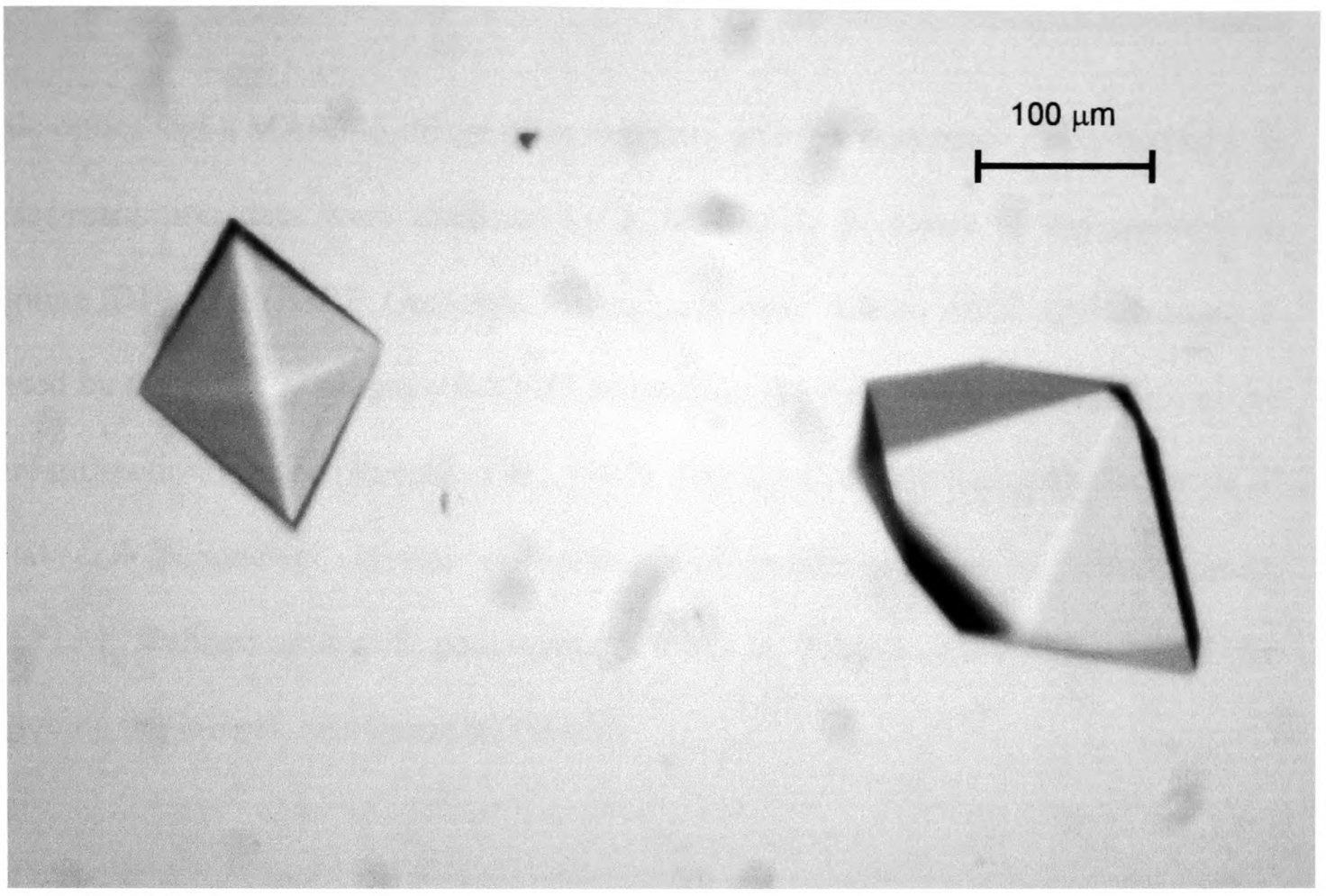
Number	Precipitant	Buffer	Salt	Outcome
1	30% v/v 2-methyl-2,4-pentanediol	0.1M CH_3COONa pH 4.6	0.02M $CaCl_2$	
2	30% w/v PEG4000	0.1M CH_3COONa pH 4.6	0.2M CH_3COONH_4	+
3	25% w/v PEG4000	0.1M CH_3COONa pH 4.6	0.2M $(NH_4)_2SO_4$	+
4	2M HCOONa	0.1M CH_3COONa pH 4.6	-	
5	2M $(NH_4)_2SO_4$	0.1M CH_3COONa pH 4.6	-	+
6	8% w/v PEG4000	0.1M CH_3COONa pH 4.6	-	+
7	30% w/v PEG4000	0.1M Na Citrate pH 5.6	0.2M CH_3COONH_4	+
8	30% v/v 2-methyl-2,4-pentanediol	0.1M Na Citrate pH 5.6	0.2M CH_3COONH_4	
9	20% v/v 2-propanol and 20% w/v PEG4000	0.1M Na Citrate pH 5.6	-	+
10	1M $(NH_4)_3PO_4$	0.1M Na Citrate pH 5.6	-	
11	20% v/v 2-propanol	0.1M CH_3COONa pH 4.6	0.02M $CaCl_2$	
12	1.4M CH_3COONa	0.1M Na Cacodylate pH 6.5	-	
13	30% v/v 2-propanol	0.1M Na Cacodylate pH 6.5	0.2M Na Citrate	
14	30% w/v PEG8000	0.1M Na Cacodylate pH 6.5	0.2M $(NH_4)_2SO_4$	+
15	20% w/v PEG8000	0.1M Na Cacodylate pH 6.5	0.2M $(CH_3COO)_2Mg$	+
16	30% v/v 2-methyl-2,4-pentanediol	0.1M Na Cacodylate pH 6.5	0.2M $(CH_3COO)_2Mg$	
17	1M CH_3COONa	0.1M Imidazole pH 6.5	-	
18	30% w/v PEG8000	0.1M Na Cacodylate pH 6.5	0.2M CH_3COONa	+
19	18% w/v PEG8000	0.1M Na Cacodylate pH 6.5	0.05M $(CH_3COO)_2Zn$	+
20	18% w/v PEG8000	0.1M Na Cacodylate pH 6.5	0.2M $(CH_3COO)_2Ca$	+
21	30% v/v 2-methyl-2,4-pentanediol	0.1M Na HEPES pH 7.5	0.2M Na Citrate	
22	30% v/v 2-propanol	0.1M Na HEPES pH 7.5	0.2M $MgCl_2$	
23	28% w/v PEG400	0.1M Na HEPES pH 7.5	0.2M $CaCl_2$	
24	30% w/v PEG400	0.1M Na HEPES pH 7.5	0.2M $MgCl_2$	
25	20% v/v 2-propanol	0.1M Na HEPES pH 7.5	0.2M Na Citrate	
26	0.8M K, Na Tartate	0.1M Na HEPES pH 7.5	-	
27	1.5M Li_2SO_4	0.1M Na HEPES pH 7.5	-	
28	1.6M K, Na Phosphate	0.1M Na HEPES pH 7.5	-	
29	1.4M Na Citrate	0.1M Na HEPES pH 7.5	-	+
30	2% v/v PEG400 and 2M $(NH_4)_2SO_4$	0.1M Na HEPES pH 7.5	-	+
31	10% v/v 2-propanol and 20% w/v PEG4000	0.1M Na HEPES pH 7.5	-	+
32	2M $(NH_4)_2SO_4$	0.1M Tris HCl pH 8.5	-	+
33	30% w/v PEG4000	0.1M Tris HCl pH 8.5	0.2M $MgCl_2$	+
34	30% w/v PEG400	0.1M Tris HCl pH 8.5	0.2M Na Citrate	
35	30% w/v PEG4000	0.1M Tris HCl pH 8.5	0.2M Li_2SO_4	
36	30% v/v 2-propanol	0.1M Tris HCl pH 8.5	0.2M CH_3COONH_4	
37	30% w/v PEG4000	0.1M Tris HCl pH 8.5	0.2M CH_3COONa	+
38	8% w/v PEG8000	0.1M Tris HCl pH 8.5	-	
39	2M $(NH_4)_3PO_4$	-	-	
40	0.4M K, Na Tartate	-	-	
41	0.4M $(NH_4)_3PO_4$	-	-	
42	30% w/v PEG8000	-	0.2M $(NH_4)_2SO_4$	+
43	30% w/v PEG4000	-	0.2M $(NH_4)_2SO_4$	+
44	2M $(NH_4)_2SO_4$	-	-	
45	4M HCOONa	-	-	
46	20% w/v PEG8000	-	0.05M KH_2PO_4	+
47	30% w/v PEG1500	-	-	
48	0.2M $(HCOO)_2Mg$	-	-	
49	2% w/v PEG8000	-	1M Li_2SO_4	
50	15% w/v PEG8000	-	0.5M Li_2SO_4	

After 3 months, around 20 medium-sized (the length of a diagonal of the hexagonal base of the bipyramid was ~150 μm) crystals (Fig.6.3) formed only at 21°C in 10% PEG 8000, 0.2 M $(NH_4)_2SO_4$ (pH 5.5) buffer. The next batch of around 50 very regular crystals grew after 5 months at 21°C in 10 μl sitting drops (8% PEG 8000 and 0.2 M $(NH_4)_2SO_4$). The average volume of such crystals was ~2 x 10^{-3} mm^3 (the length of a diagonal was 150-220 μm, half of this length was assumed as the pyramid height). However, the crystals were extremely fragile and grew only either attached to the cover slip or to the PEG film formed on the drop surface. Therefore the potential for manual manipulation of the crystals was limited and the largest crystals appeared almost impossible to isolate from the drop. To speed up the crystallization process, micro- and macroseeding (Stura & Wilson, 1992) were attempted, after prior equilibration of a sitting drop with the buffer in the reservoir for 48 h. Microseeding with crushed crystal fragments did not result in crystal growth; small crystals used for macroseeding dissolved after 48 h and after 2 months needle-shaped crystals reappeared in drops.

6.3. Data collection and structure determination

All the data were collected under cryo-conditions at 100 K (Garman & Schneider, 1997). Prior to the data collection, a single crystal was transferred for 30-60 s to a cryo-protectant solution containing the crystallization buffer supplemented with 30% v/v glycerol (which replaced water in the buffer). After soaking, the crystal was mounted in a nylon loop and then flash-cooled in liquid nitrogen (77 K). The diffraction power of the crystals was checked on the home-based Rigaku rotating anode generator equipped with

a.



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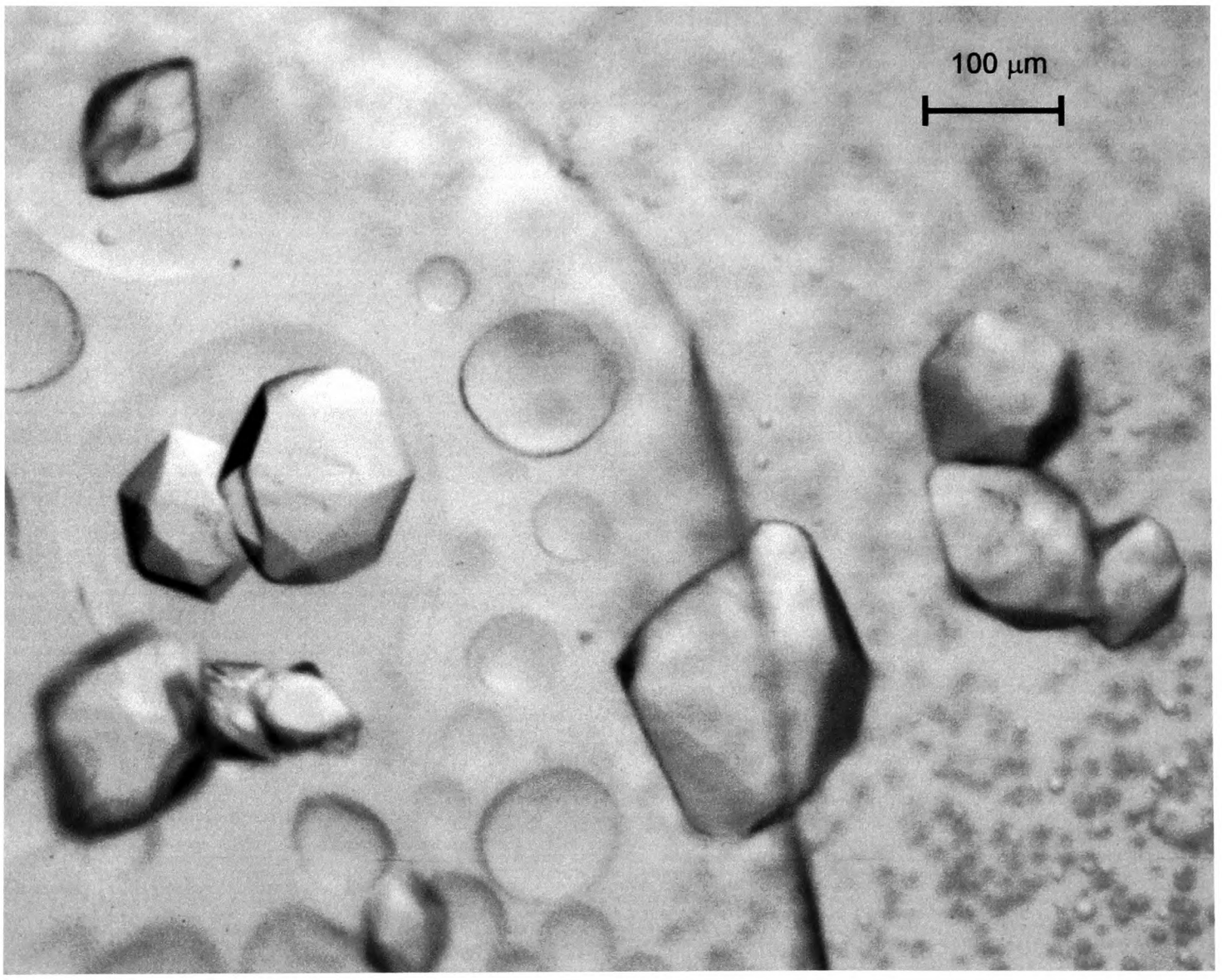


Fig.6.3. ^{4}F1^{5}F1/D3 crystals grown in 10% (**a**) and 9% (**b**) v/v PEG 8000 and 0.2 M $(NH_4)_2SO_4$.

osmic optics and a MAR345 image plate detector, giving a maximum resolution of 4 Å. Higher-resolution data were obtained by J. Murray & E. Lowe at the synchrotron beamline ID14-EH1 (ESRF, Grenoble, France), equipped with an ADSC Q4R detector as advised by the Strategy program (MOSFLM package (Leslie, 1990)) used to analyze the initial diffraction images (Ravelli *et al.*, 1997). The autoindexing and postrefinement of crystal cell parameters, crystal orientation and mosaicity were performed using MOSFLM. Refined unit cell parameters (shown in Table.6.2) were then used for integrating the images, also using MOSFLM.

Table.6.2. High-resolution data set parameters	
Maximum resolution	2.8 Å
Crystal-to-detector distance	200 mm
Number of diffraction images	180
Oscillation range ($\Delta\varphi$)	0.5°
Total φ range	90°
Exposure time	2 s
Wavelength	0.98 Å
Space group	$P6_122$ or $P6_522$
Unit cell dimensions	a = b = 91.4 Å, c = 126.6 Å
Unit cell angles	$\alpha = \beta = 90°, \gamma = 120°$
Unit cell volume	952072 $Å^3$
Mosaicity	0.5°

Table.6.3 shows the merging statistics for all space-groups suggested by MOSFLM. The agreement between observed and calculated amplitudes is called the R factor. R_{merge}

(merging R factor) is the sum of the differences of all measurements from the average value of the measurement divided by the sum of all measurements.

Table.6.3. High-resolution data set analysis for all possible space-groups, sorted by a number of rejections

Space-group	Rejections	R_{merge} (resolution(Å))	R factor	Completeness
p321	340	37.8% (2.8), 27.1% (2.85)	8%	90%
$p3_221$	343	38.3% (2.8), 27.4% (2.85)	8%	90%
$p3_121$	343	38% (2.8), 27.3% (2.85)	8%	90%
$p3_1$	446	29.5% (2.8)	7.70%	86.50%
$p3_2$	446	29.5% (2.8)	7.70%	86.50%
p3	460	29.5% (2.8)	7.90%	86.50%
$p3_112$	614	40.4% (2.8), 27.7% (2.85)	7.80%	88.30%
p312	734	40.9% (2.8), 27.7% (2.85)	7.70%	88.30%
$p3_212$	734	40.9% (2.8), 27.7% (2.85)	7.70%	88.30%
p6	316	39.5% (2.8), 27.4% (2.85)	8.20%	90.20%
$p6_5$	316	39.5% (2.8), 27.4% (2.85)	8.20%	90.20%
$p6_1$	362	39.5% (2.8), 27.2% (2.85)	8.20%	90.20%
$p6_2$	362	39.5% (2.8), 27.2% (2.85)	8.20%	90.20%
$p6_3$	362	39.5% (2.8), 27.2% (2.85)	8.20%	90.20%
$p6_4$	362	39.5% (2.8), 27.2% (2.85)	8.20%	90.20%
p622	401	43.7% (2.8), 31.3% (2.85)	8.50%	90.70%
$p6_122$	419	44.3% (2.8), 31.3% (2.85)	8.50%	90.70%
$p6_222$	419	44.3% (2.8), 31.1% (2.85)	8.50%	90.70%
$p6_322$	419	44.3% (2.8), 31.3% (2.85)	8.50%	90.70%
$p6_422$	419	44.3% (2.8), 31.1% (2.85)	8.50%	90.70%
$p6_522$	419	44.3% (2.8), 31.3% (2.85)	8.50%	90.70%

As the highest possible symmetry did not result in a much higher number of rejections and the R_{merge} value remained reasonable, two solutions were chosen: $P6_122$ and $P6_522$. These space-groups differ only in the direction of the screw-axis, which can be defined only after the structure determination. If the asymmetric unit characterized by either $P6_122$ or $P6_522$ space group contains 1 molecule of the $^4F1^5F1$/D3 complex, the estimated water content is ~80%, ~60% if 2 molecules are present, and ~40% for 3 molecules within a unit.

Further processing was carried out using the CCP4 suite (1994). After sorting of the reflections (SORT, CCP4), scale factors were estimated and images merged using SCALA (CCP4), giving 100% completeness up to a resolution of 2.8 Å (Table.6.4). However, as the data above the resolution of 2.92 Å were very weak with $I/\sigma I \leq 1$ (ratio of integrated intensity to its standard deviation) and high R_{merge}, they were excluded from the structure determination. The incompleteness of the 2.73 - 2.45 Å data is due to the use of a square detector plate, which only partially covered the reciprocal lattice at this resolution.

The first attempt to solve the crystal structure was made using MR with the structure of the $^4F1^5F1$ module pair in a complex with D3, determined by NMR (**Chapter 5**). Three different models were used: the average structure, a single structure which was the closest to the average one, and an ensemble of 10 of the lowest energy structures. The models were prepared according to the previously published protocols (Chen, 2001): all the flexible regions were removed and three different sets of structures prepared: all-Gly,

Ala-Gly-Ser (the side-chains other than Ala and Gly were replaced by Ser) and Ala-Gly-Ser with unmodified residues in the hydrophobic cores. The ensemble of 10 structures was superimposed on the backbone atoms of residues 3-91 prior to modifications. Similar models were prepared for an ensemble of 10 of the lowest energy structures of the unbound $^{4}F1^{5}F1$, as they exhibited higher precision in comparison with $^{4}F1^{5}F1$ bound to D3.

Table.6.4. Summary of the scaled data set taken from the SCALA output

Resolution shell (Å)	No. of unique reflections	<I/σI>	Completeness (%)	R_{merge}	Multiplicity
22.92 – 7.73	454	9.9	96.2	0.052	7.4
7.73 – 5.47	779	9.8	100	0.058	8.6
5.47 – 4.47	969	9.4	100	0.064	8.9
4.47 – 3.87	1138	7.6	100	0.080	9.1
3.87 – 3.46	1260	6.0	100	0.111	9.2
3.46 – 3.16	1376	3.9	100	0.182	9.4
3.16 – 2.92	1498	1.9	100	0.390	9.5
22.92 – 2.92	**7474**	**6.9**	**99.5**	**0.134**	**8.9**
2.92 – 2.73	1606	1.0	100	0.749	9.6
2.73 – 2.58	1611	0.7	98.2	1.037	6.9
2.58 – 2.45	833	0.6	61.2	1.195	3.2

The following MR programs were used: AMORE (Navaza, 1994), MOLREP (Vagin & Teplyakov, 1997) and BEAST (Read, 2001). To provide an independent control, 5-10% of the reflections are excluded from the model calculation. The degree of their subsequent agreement with the calculated model is called R_{free}. To consider a refined structure as valid, the R value should not exceed 25% and R_{free}, always slightly higher, 30%. In all the

cases of MR tested here, the peaks from the rotation function were very similar in height, failing to provide a clear solution. The R values directly after MR oscillated around 55-60% (Table.6.5).

Table.6.5. Examples of top 5 solutions (rotation function) and top 2 solutions (translation function) from the MR protocol (searching for 15 translations solutions for each of top 20 rotation functions)

Rotation solution	R factor	R/σ
1	134.5	3.64
2	134.5	3.64
3	123.8	3.35
4	123.8	3.35
5	114.0	3.08
Translation solution	R factor	Correlation
1_7	0.643	0.243
1_14	0.645	0.238
2_12	0.640	0.245
2_11	0.642	0.242
3_9	0.596	0.353
3_12	0.596	0.352
4_6	0.594	0.358
4_7	0.599	0.359
5_1	0.601	0.348
5_9	0.601	0.336

Refinement with REFMAC (CCP4, Murshudov *et al.*, 1997) was attempted using either the rigid-body or the restrained maximum likelihood method, but it did not improve the R factors significantly and the electron density maps did not show any additional elements

except the backbone of the ^{4}F1^{5}F1 module pair. A reason for this probably lies in the nature of the complex. The ^{4}F1^{5}F1/D3 complex is elongated in shape, therefore intra- and intermolecular vectors are very similar and it is difficult to distinguish between them during MR, especially when, as in this case, the quality of the data was not the highest. Also, the structure itself is a difficult case to solve as it contains two almost identical modules and a peptide string, with the lack of structural data for the latter. The whole complex is practically a series of β-sheets folded on top of each other, which also does not provide enough sampling to distinguish the correct orientation and identity of its elements. The native set of data was rather weak and did not reach very high-resolution limits, therefore an additional source of phases apart from MR is required. Trials with heavy-atom derivatives are being carried out with a view to obtaining the missing information.

As the ^{4}F1^{5}F1/D3 complex appeared difficult for both crystallizing and data acquisition, alternative solutions have been sought. The most convenient way to obtain phases in this case would be by expressing a ^{Se}Met derivative of one of the complex components. Unfortunately the D3 peptide, which would be relatively easy to produce in *Escherichia coli*, does not possess any Met in its sequence. Similar expression of ^{4}F1^{5}F1 in *P. pastoris* is more problematic due to a high toxicity of such a process and there have been few reports of using this approach. Moreover, the two Met residues in ^{4}F1^{5}F1 are positioned next to each other, which would make an interpretation of the diffraction data very difficult.

A larger protein complex might be easier (and faster) to crystallize. Thus, attention was focused on the whole N-terminal domain of Fn and its bacterial ligand. Work is currently underway to identify the regions of FnbpA and SfbI, which bind $^{1\text{-}5}$F1 (**Chapter 7**). When these peptides have been identified, the ^{Se}Met derivative will be expressed and crystallization with the N-terminal domain of Fn will be attempted.

7. BINDING OF PEPTIDES FROM STAPHYLOCOCCAL AND STREPTOCOCCAL FNBPS TO THE ^{4}F1^{5}F1 MODULE PAIR OF HUMAN FIBRONECTIN

7.1. Introduction

Kelly *et al.* (1999) suggested that using synthetic peptide epitopes from bacterial adhesins could be an effective approach for prevention of microbial infections. They showed that a single adhesion-blocking agent could prevent the pathogen from colonizing the host tissue. As non-attached bacterial cells are expelled from the host, the selective pressure towards the development of microbial resistance is much weaker than in the case of antibiotics. FnBPs have also been shown to mediate internalisation of bacteria into host cells where they may evade the host immune system or administered antibiotics. Thus, blocking internalisation may improve the effectiveness of current antibiotics. The precise identification of binding sites on Fn and Fn-binding repeats will aid the development of such adhesion/internalisation-blocking therapeutic approaches to the treatment of staphylococcal and streptococcal infections.

One of the most striking features of the first identified FnBP, protein FnbpA from *S. aureus*, is the complete lack of any secondary structure in the Fn-binding region (Penkett *et al.*, 1998). This finding confirmed previous results from circular dichroism (CD), which suggested the Fn-binding regions of *S. aureus*, *S. dysgalactiae* and *S. pyogenes* were largely unstructured (House-Pompeo *et al.*, 1996). Due to this lack of structure, the definition of the Fn-binding repeats (Fig.1.5) cannot be based on structural elements. In addition, sequence comparison could not predict the binding properties of apparently homologous repeats or the cross-reactivity observed in inhibition studies with Fn-binding repeats of different species – eg. the B repeat region from *S. dysgalactiae* FnBB was the strongest inhibitor of bacterial adhesion to Fn not only for *S. dysgalactiae* itself, but also for *S. aureus* and *S. pyogenes* (Joh *et al.*, 1994).

Huff *et al.* (1994) and Penkett *et al.* (2000) obtained results suggesting that the D1-D4 sequence contains more than one binding site for $^{4}F1^{5}F1$. As the C-terminal part of each of the D repeats was most affected by the addition of $^{4}F1^{5}F1$, these C-terminal regions are likely to contain the main binding site. McGavin *et al.* (1991) have also shown, through inhibition studies with fragments of D3 and trypsin/chymotrypsin-digested fragments of Fn, that D3 contains two Fn-binding sites.

Both the bacterial FnBPs and their binding site on Fn (i.e. the N-terminal domain) are repetitive in nature. The FnBPs contain peptide repeats of similar sequence, the N-terminal domain of Fn contains five F1 modules, each with a similar structure. Using

a peptide (B3) from *S. dysgalactiae* FnBB, work in this laboratory dissected this peptide into segments that bound specifically to ^{1}F1 and ^{2}F1. Thus a hypothesis was formed that FnBPs might contain specific sequences which bind to each of five F1 modules in the N-terminal domain. Based on this hypothesis, a comprehensive sequence analysis, and the structure of the ^{1}F1^{2}F1/B3 complex (J. Potts – manuscript in preparation), putative F1-binding peptides from both streptococcal and staphylococcal FnBPs (of ~10 amino acids) were identified (Fig.7.1). Each motif is predicted to bind to a specific F1 module.

The pattern of Fn-binding motifs suggests continuous binding to the $^{1\text{-}5}$F1 modules. According to this model, in most streptococcal FnBPs there is a single ^{1}F1-binding motif at the C-terminus, adjacent to the wall-spanning domain. Moving towards the N-terminus there are further putative $^{2\text{-}5}$F1-binding sequences, repeated several times, the number of repetitions varying between different FnBPs. Thus each FnBP could bind several N-terminal domains of Fn. The polyvalent protein-protein interaction between each string of F1-binding motifs and an individual $^{1\text{-}5}$F1 domain has been named a 'β-zipper' (Fig.7.2). The β-zipper model has not yet been confirmed for staphylococcal FnBPs, but potential ^{2}F1- and ^{4}F1-binding motifs were identified by a sequence alignment (Fig.7.1 - J. Briggs - unpublished results). The ^{1}F1-binding sequence seems to be missing, and the identification of motifs recognizing ^{3}F1 and ^{5}F1 remains problematic due to a lack of sequence conservation in these regions. The studies presented here aim to test the β-zipper hypothesis by testing the binding of bacterial FnBP motifs to their predicted binding partner (F1 module) in the N-terminal domain of Fn.

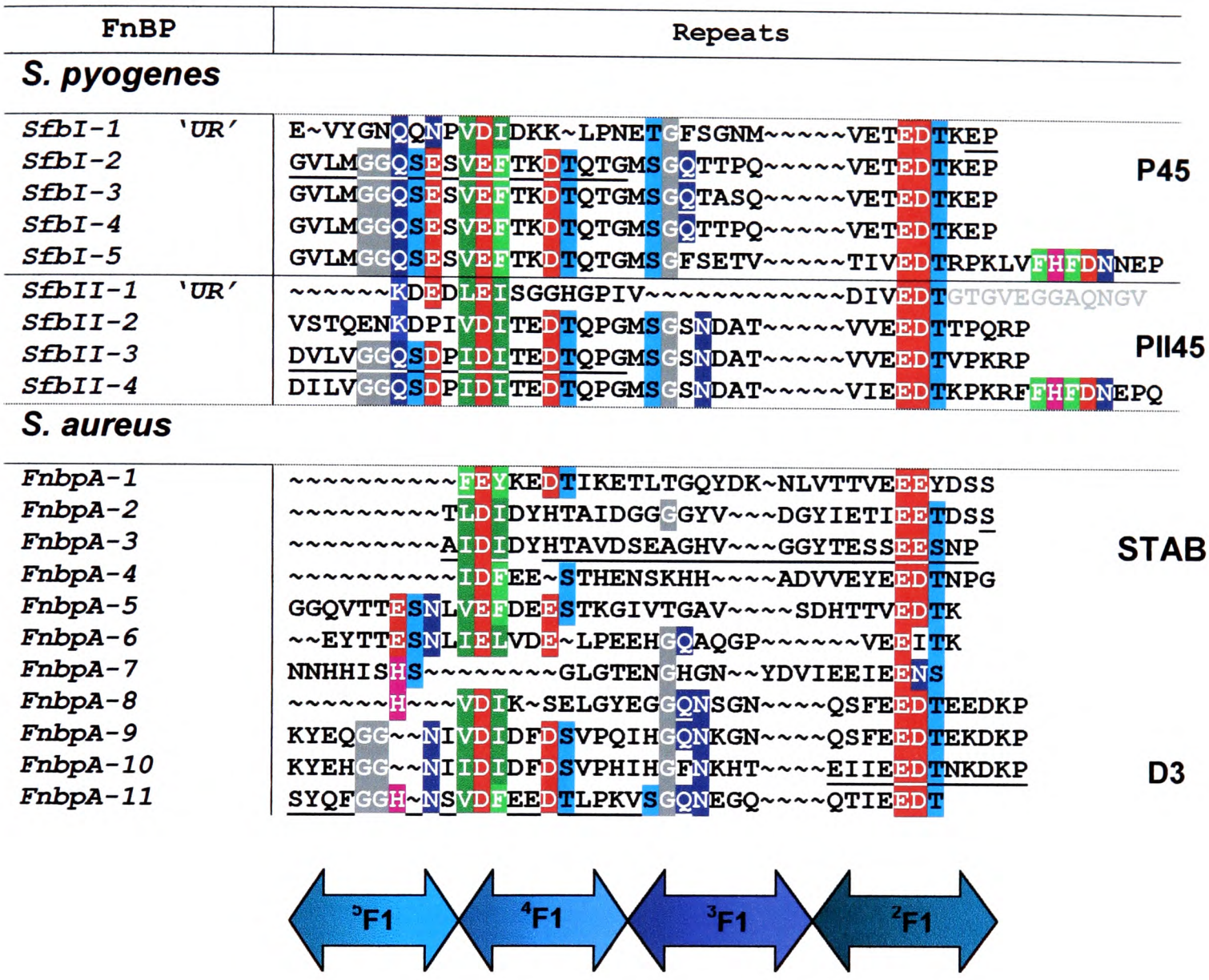


Fig.7.1. New sequence alignment of Fn-binding repeats. Three FnBPs are presented: SfbI and SfbII from *S. pyogenes* and FnbpA from *S. aureus*. This model identifies short repeated units, each potentially capable of binding the $^{2\text{-}5}$F1 N-terminal domain of fibronectin (FnBPs from *S. pyogenes* also can bind the ^{1}F1 module). Based on U. Schwarz-Linek *et al.* (manuscript in preparation). The sequences of Fn-binding motifs used in these studies are underlined.

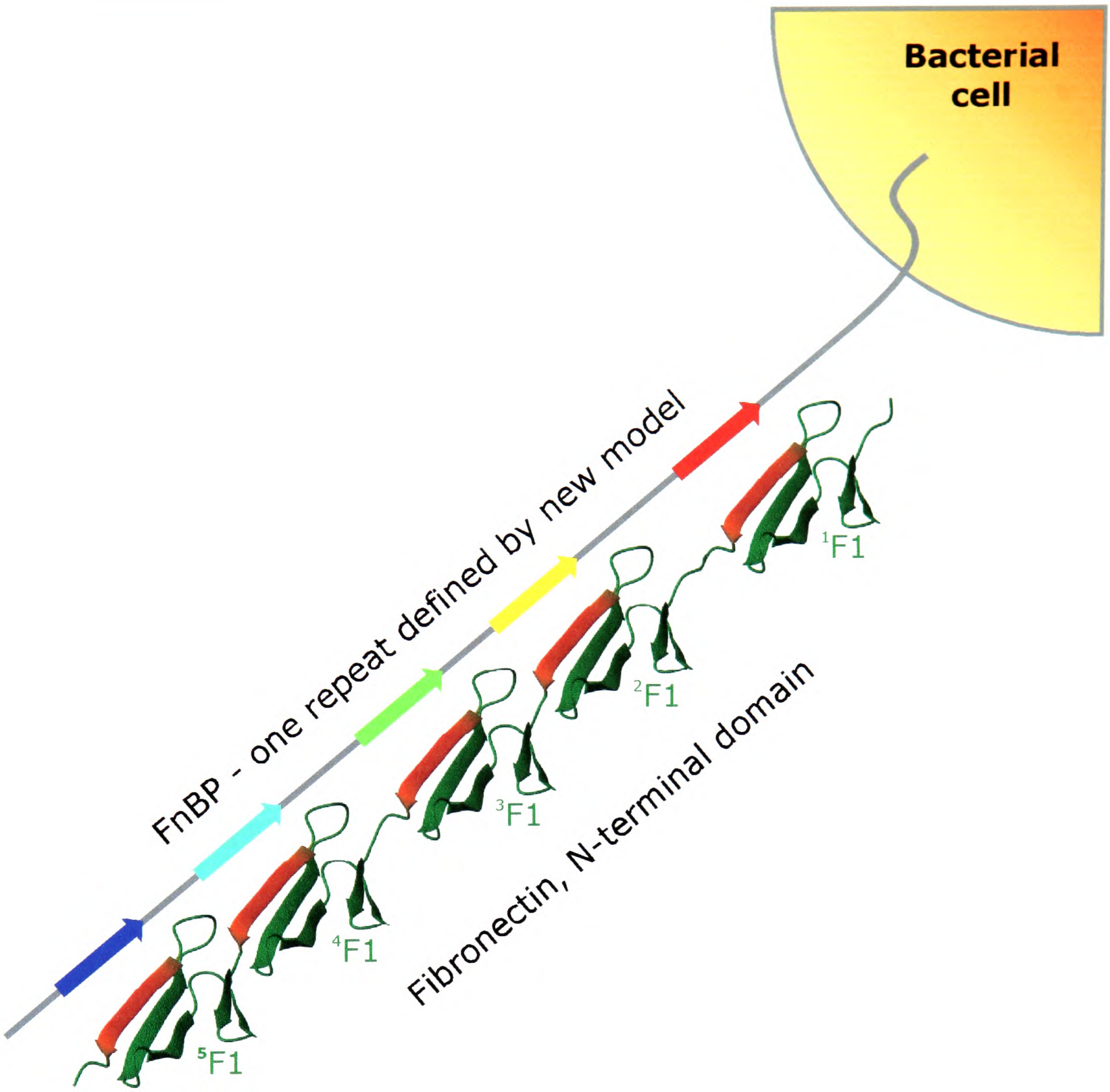


Fig.7.2. Representation of the 'β-zipper' model. The E strand of each F1 module is shown in red. Short peptides within the FnBP are predicted to form an additional antiparallel β-strand along each E strand. The figure was kindly provided by U. Schwarz-Linek.

The UR domain of SfbI has been shown to cooperate with the C-terminal Fn-binding repeats in binding of intact Fn as well as its 70 kDa fragment (Ozeri *et al.*, 1996), which encompasses the N-terminal domain and the gelatin-binding domain. As the UR domain has also been shown to block Fn matrix assembly, it has been postulated, that it contains a binding site for the 70 kDa domain of Fn (Tomasini-Johansson *et al.*, 2001). UR was shown to be crucial in streptococcal invasion of respiratory epithelial cells (Talay *et al.*, 2000). As an isolated peptide, UR is also capable of inhibiting streptococcal invasion of host cells (Ozeri *et al.*, 1998). In binding studies with deletion mutants of the 70 kDa domain (Ensenberg *et al.*, 2001), deletion of $^{1\text{-}3}$F1 from the N-terminus resulted in loss of binding. In general the binding was weaker when any two adjacent F1 modules were deleted. The studies presented here aim to identify the presence of F1-binding sites in UR.

7.2. Methods

Materials

^{15}N-labelled and unlabelled ^{4}F1^{5}F1 were recombinantly expressed and purified as described in **Section 2.1**. Except where stated otherwise, the peptides were purchased from Alta Bioscence (Birmingham, UK) or prepared in the peptide synthesis facility in the Oxford Centre for Molecular Sciences in a capped form (acetylated N-terminus and amide group on the C-terminus). The Df5 peptide was uncapped to improve its solubility. A2, UR and SUR were provided by M. Höök; UR and SUR were recombinantly expressed as described previously (Joh *et al.*, 1998).

Isothermal titration calorimetry

Isothermal titration calorimetry (ITC) is one of the most accurate methods for the determination of dissociation constants in the nM – μM range (Doyle, 1997). In the ITC experiment, after each injection of a precise amount of ligand into the cell (which contains the macromolecule), heat is released or absorbed as a result of the interaction between ligand and macromolecule. A pair of identical cells is enclosed in an adiabatic inner shield inside an adiabatic outer shield (Fig.7.3). A spinning syringe is utilized for injecting and subsequent mixing. Temperature differences between the reference cell and the sample cell are measured and calibrated to power units. An injection which results in the release of heat (exothermic) within the sample cell causes a negative change in the DP (differential power) since the heat evolved provides heat that the DP feedback is no longer required to provide. The opposite is true for endothermic reactions.

For a ligand L binding to a macromolecule/protein P the single-site binding constant for complex PL is

$$K_B=[PL]/[P][L] \qquad (7.1) \quad \text{and}$$

$$\Delta G = -RT\ln K = \Delta H - T\Delta S \qquad (7.2)$$

Where ΔG, ΔH and ΔS are the free energy, enthalpy, and entropy change for single-site binding. By a non-linear least squares fit of calorimetric titration data, the parameters K_B, ΔH, and n are determined directly in a single experiment and ΔG and ΔS may then be calculated (VP-ITC MicroCalorimeter User's Manual).

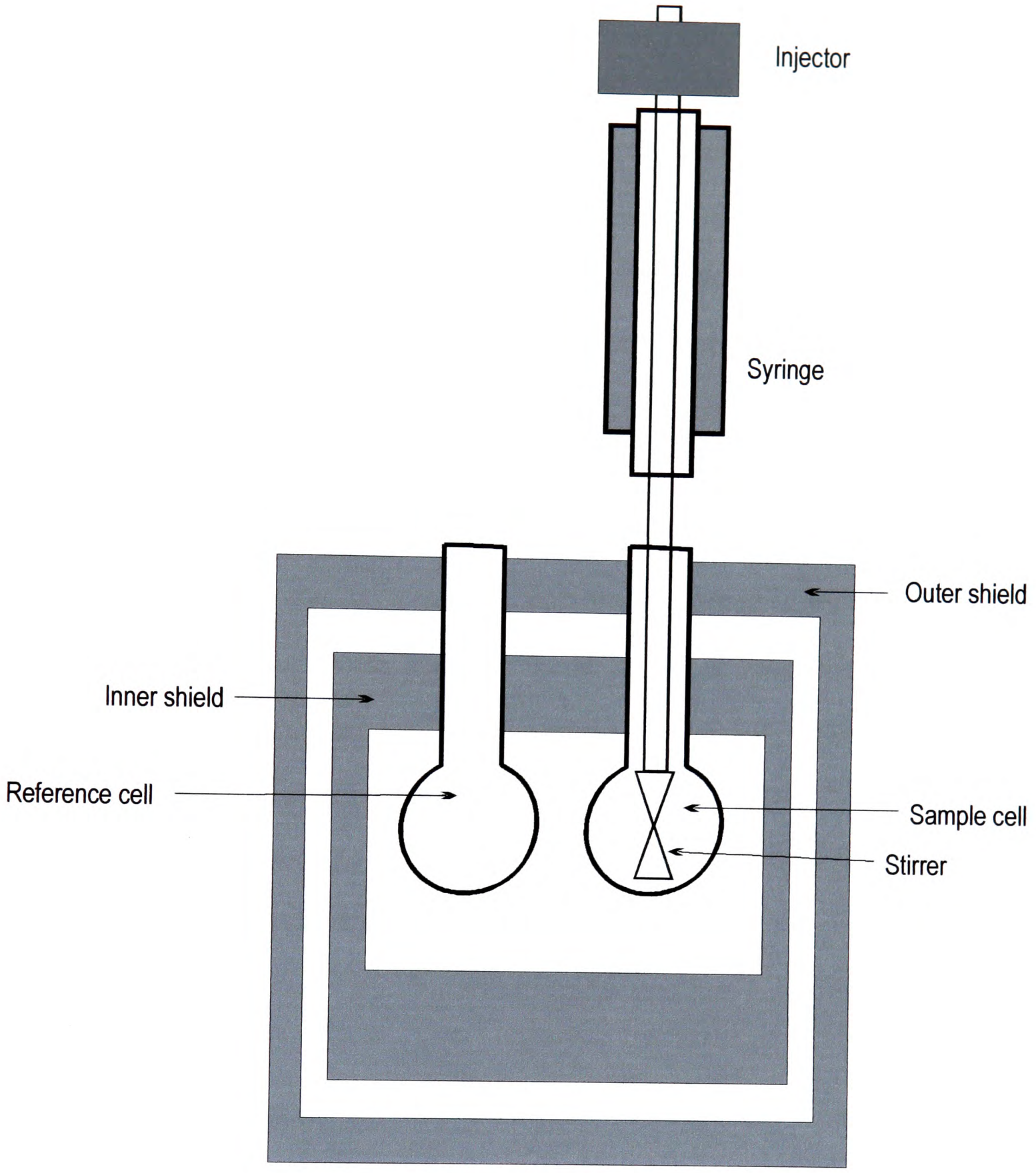


Fig.7.3. Schematic representation of the ITC apparatus. Both cells are placed in a well-isolated heating jacket. The ligand injections are performed into the sample cell with constant stirring and the change in power required to keep both cells at the same temperature is measured.

ITC experiments were carried out on a VP-ITC microcalorimeter (MicroCal Inc.) at 25°C and 30°C. In a typical experiment, the cell contained 0.1 mM $^{4}F1^{5}F1$ (1.4 ml) and the syringe contained 1.2-1.4 mM ligand (290 µl). In the 'reverse' experiment for the A2 peptide, the cell contained 0.1 mM A2 and the syringe contained 1.2 mM $^{4}F1^{5}F1$. Both solutions were buffered with 10 mM sodium acetate buffer (pH 5.0) and degassed for 20 min at 15°C. The titration consisted of a preliminary injection of 2 µl and 30-40 injections of 4 or 5 µl with an injection speed of 0.5 µl/sec, a stirring speed of 310 rpm and delay time between injections of 4 min.

To account for the heat of dilution, a blank titration was performed in which the ligand solution was injected into buffer in the cell (in the absence of macromolecule). The averaged heat of dilution from 15-20 injections was subtracted from the main experiment. The data were analysed using MicroCal Origin software (version 5.0) and fitted with a non-linear function to a single-site model. The exception was the data for the PII45 peptide, which was also fitted to a two-site model.

NMR experiments

For NMR studies of the macromolecule-ligand complexes it is important to know the exact amounts of bound and unbound species. The concentration of bound macromolecule in a sample containing the macromolecule and a binding peptide can be determined, if the dissociation constant (K_D) for the reaction is known.

$$[PL]=\tfrac{1}{2}\{K_D+[P_T]+[L_T]-((K_D+[P_T]+[L_T])^2-4[P_T][L_T])^{1/2}\} \qquad (7.3)$$

[PL] - complex concentration

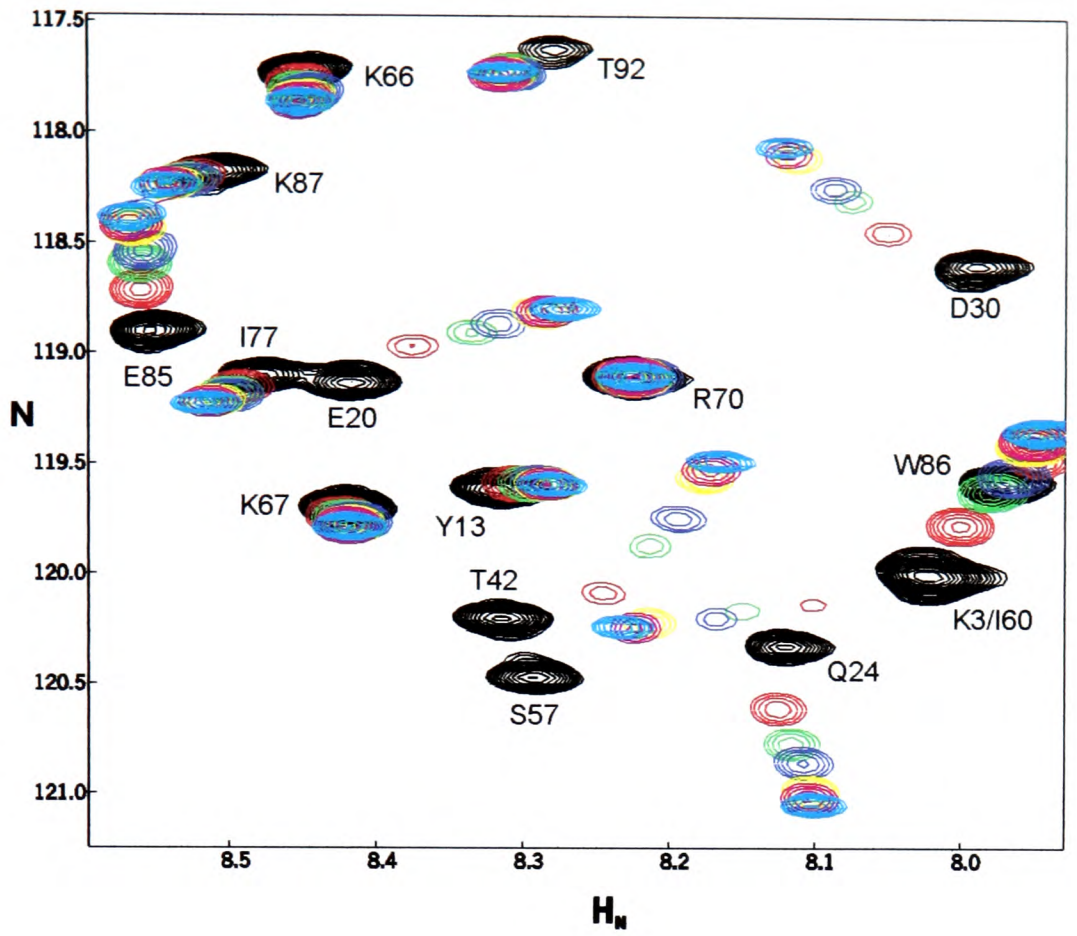
[P_T] – total macromolecule concentration

[L_T] – total ligand (peptide) concentration

NMR spectra were acquired at ^1H frequencies of 500 and 600 MHz on spectrometers built in-house at the Oxford Centre for Molecular Sciences and incorporating Oxford Instruments Magnets and GE/Omega computers. Spectra were acquired at 37°C unless stated otherwise. Chemical shift changes were measured by acquiring a series of ^{15}N-^1H HSQC (Bodenhausen & Ruben, 1980) spectra typically with 0.1 or 0.2 mM ^{15}N-$^4F1^5F1$, or 0.1 mM ^{15}N-$^1F1^2F1$, or 0.1 mM ^{15}N-$^2F1^3F1$ with addition of identical ligand aliquots to the point of saturation, if possible. The acquisition parameters for the $^4F1^5F1$ experiments were 1024 complex points for 10000 Hz and 512 total points for 1263 Hz or 1515 Hz in t_2 and t_1, respectively. For $^1F1^2F1$ and $^2F1^3F1$, the experiments were carried out at 25°C with the acquisition parameters: 1024 complex points for 10000 Hz and 512 total points for 1603 Hz or 1923 Hz in t_2 and t_1, respectively.

In an HSQC spectrum, a single peak is observed for each $^1H^{15}N$ group in a ^{15}N-labelled macromolecule. Upon addition of the ligand, changes in the chemical shift of some peaks of the macromolecule will be observed due to the ligand binding (Fig.7.4a). When the amount of ligand added is sufficient to saturate the binding sites, the final chemical shifts correspond to those of bound macromolecule. If the peaks corresponding to the bound and unbound species are in fast exchange on the NMR timescale, the chemical shifts for each addition of the ligand can be extracted and the

a.



b.

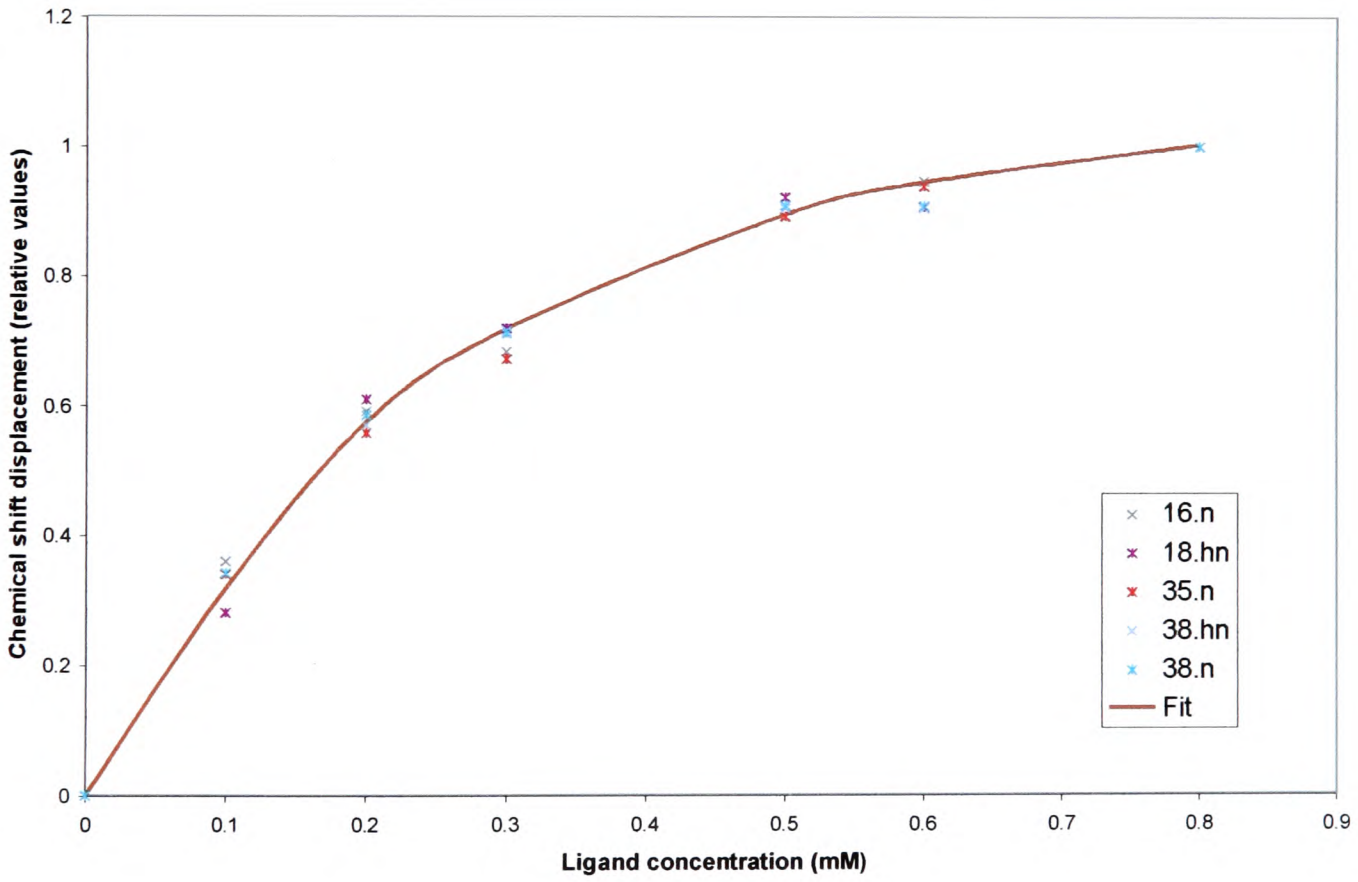


Fig.7.4.

a. Overlays of HSQC spectra of 0.2 mM ^{15}N-$^{4}F1^{5}F1$ with Df4 (protein:peptide ratios of 1:0, 1:0.5, [illegible], 1:1.5, [illegible], 1:3, 1:4), at 37°C, pH 5.0. The first experiment (in black) and the last (in blue) were recorded with 64 scans compared with 16 scans for other spectra.

b. An example of the fit of $^{1}H^{N}$ and ^{15}N chemical shift changes to estimate the binding affinity (determined K_D = 0.1 ± 0.01 mM). The curve shown was calculated using the data for H^{N} of G35.

K_D determined by a non-linear fit (equation 7.4 - Morton *et al.*, 1996) for each bound residue separately. An example of such a fit is shown in Fig.7.4b.

$$\Delta = (K_D+L+P-((K_D+L+P)^2-4LP)^{0.5}\delta_{max}0.5/P \qquad (7.4)$$

Δ – the chemical shift difference between unbound and partially bound states

L – total ligand concentration

P – total protein concentration

δ_{max} – chemical shift at saturation (i.e. chemical shift difference between unbound and fully bound states)

7.3. Results & Discussion

For studies of *S. aureus* binding to Fn, the strongest Fn-binding repeat, D3 from FnbpA, together with smaller fragments from this repeat were chosen (Fig.7.1: FnbpA-10 and –11), as well as the peptides from the FnbpA B region (Fig.1.5, 7.1: FnbpA-2 and –3):

D3 EIIEEDTNKDKPSYQFGGHNSVDFEEDTLPKV

D3t KPSYQFGGHNSVDFEEDTLPKV

Df4 NSVDFEEDTLPK

Df5 SYQFGGHNSVD

Df2 TEIIEEDTNKDK

B region

STAB SAIDIDYHTAVDSEAGHVGGYTESSEESNP

N-STAB SAIDIDYHTAVDSE

C-STAB AGHVGGYTESSEESNP

For studies of *S. pyogenes* binding to Fn, peptides from the C-terminus (previously P repeats – Fig.1.5, now SfbI-1 and –2 - Fig.7.1) and UR region of SfbI were used. A peptide from the C-terminus of SfbII (here called PII45 - Fig.7.1: SfbII-3) was also used. One definition of a functional UR domain (Ensenberg *et al.*, 2001) encompassed the first six residues of the Fn-binding repeat P1 (Ozeri *et al.*, 1996). The alignment, presented in Fig.7.1, identifies these residues as part of a putative ^{2}F1-binding motif. Therefore, a truncated version of UR without these residues (short UR or SUR) was also studied here. The A2 motif from *S. dysgalactiae* FnbB was used for affinity studies with the ^{4}F1^{5}F1 module pair of Fn using ITC.

P45 EPGVLMGGQSESVEFTKDTQTG

P4 ESVEFTKDTQTG

P5 EPGVLMGGQSE

UR KDQSPLAGESGETEYITEVYGNQQNPVDIDKKLPNETGFSGNMVETEDT

SUR KDQSPLAGESGETEYITEVYGNQQNPVDIDKKLPNETGFSGNM

PII45 DVLVGGQSDPIDITEDTQPG

A2 VIIGGQGQVIDFTEDTQSG

Significance of studied affinities and chemical shift changes

The main aim of these studies was the identification of short sequences from the peptides shown above, that bind specifically to a single F1 module. The affinities obtained are frequently in the mM range which, on their own, may not seem of sufficiently high affinity to indicate importance *in vivo*. However, a ~10-fold increase in affinity is observed when two Fn-binding motifs linked together bind a module pair (see below), indicating that the full-length N-terminal domain-binding sequence should bind $^{2\text{-}5}$F1 or $^{1\text{-}5}$F1 with a K_D in the nM range. Thus, the detection of this weak binding is required to investigate this very high-affinity interaction. In addition, the ^{1}H chemical shift changes discussed below often are lower than 0.1 ppm. Normally this magnitude of change might not be considered as significant, although there have been reports of such changes as an indication of binding in literature, eg. by Worrall *et al.* (2002). Here, by comparing the binding of different ligands to ^{4}F1^{5}F1 (Fig.7.5) it is apparent that for some peptides (eg. Df5 and P5) the ^{1}H chemical shift changes induced in ^{4}F1^{5}F1 do not exceed 0.05 ppm, supporting the interpretation of 0.1 ppm changes in the light of the model in Fig.7.1.

Identification of ^{4}F1- and ^{5}F1-binding motifs in *S. aureus* FnbpA

Df4 was predicted, according to a model presented earlier (Fig.7.1), to consist of a

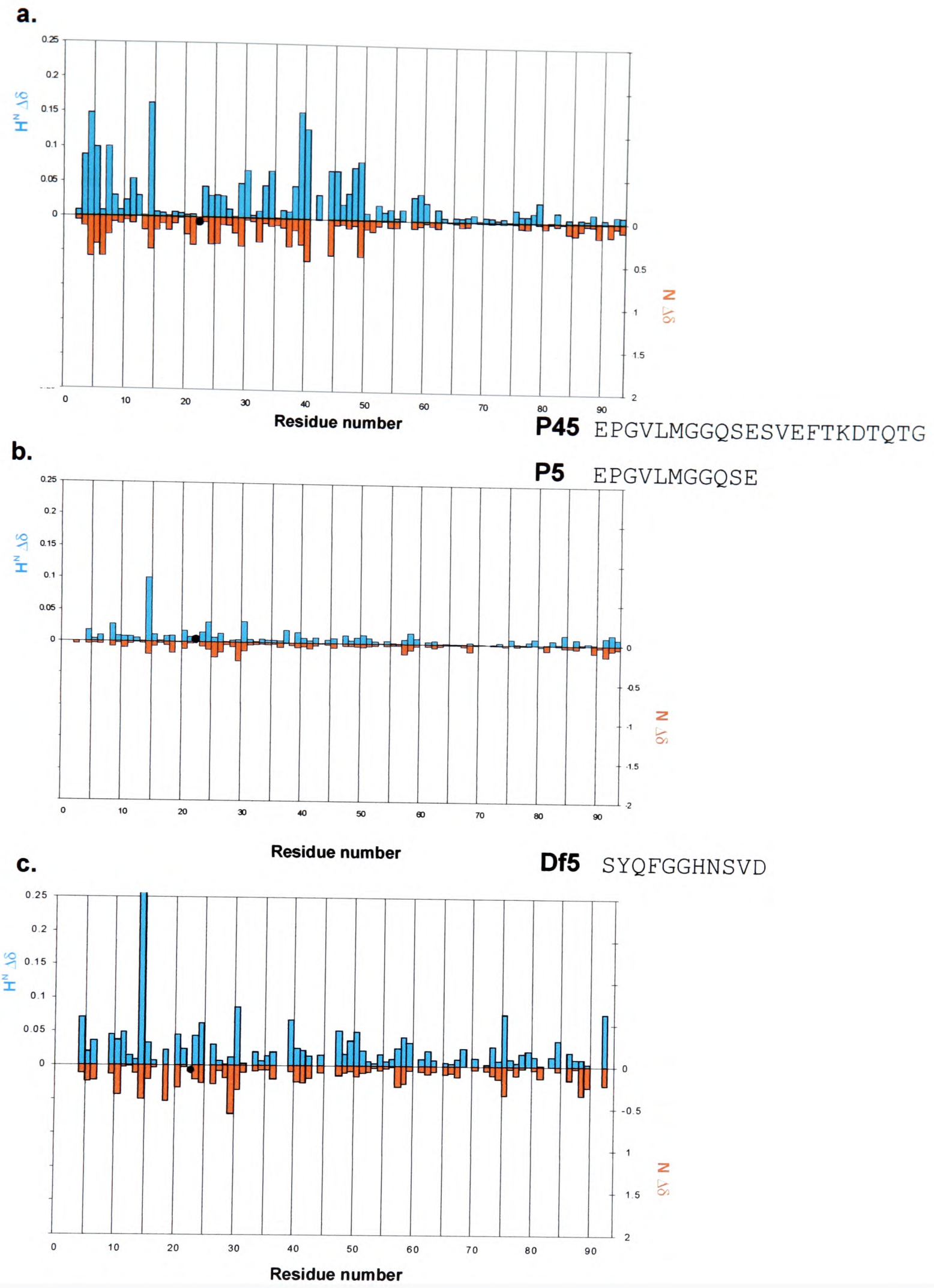


Fig.7.5. The magnitude of backbone $^{1}H^{N}$ and ^{15}N chemical shift changes in $^{4}F1^{5}F1$ upon P4, P5 and Df5 binding ($\Delta\delta = \delta^{bound} - \delta^{free}$) at 25°C. **a.** P4 - $\Delta\delta$ at the protein-peptide ratio of 1:10 (saturation), **b.** P5 - $\Delta\delta$ at the protein-peptide ratio of 1:3 - no saturation reached. **c.** Df5 - $\Delta\delta$ at the protein-peptide ratio of 1:2 – no saturation reached. • - proline residue.

single ^{4}F1-binding motif. The accuracy of this prediction can be assessed by comparing the chemical shift changes caused by Df4 with those caused by longer peptides (D3t and D3), containing Df4. On Df4 binding (Fig.7.6c, 7.7a), the affected ^{4}F1^{5}F1 residues are situated primarily on the A and E β-strands of ^{4}F1 and in the D-E loop in ^{4}F1. Residues of the intermodule interface (W26, R46, R48, N50, R55, S57, G80, N81, G82, R83, G84 and E85 - Fig.7.8) are also affected. The chemical shift changes in ^{4}F1 caused by D3 binding (Fig.7.6a) are almost identical to those caused by Df4. These results indicate that Df4 contains a complete ^{4}F1-binding motif, which binds to ^{4}F1 in the same way as full-length D3. The length of this motif is comparable with previously identified ^{1}F1- and ^{2}F1-binding motifs from *S. dysgalactiae* FnbB (U. Schwarz-Linek & J. Potts – manuscript in preparation), which further supports the finding.

Although the ^{4}F1^{5}F1 chemical shift maps upon Df4, D3t and D3 binding looked very similar (Fig.7.6), the affinities of peptides varied. The affinity of Df4 binding to ^{4}F1^{5}F1 was too low to be reliably measured using ITC. Therefore the K_D was determined from a series of HSQC experiments acquired with an increasing molar ratio of protein:peptide (as described above). Using this technique the affinity of Df4 binding to ^{4}F1^{5}F1 was estimated to be ~100 μM. K_D values were determined for the interaction of ^{4}F1^{5}F1 with D3 and ^{4}F1^{5}F1 with D3t using ITC. A K_D of 1.8 μM was obtained for the ^{4}F1^{5}F1-D3 interaction at pH 5.0 and 25°C (Fig.7.9a), and a K_D of 8 μM was obtained at the same conditions for the interaction between ^{4}F1^{5}F1 and D3t (Fig.7.9b). The experimental data in both cases are consistent with a single binding site and therefore with a 1:1 complex.

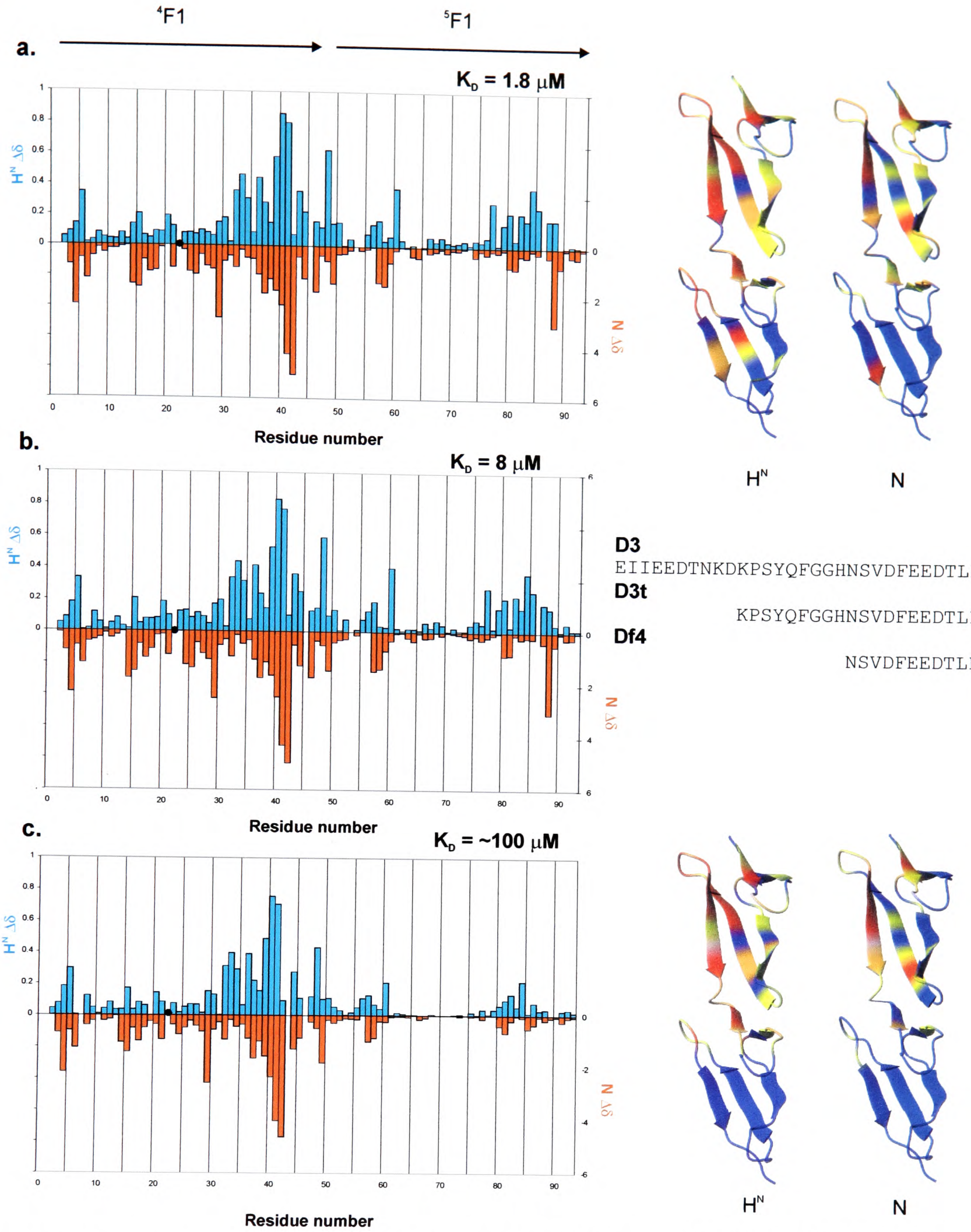


Fig.7.6. The magnitude of backbone $^1H^N$ and ^{15}N chemical shift changes in $^4F1^5F1$ upon D3, D3t and Df4 binding ($\Delta\delta = \delta^{bound} - \delta^{free}$) at 37°C, presented in a graph form and mapped onto the average structure of $^4F1^5F1$ bound to D3. K_D values were determined at 25°C. **a.** D3 - $\Delta\delta$ at the protein-peptide ratio of 1:2 (saturation), **b.** D3t - $\Delta\delta$ at the protein-peptide ratio of 1:3 (saturation). **c.** Df4 - $\Delta\delta$ at the protein-peptide ratio of 1:4 (saturation). •- proline residue. indicates $\Delta\delta$ > 0.05 ppm for H^N and > 0.5 ppm for N, orange $\Delta\delta$ > 0.1 ppm for H^N and > 1 ppm for N, and red $\Delta\delta$ > 0.2 ppm for H^N and > 2 ppm for N. indicates missing HSQC peaks, also very weak in HSQC of unbound $^4F1^5F1$. All other regions are shown in blue.

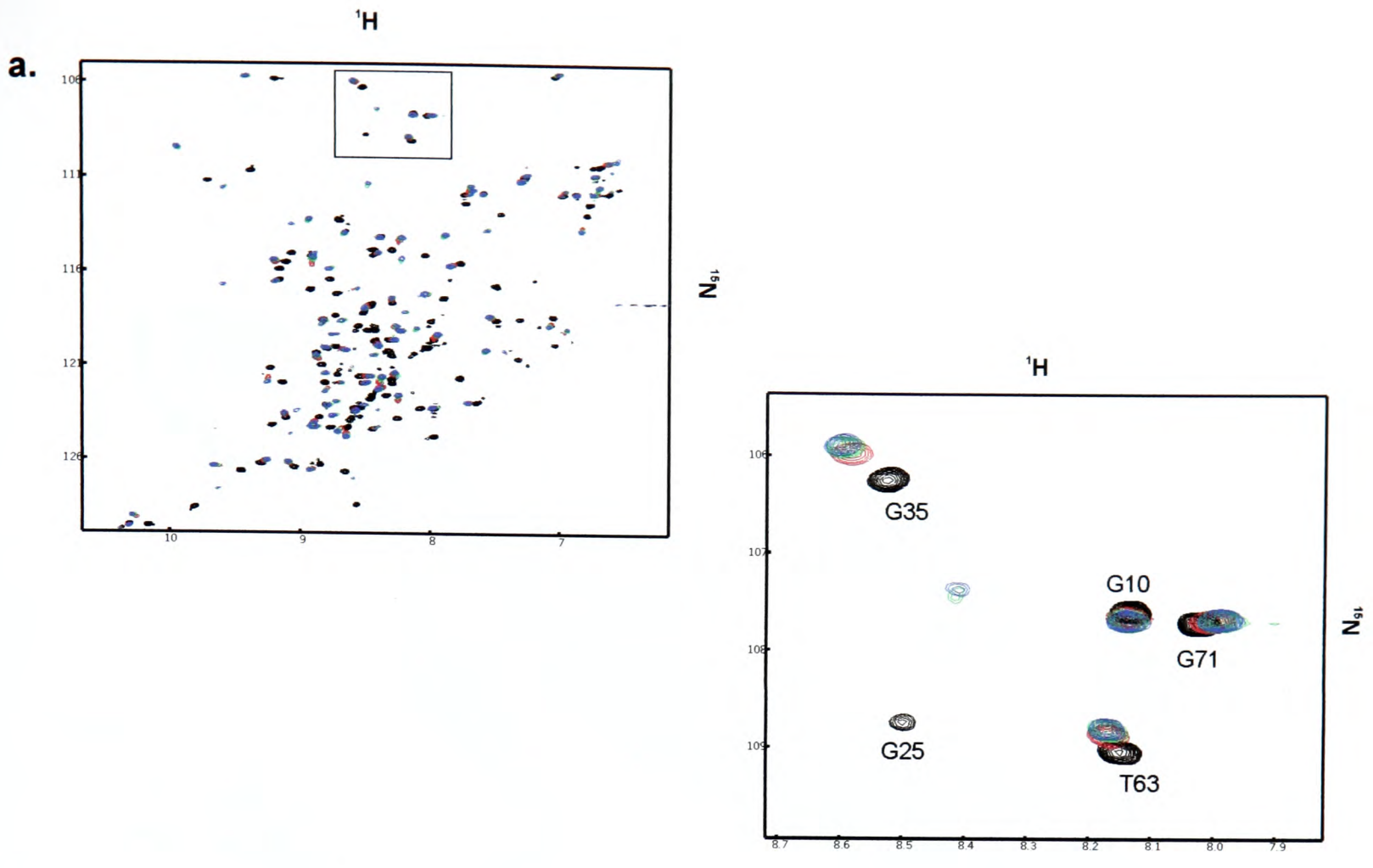


Fig.7.7. Overlays of HSQC spectra of 0.2 mM ^{15}N-^{4}F1^{5}F1 with ligands, performed at 37°C, pH 5.0. The areas in squares are zoomed in.

a. D3t - an overlay of 4 stages of an HSQC titration (protein:peptide ratios of 1:0, 1:1, [illegible], 1:4),

b. Df4 - an overlay of 4 stages of an HSQC titration (protein:peptide ratios of 1:0, 1:1, [illegible], 1:4).

The chemical shifts of ^{1}H^{15}N of G25 change less than in case of D3t binding, which is probably due to a difference in the change of intermodular interactions upon binding of both ligands.

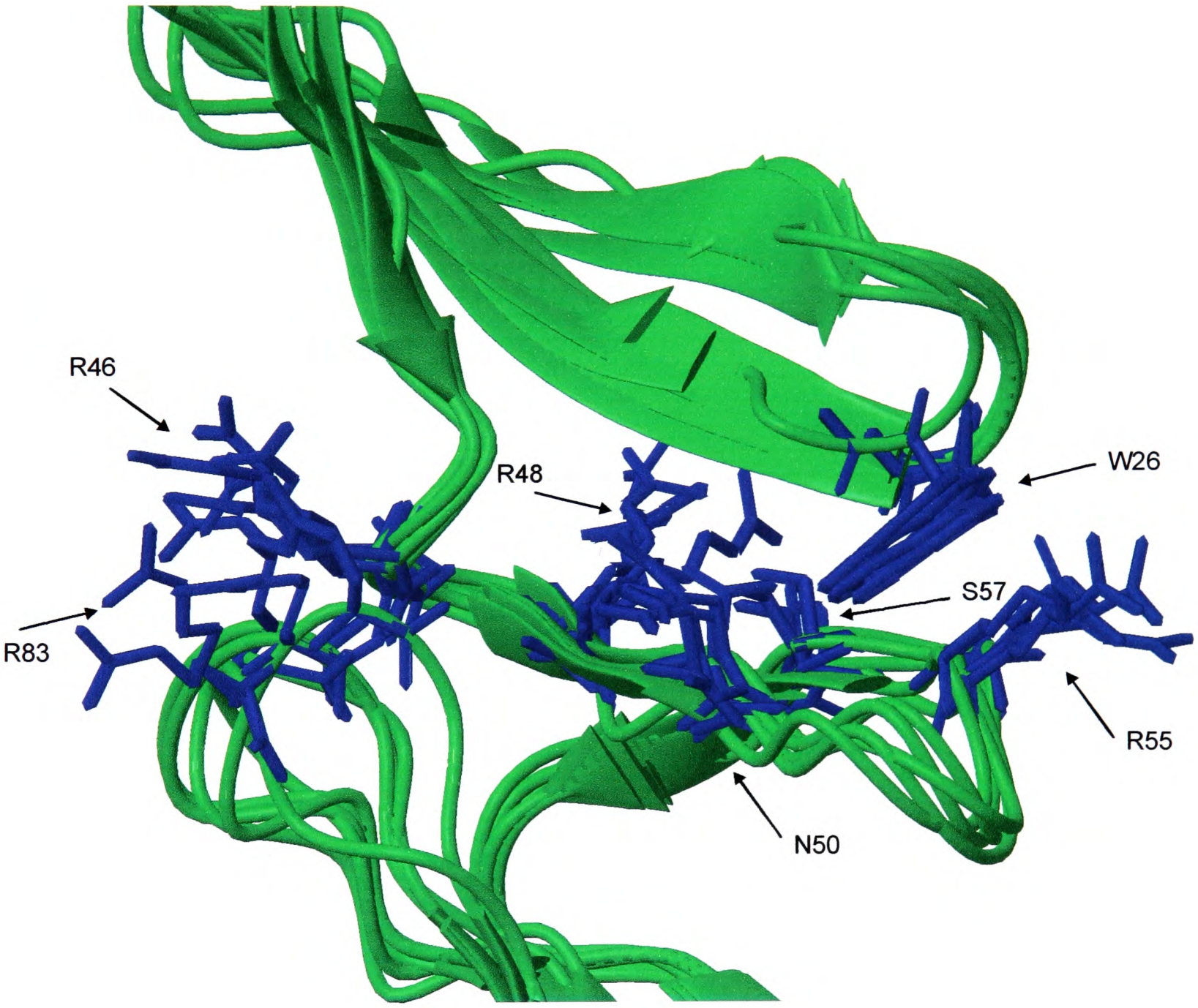


Fig.7.8. An expanded view of an overlay of five of the lowest energy structures of ^{4}F1^{5}F1 module pair in a complex with D3. Side-chains of residues stabilizing the intermodule orientation are indicated.

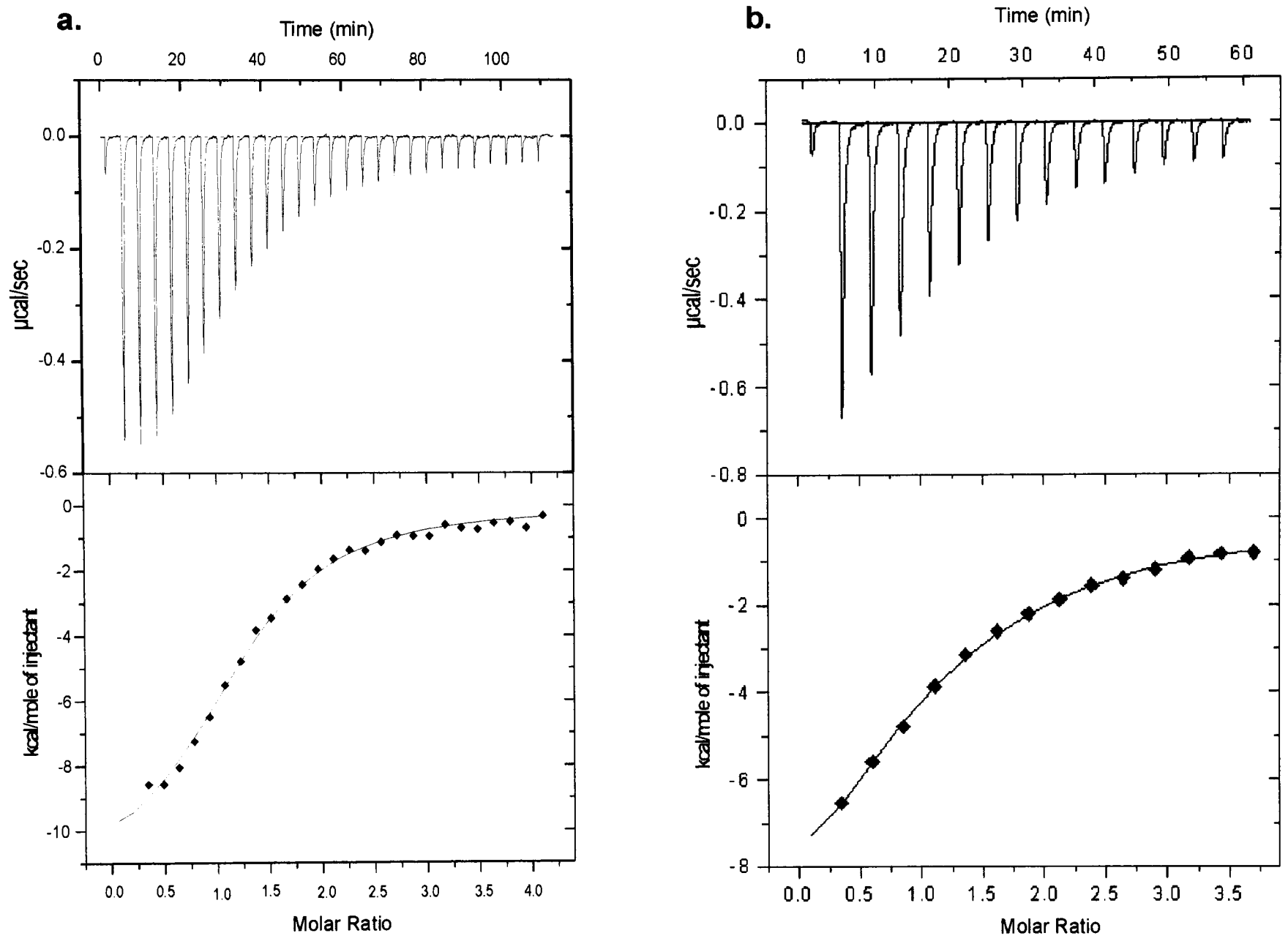


Fig.7.9. ITC experiments of $^{4}F1^{5}F1$ with D3/D3t. The solid lines describe the best fit of experimental points. **a.** D3 - 25°C. Heat of interaction obtained for 28 injections. Data were fitted using a one-site model resulting in: stoichiometry n=1.02 ± 0.028; K_D=1.8 ± 0.15 µM; ΔH=-11760 cal/mol; ΔS=-13.78 cal/mol·K. **b.** D3t - 25°C. Heat of interaction obtained for 16 injections. Data were fitted using a one-site model resulting in: stoichiometry n=0.94 ± 0.048; K_D=8 ± 0.51 µM; ΔH=-13970 cal/mol; ΔS=-23.58 cal/mol·K.

The ^{4}F1^{5}F1 chemical shift changes observed on D3 and D3t binding are very similar (Fig.7.6a&b), indicating that peptides bind in an almost identical way to this module pair. The first fourteen residues of D3 could be assigned in the spectra of the ^{4}F1^{5}F1/D3 complex in positions identical to unbound D3 (**Section 5.1**), and D3t is only ten residues shorter than D3. There is also no evidence from the peptide chemical shifts that D3 contains an additional binding site for ^{4}F1^{5}F1 (Penkett *et al.*, 2000). The results (Fig.7.6) indicate that Df4 binds to ^{4}F1 in a way identical to D3t and D3 however, there are differences in the shifts induced in ^{5}F1. Thus, the lower affinity observed for Df4 binding to ^{4}F1^{5}F1 (approx. 10-fold lower when compared to D3t binding to ^{4}F1^{5}F1) could be due to a lack of residues involved directly in ^{5}F1 binding.

Residues in ^{5}F1 that undergo significant chemical shift changes on binding of both D3 and Df4 are limited to those near the intermodule interface (Fig.7.6c). This could either indicate that Df4 is longer than the precise ^{4}F1-binding motif or that changes in ^{5}F1 on Df4 binding to ^{4}F1^{5}F1 are due to small differences in the intermodule interface (Fig.7.8). Fig.7.10 shows a space-filling model of the ^{4}F1^{5}F1 module pair bound to D3, were the backbone $^{1}H^{N}$, $^{1}H^{\alpha}$ and ^{15}N chemical shift changes data are complemented with the largest chemical shift changes of side-chain protons. The largest changes are concentrated on the one face of the molecule, around the 'groove' formed by both modules, indicating that this is the likely binding site for bacterial peptides.

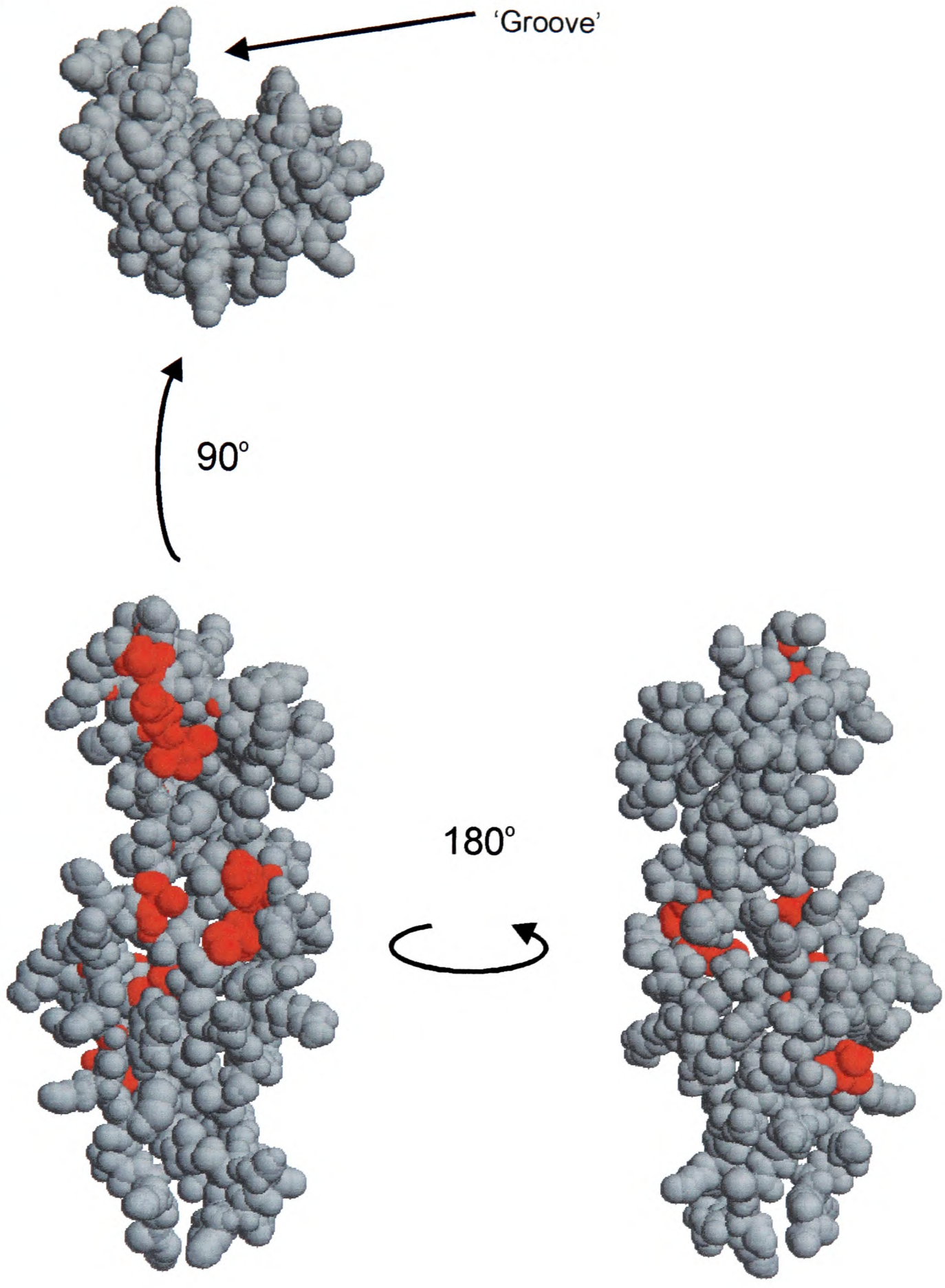


Fig.7.10. The largest proton (H^N, H^α, side-chain) chemical shift changes (red - $\Delta\delta = \delta^{bound} - \delta^{free} \geq 0.2$ ppm) mapped onto the space-filling model of the average structure of $^4F1^5F1$ bound to D3. All other regions are shown in grey.

The residues in ^{5}F1 affected by D3 (Fig.7.6a), but not by Df4 (Fig.7.6c) binding are primarily found in the E strand of ^{5}F1 (residues 85-88). As this is far from the intermodule interface, this suggests that the region of D3 N-terminal to Df4 interacts directly with this part of ^{5}F1. In addition, the orientation of the binding fragments of D3 for ^{4}F1 and ^{5}F1 is consistent with an antiparallel orientation of binding of ^{4}F1^{5}F1 and D3, as was observed for binding of ^{1}F1^{2}F1 to B3. The three-dimensional NMR solution structure of the complex of B3 from *S. dysgalactiae* with ^{1}F1^{2}F1 reveals an antiparallel orientation of the binding partners. The B3 peptide forms additional β-strands antiparallel to last β-strands from ^{1}F1 and ^{2}F1 Fn modules (J. Potts – manuscript in preparation), consistent with the observation of chemical shift changes in the E strands of ^{4}F1 and ^{5}F1.

Table.7.1. Comparison of minimised average structures of ^{4}F1^{5}F1 in unbound and bound state.

superposition of backbone atoms	rmsd (Å)
residues 3-45	0.98
residues 48-91	1.32
residues 3-91	2.05

Table 7.1 shows the comparison of minimised average structures of ^{4}F1^{5}F1 in unbound and bound state. The rmsd values for individual modules are within the range for the ensemble of structures of bound ^{4}F1^{5}F1. The maximum rmsd for both modules would be 1.5 Å, hence the value of 2 Å indicates a change in the intermodule orientation upon D3 binding. However, structures from two ensembles (unbound and bound state) overlap (Fig.7.11a), therefore no direct conclusion can be drawn. Unfortunately, all the orientational restraints used in the structure refinement refer to the backbone N-H bonds and thus did not improve the side-chain definition.

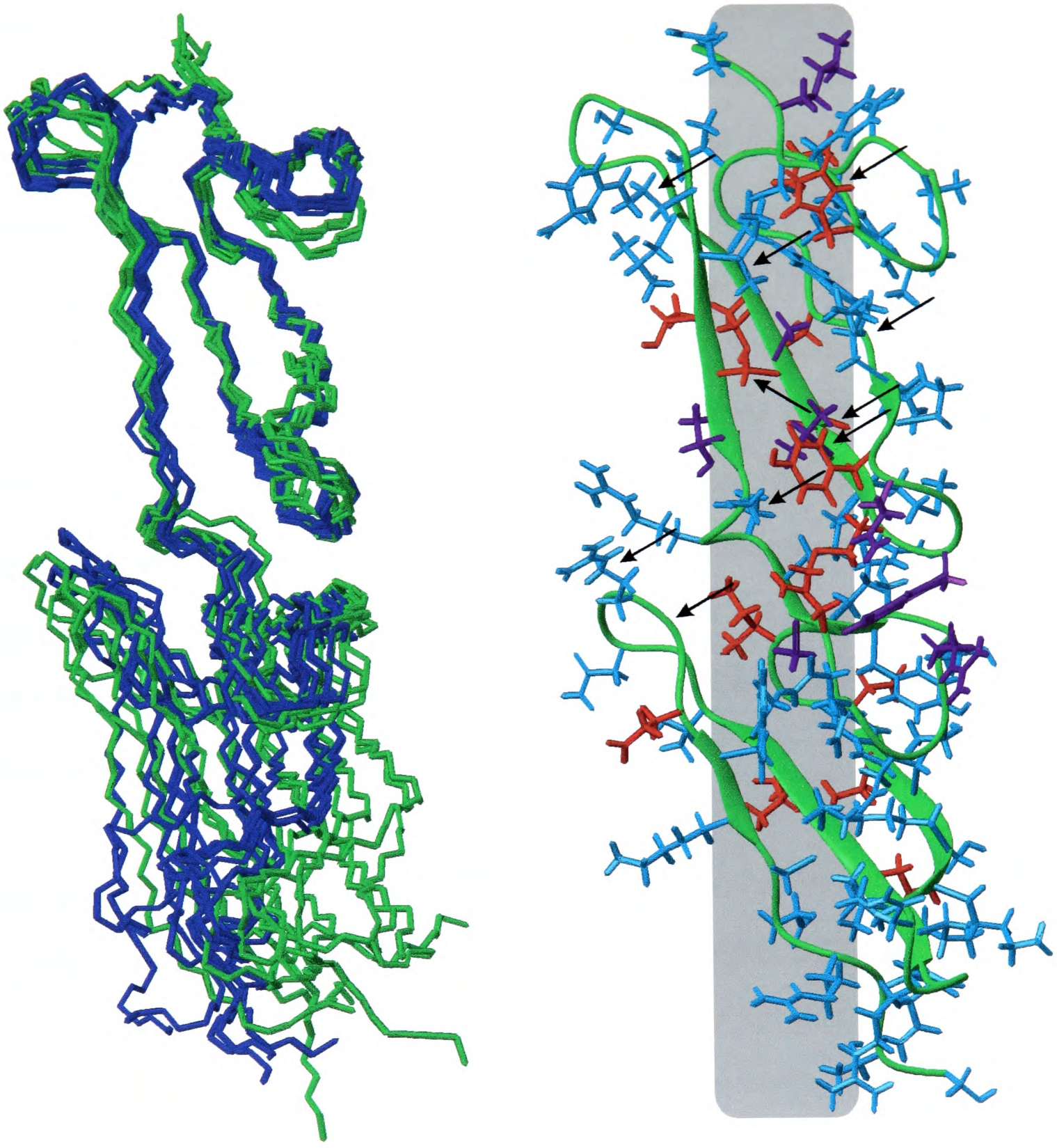


Fig.7.11.

a. The superposition of five the lowest energy structures from the unbound and family (res. 3-45).

b. The minimised average structure of ^{4}F1^{5}F1 in the complex with D3 with displayed side-chains. Residues that have lower side-chain atoms rmsd in comparison to the unbound module pair are shown in purple, lower rmsd and higher angular order parameter for χ1 and χ2 in red. Other side-chains are shown in . The arrows indicate residues with higher proportion of surface buried in the bound structure. The grey indicates the orientation of the 'groove' in the structure.

However, it is still possible to check if side-chains of residues that might be involved in binding the D3 peptide become more rigid in comparison to the free state. A comparison was carried out for the heavy-atom rmsd of residues in average structures, for a percentage of solvent-exposed surface of these residues, and for the angular order parameter for side-chain $\chi 1$ and $\chi 2$ angles. The results are shown in Table 7.2 and Fig.7.11b. The better defined positions of some of the side-chains in the bound ^{4}F1^{5}F1 indicate a higher number of NOEs involving protons from these side-chains. This might be relevant to the ligand binding, especially as most of changed residues lay within the region of the molecular 'groove' (Fig.7.10, Fig.7.11b). The backbone amide groups of residues situated along the 'groove' undergo the motions on a slow time-scale, according to ^{15}N-relaxation measurements (W19, M28, R46, R55, S57, E85 and K87 – **Section 5.1**). All this suggests the involvement of this region of the module pair into D3 binding.

The final minimised average structure of the bound module pair was used for docking of ^{4}F1-binding motif from D3. The docking of seven residues belonging to Df4 in the structure of bound ^{4}F1^{5}F1 was carried out (the CHARMM protocol was provided and run by A. Almond). The β-strand of the peptide was fitted into the groove with implementation of the hydrogen bonds predicted on the basis of the secondary structure of the ^{1}F1^{2}F1/B3 complex (J. Potts – pers. com.) and of the H^{α} chemical shift information for the E strand of ^{4}F1. The down-field chemical shift change for H^{α} of R40, T42 and T44 upon D3 binding (**Appendix 2A**) supported the prediction that this β-strand interacts with the ligand forming a β-sheet. The model of the ^{4}F1^{5}F1/Df4 complex obtained from docking is shown in Fig.7.12. The model shows that V29, that

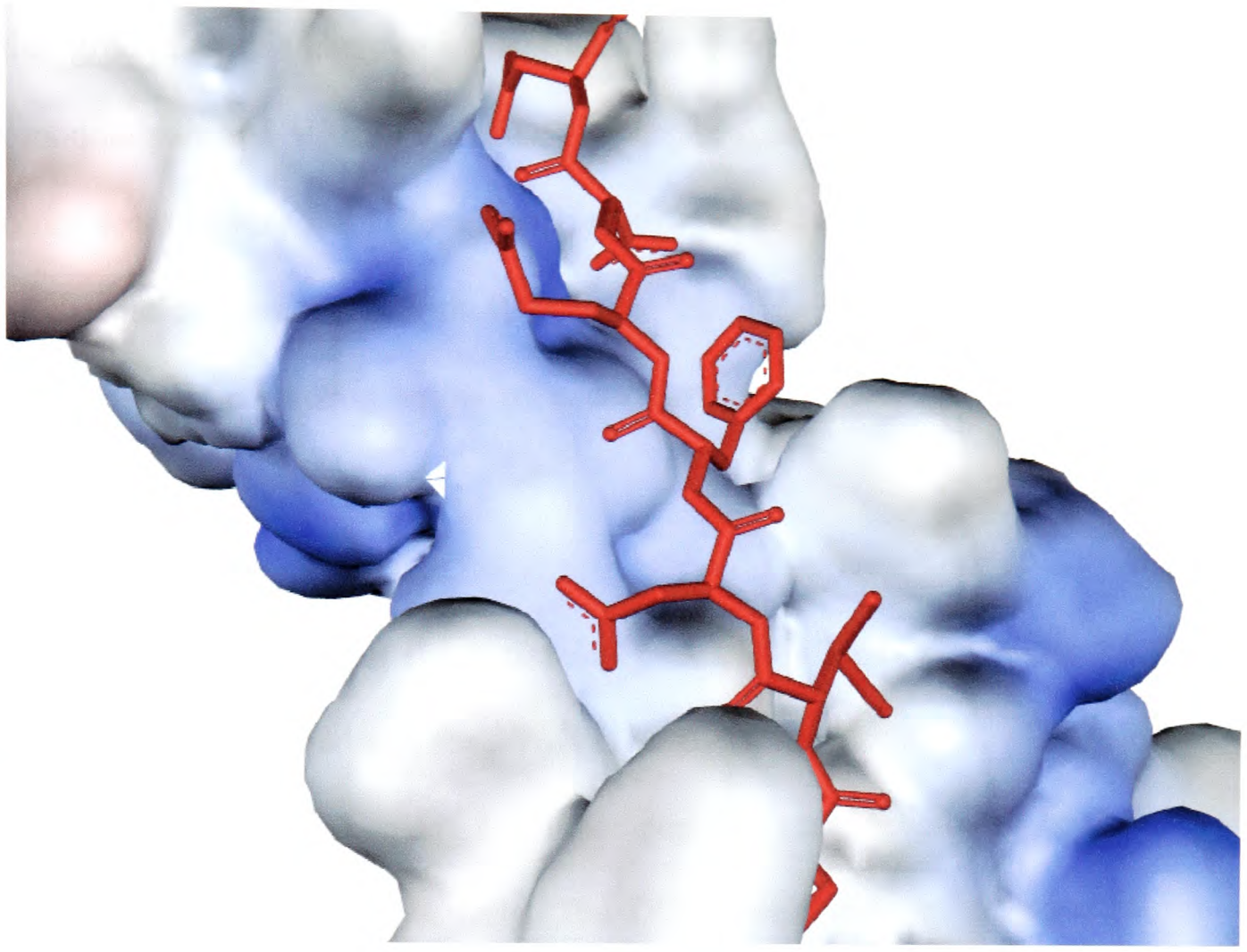
Table.7.2. The comparison of average structures of bound and unbound $^{4}F1^{5}F1$

Residue	Δ_{rmsd} [a]	Surface [b]	$S_{\chi1}$, $S_{\chi2}$ [c]	Residue	Δ_{rmsd} [a]	Surface [b]	$S_{\chi1}$, $S_{\chi2}$ [c]
K3	**-0.29**			R48	0.88		
C4	0.53			C49	**-0.16**		
P5	0.34			N50	**-0.20**		+
D6	0.01			D51	-0.08		+
H7	**-0.30**	44 (65)%	+	Q52	-0.03		+
A8	0.03			D53	0.39		+
A9	0.03			T54	0.49		
G10	-0.05			R55	**-0.29**		
T11	-0.01			T56	0.95		
S12	-0.07			S57	0.01		
Y13	0.09		+	Y58	0.34		
V14	**-0.70**		+	R59	-0.01		
V15	0.04		+	I60	**-0.17**		+
G16	**-0.35**			G61	**-0.19**		
E17	-0.07		+	D62	**-0.11**		+
T18	**-0.21**		+	T63	**-0.18**		+
W19	0.03			W64	0.02		
E20	0.01			S65	-0.05		
K21	0.48	6 (16)%	+	K66	0.24		
P22	0.30			K67	0.50		
Y23	**-0.11**	29 (41)%	+	D68	0.11		+
Q24	**-0.29**			N69	0.33		
G25	-0.08			R70	0.37		+
W26	**-0.14**			G71	0.42		
M27	**-0.24**		+	N72	0.65		
M28	0.25			L73	0.35		
V29	**-0.36**	4 (12)%		L74	0.19		
D30	**-0.19**		+	Q75	**-0.46**		+
C31	**-0.31**			C76	0.17		
T32	**-0.14**		+	I77	**-0.35**		+
C33	0.13			C78	0.29		
L34	0.13			T79	0.17		+
G35	0.33			G80	0.77		
E36	0.99		+	N81	1.45		+
G37	0.66			G82	0.64		
S38	1.43		+	R83	0.79	50 (63)%	
G39	0.36			G84	**-0.30**	10 (19)%	
R40	1.24	39 (53)%	+	E85	**-0.36**		+
I41	0.56	0.4 (25)%	+	W86	0.48		
T42	0.12		+	K87	0.30		+
C43	**-0.27**	2 (24)%	+	C88	0.10		
T44	**-0.19**			E89	0.27		+
S45	**-0.42**	2 (28)%	+	R90	0.82		
R46	0.10			H91	1.51		+
N47	0.38						

[a] $bound_{rmsd}$ - $unbound_{rmsd}$ (all heavy atoms of side-chains)
[b] percentage of solvent-exposed surface in bound (unbound) minimised average structure
[c] plus indicates residues with larger order parameter S in the bound structure

a.

Df4 NSVDFEEDTLPK



b.

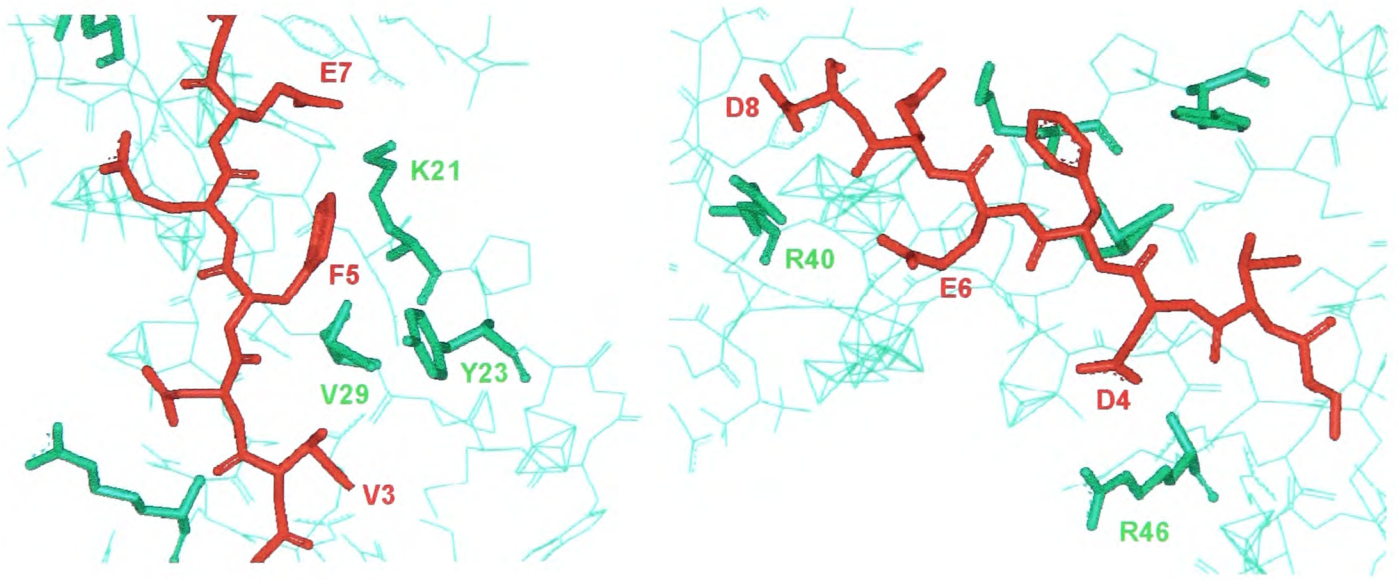


Fig.7.12. Model created by docking the seven residues of Df4 (indicated in red) into the minimised average structure of ^{4}F1^{5}F1, without altering the module pair. **a.** the position of the peptide on the surface of the module pair. **b.** the close-up on residues involved in protein-protein interactions. Df4 residues are shown in red, ^{4}F1^{5}F1 residues in .

underwent a large change on Df4 binding in the NMR experiment, occupies the bottom of the 'groove' and is likely to interact with the hydrophobic patch formed by Y23 of the module pair and V3 and F5 of Df4. The positions of side-chains of Y23 and V29 do not seem to vary from the unbound state. Also, Df4 residues D4, E6 and D8 form electrostatic interactions with R40 from ^{4}F1 and R46 from the linker. Additional electrostatic interaction seems to occur between E7 from Df4 and K21 from ^{4}F1.

In order to test the hypothesis that the N-terminal part of D3t binds directly to ^{5}F1, HSQC experiments were carried out for ^{15}N-^{4}F1^{5}F1 and fragments of D3: Df5 and Df2. According to the model from Fig.7.1, these peptides are predicted to contain binding sites for ^{5}F1 and ^{2}F1, respectively. The Df5 peptide contains residues N-terminal to Df4 in the D3 sequence. Fig.7.5c shows, that only very small chemical shift changes were observed for the backbone $^{1}H^{N}$ and ^{15}N atoms in ^{4}F1^{5}F1 on addition of Df5. The only exception is V14, however the $^{1}H^{15}N$ chemical shifts of this residue have been observed in many other spectra to be particularly sensitive to small changes in sample conditions thus, on their own, these changes are not considered to be due to binding. In support of this, V14 is located far from the estimated Df5-binding site, which makes it unlikely that this residue would be directly affected by the peptide. The other chemical shift changes in ^{4}F1^{5}F1 caused by Df5 are too small to interpret in terms of binding. To conclude then, D3t, which contains the sequences of both Df4 and Df5, shows much higher (10-fold) affinity than Df4 itself. Also, chemical shift changes are observed in ^{5}F1 upon D3t, but not Df4 binding. Thus, Df5 appears to interact directly with ^{5}F1. However, it was impossible to confirm its

binding to ^{4}F1^{5}F1 as an isolated motif and this is probably due to very weak binding of the isolated Df5 motif to ^{5}F1.

Identification of the ^{4}F1-binding motif in the B region of *S. aureus* FnbpA

The model presented in Fig.7.1, and recent studies (Massey *et al.*, 2001), suggest that the B region of FnbpA from *S. aureus* also binds the N-terminal domain of Fn. Peptides encompassing the sequence from this region were selected for binding studies, according to the prediction based on a model in Fig.7.1. The results of a series of HSQC experiments with an increasing molar ratio of ^{15}N-^{4}F1^{5}F1:N-STAB (the peptide possibly containing a putative ^{4}F1-binding motif) are shown in Fig.7.13a (H. Jenkins – unpublished results). The comparison of these results with the ^{4}F1^{5}F1 shift map upon Df4 binding (Fig.7.13b) reveals that the patterns of chemical shift changes are similar, although the magnitude of changes caused by N-STAB is approximately half that of the changes caused by Df4 at saturation. In both cases the residues of the ^{4}F1 module, belonging to A and E β-strand and the interface, are affected, as well as interface residues and the D-E loop from ^{5}F1. The similarity between these peptides could only be detected in the context of the overall alignment of F1-binding motifs, and their putative functional similarity in ^{4}F1 binding has been now confirmed empirically (for a detailed discussion of the binding see **Chapter 8**).

Interaction of D3 with the ^{1}F1^{2}F1 module pair

D3 EIIEEDTNKDKPSYQFGGHNSVDFEEDTLPKV

Df4 NSVDFEEDTLPK

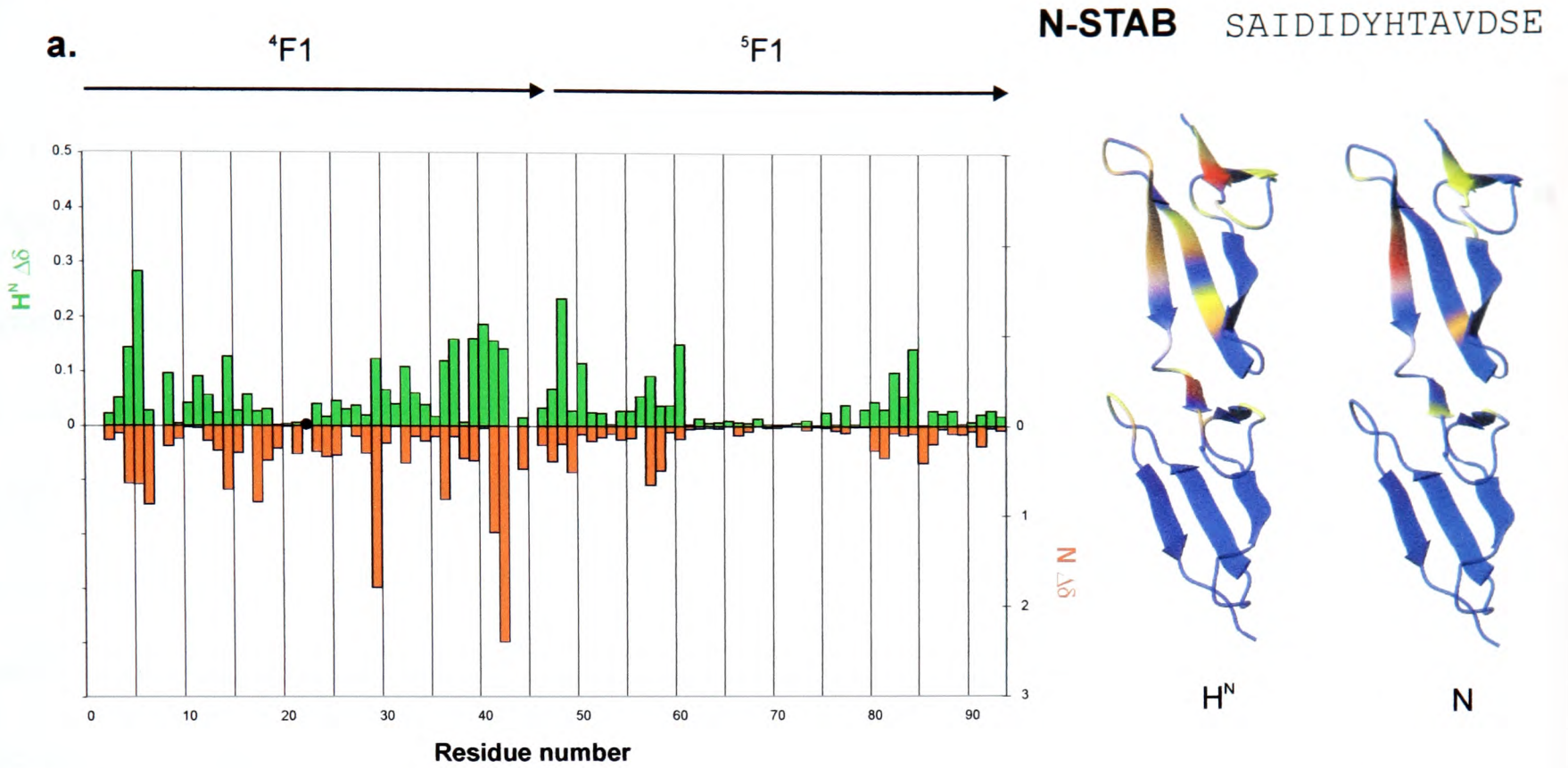


Fig.7.13. The magnitude of backbone $^1H^N$ and ^{15}N chemical shift changes in $^4F1^5F1$ upon N-STAB and Df4 binding ($\Delta\delta = \delta^{bound} - \delta^{free}$) at 37°C, presented in a graph form and mapped onto the average structure of $^4F1^5F1$ bound to D3. **a.** N-STAB - $\Delta\delta$ at the protein-peptide ratio of 1:5.4 (saturation), **b.** Df4 - $\Delta\delta$ at the protein-peptide ratio of 1:4 (saturation). • - proline residue. indicates $\Delta\delta$ > 0.05 ppm for H^N and > 0.5 ppm for N, orange $\Delta\delta$ > 0.1 ppm for H^N and > 1 ppm for N, and red $\Delta\delta$ > 0.2 ppm for H^N and > 2 ppm for N. indicates missing HSQC peaks, also very weak in the HSQC of the unbound $^4F1^5F1$. All other regions are shown in blue.

Df2 TEIIEEDTNKDK

D3, Df4 and Df2 were also tested for binding to ^{1}F1^{2}F1. Df2, which is predicted to be a ^{2}F1-binding motif (Fig.7.1), only interacted weakly (no saturation reached at protein:peptide ratio of 1:4) with the ^{1}F1^{2}F1 module pair (Fig.7.14a). It also appeared to interact weakly (no saturation reached at protein:peptide ratio of 1:4) with ^{4}F1^{5}F1 (Fig.7.14b). When the sequences of the Fn F1 modules are compared, ^{2}F1 and ^{4}F1 are 50% identical and this may explain the lack of complete specificity of the ^{2}F1-binding and ^{4}F1-binding peptides. Interestingly, when D3 was tested with ^{1}F1^{2}F1 (Fig.7.15a), the pattern of chemical shift changes in ^{2}F1 was almost identical to those caused by Df4 (Fig.7.15b) and different to that caused by Df2 binding to ^{1}F1^{2}F1. This indicates that, when D3 is tested, the sequence containing Df4, not Df2, binds to ^{2}F1. It is clear from Fig.7.1 that Df4 is quite different to other predicted ^{4}F1-binding motifs having more similarity to a ^{2}F1-binding sequence. These results suggest that D3 might contain no specific ^{2}F1-binding motif. For further summary see **Chapter 8**.

Identification of ^{4}F1 and ^{5}F1 binding motifs in *S. pyogenes* SfbI

P45 EPGVLMGGQSESVEFTKDTQTG

P4 ESVEFTKDTQTG

P5 EPGVLMGGQSE

The sequence of P45 was chosen as it was predicted to contain both ^{4}F1- and ^{5}F1-binding motifs (Fig.7.1), highly conserved among $^{1\text{-}5}$F1-binding motifs in SfbI. The

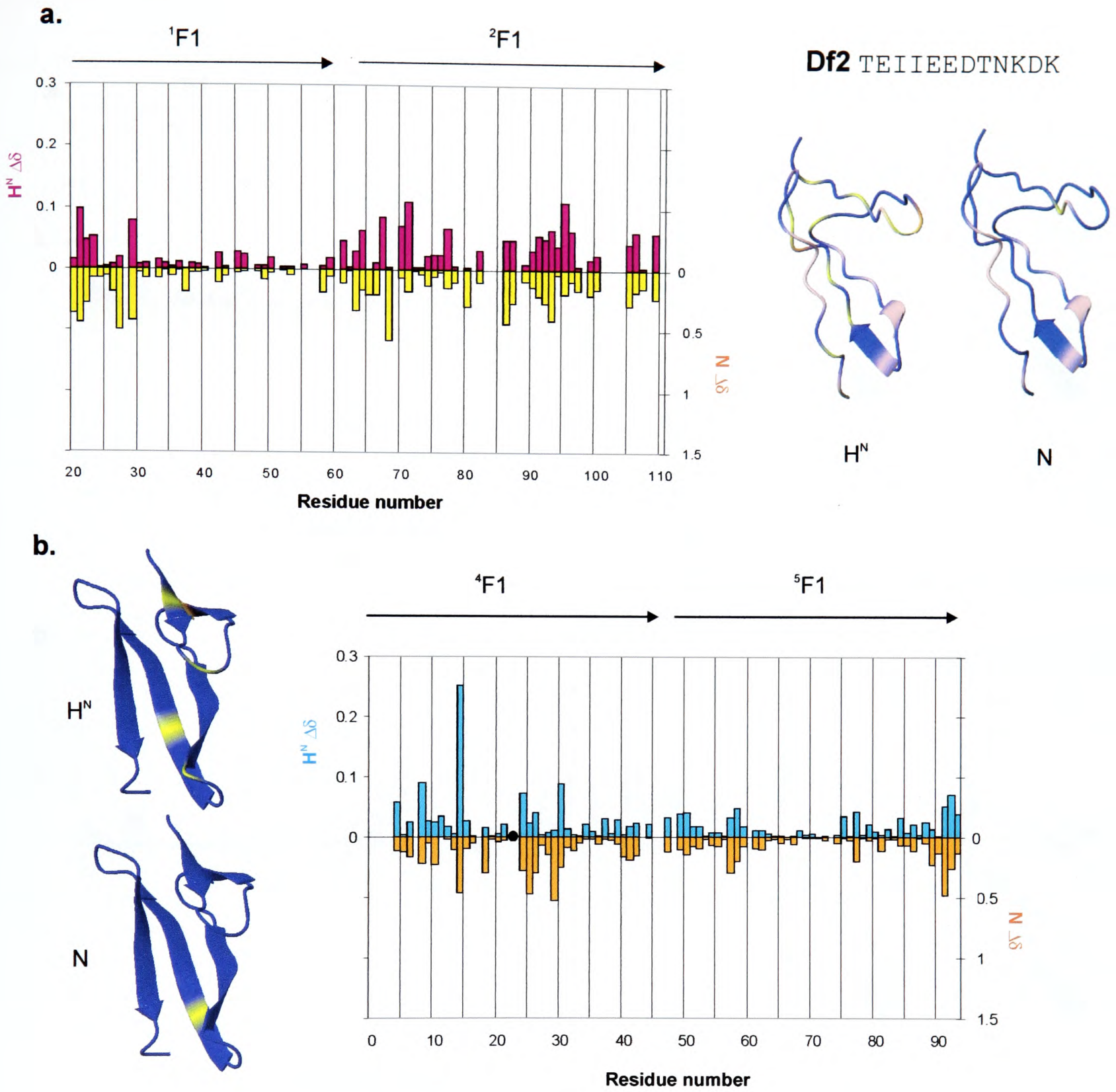
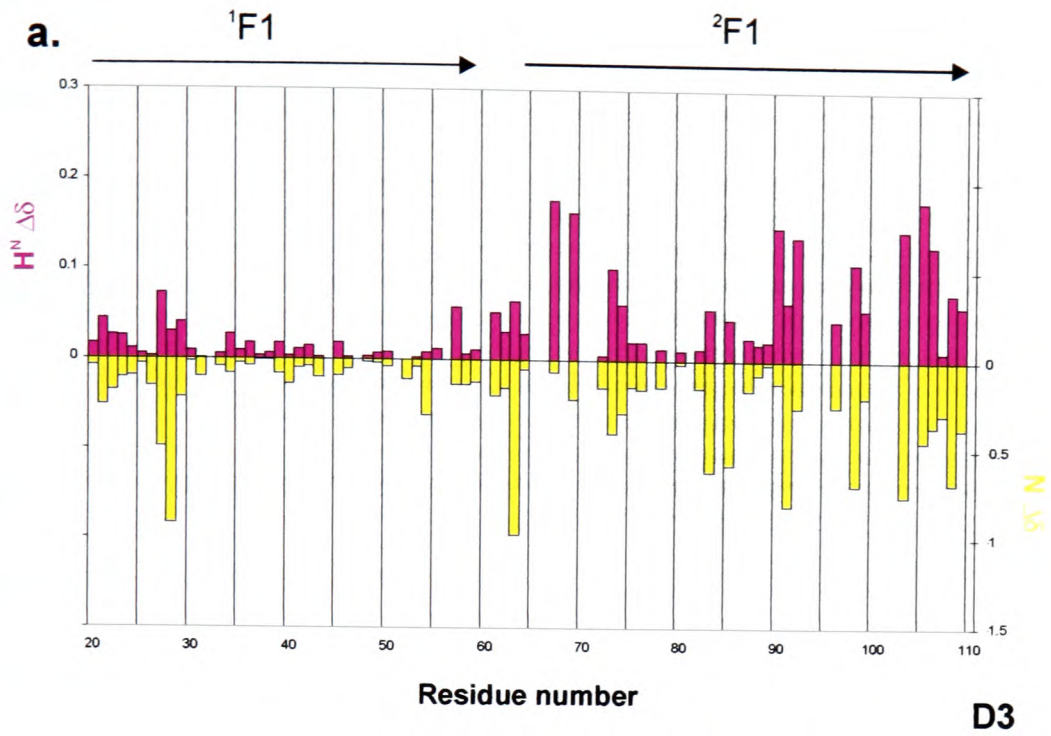
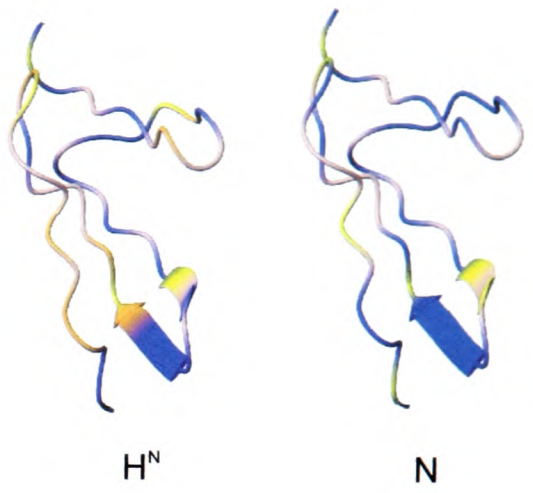


Fig.7.14. a. The magnitude of backbone $^{1}H^{N}$ and ^{15}N chemical shift changes in $^{1}F1^{2}F1$ upon Df2 binding ($\Delta\delta = \delta^{bound} - \delta^{free}$) at 25°C, presented in a graph form and mapped onto the average structure of $^{2}F1$ bound to B3 (protein-peptide ratio of 1:4 - no saturation reached). **b.** The magnitude of backbone amide proton and nitrogen chemical shift changes in $^{4}F1^{5}F1$ upon Df2 binding ($\Delta\delta = \delta^{bound} - \delta^{free}$) at 37°C, presented in a graph form and mapped onto the average structure of $^{4}F1$ bound to D3 (protein-peptide ratio of 1:4 - no saturation reached). • - proline residue. indicates $\Delta\delta$ > 0.05 ppm for H^{N} and > 0.5 ppm for N and orange $\Delta\delta$ > 0.1 ppm for H^{N} and > 1 ppm for N. indicates missing HSQC peaks, probably due to line broadening suggesting large $\Delta\delta$. All other regions are shown in blue.





D3

EIIEEDTNKDKPSYQFGGHNSVDFEEDTLPKV

Df4

NSVDFEEDTLPK

Fig.7.15. The magnitude of backbone $^{1}H^{N}$ and ^{15}N chemical shift changes in $^{1}F1^{2}F1$ upon D3 and Df4 binding ($\Delta\delta = \delta^{bound} - \delta^{free}$) at 25°C, presented in a graph form and mapped onto the average structure of $^{2}F1$ bound to B3. Df4 sequence homologous to two places in D3 has been aligned. **a.** D3 - $\Delta\delta$ at the protein-peptide ratio of 1:3, **b.** Df4 - $\Delta\delta$ at the protein-peptide ratio of 1:3. indicates $\Delta\delta$ > 0.05 ppm for H^{N} and > 0.5 ppm for N, and orange $\Delta\delta$ > 0.1 ppm for H^{N} and > 1 ppm for N. indicates missing HSQC peaks, probably due to line broadening suggesting large $\Delta\delta$. All other regions are shown in blue.

chemical shift changes in $^{4}F1^{5}F1$ observed on binding of P45 (Fig.7.16a, 7.17a) were much smaller than those observed on D3 binding (see above). The chemical shift changes caused by P45, as in the case of D3, occur in both modules, involving $^{4}F1$, the interface and the E β-strand of $^{5}F1$. The 'dissection' of P45 into $^{4}F1$- (P4) and $^{5}F1$-binding (P5) motifs has been successful. Again, removal of the N-terminal region (P5) confirms the anti-parallel orientation of binding. When P4 is bound to $^{4}F1^{5}F1$ (Fig.7.16b, 7.17b), the D-E loop of $^{5}F1$ and the E β-strand are unchanged, unlike when Df4 is bound to this module pair (Fig.7.6c). The pattern of changes in $^{4}F1$ upon P4 binding is not identical to P45 as in case of D3 and Df4, although it is similar. That might indicate that the binding supported by the N-terminal part of P45 is different then binding of P4 by itself.

As the binding appeared to be too weak for ITC, even in the case of P45, the K_D values for P45 and P4 were estimated from the chemical shift changes in a series of HSQC experiments acquired with an increasing molar ratio of protein:peptide (as described above). Surprisingly, it appeared that the P45 affinity for $^{4}F1^{5}F1$ is rather low (K_D ~100 μM), similar to a single $^{4}F1$ module-binding motif from *S. aureus* FnbpA i.e. Df4. However, the affinity of P4 by itself is again approx. 10-fold lower (K_D ~900 μM), which indicates the presence of an additional binding site in the full-length P45 peptide. For a further discussion, see **Chapter 8**. Fig.7.5b shows, that only very small chemical shift changes were observed for the H^N and N atoms of the $^{4}F1^{5}F1$ backbone on addition of P5.

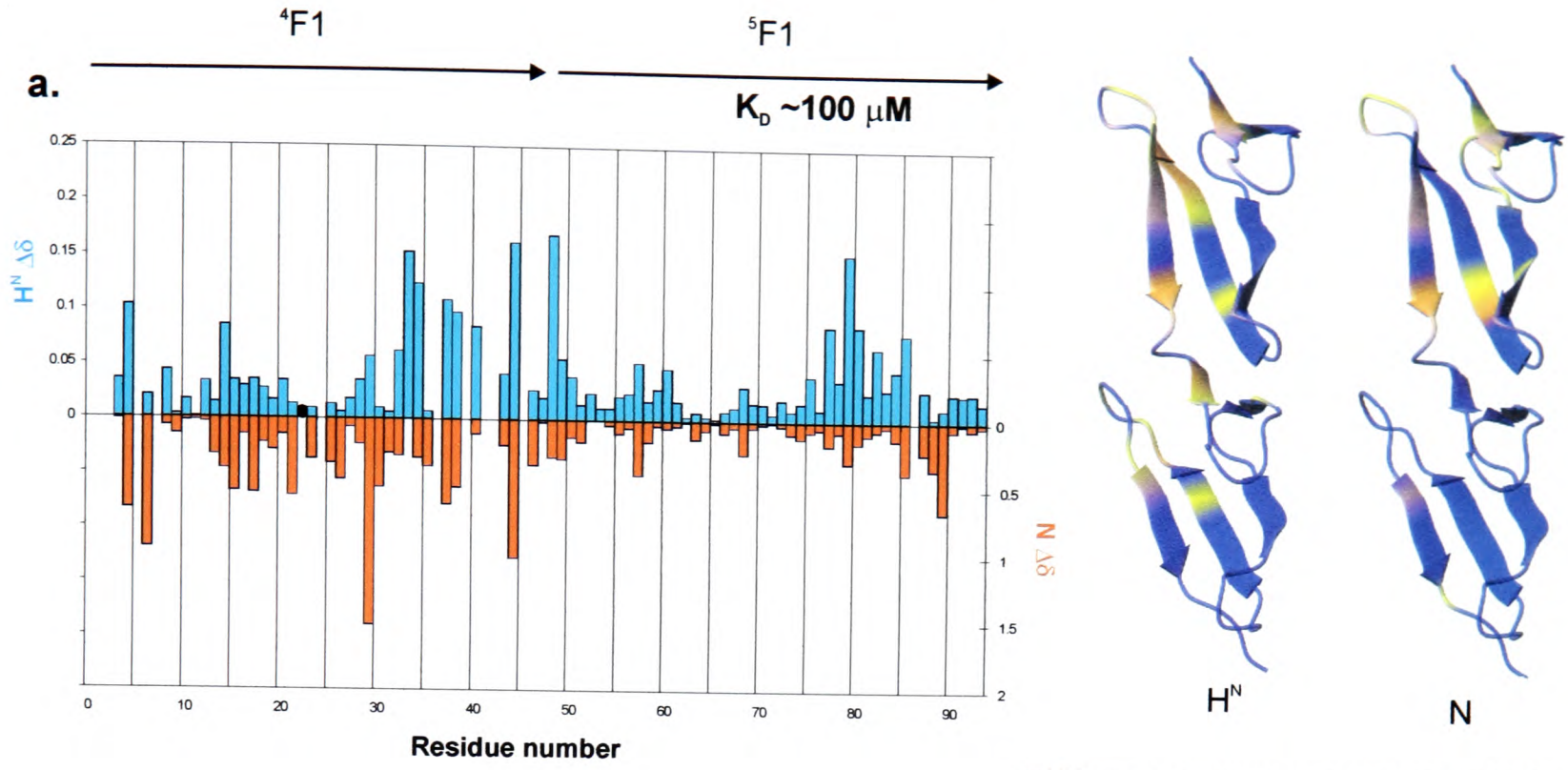


Fig.7.16. The magnitude of backbone $^1H^N$ and ^{15}N chemical shift changes in $^4F1^5F1$ upon P45 and P4 binding ($\Delta\delta = \delta^{bound} - \delta^{free}$) at 25°C, presented in a graph form and mapped onto the average structure of $^4F1^5F1$ bound to D3. **a.** P45 - $\Delta\delta$ at the protein-peptide ratio of 1:5 (saturation), **b.** P4 - $\Delta\delta$ at the protein-peptide ratio of 1:10 (saturation). • - proline residue. indicates $\Delta\delta$ > 0.05 ppm for H^N and > 0.5 ppm for N, and orange $\Delta\delta$ > 0.1 ppm for H^N and > 1 ppm for N. indicates missing HSQC peaks, probably due to line broadening suggesting large $\Delta\delta$. All other regions are shown in blue.

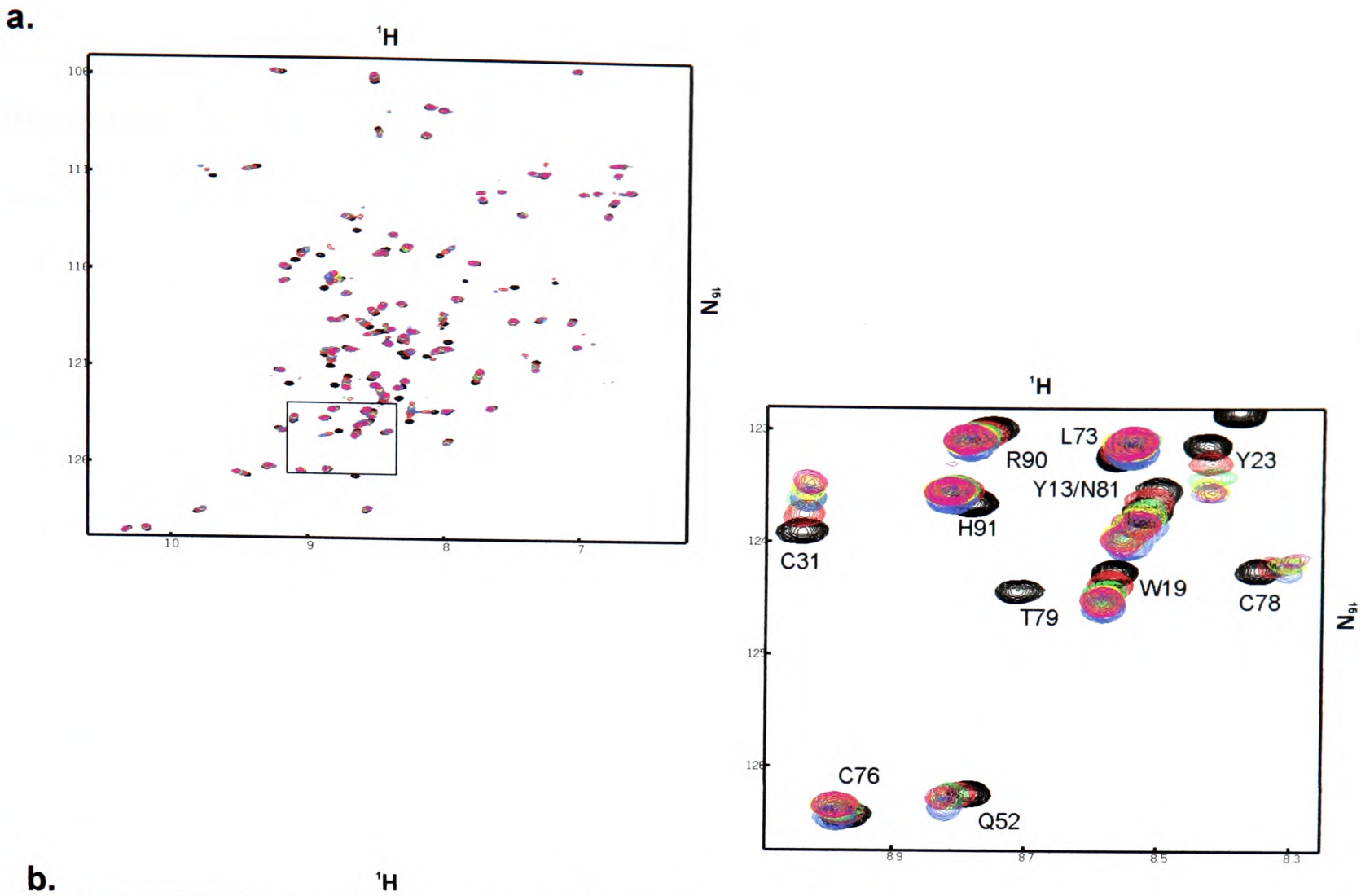


Fig.7.17. Overlays of HSQC spectra of 0.1 mM ^{15}N-^{4}F1^{5}F1 with P45 and P4, performed at 25°C, pH 5.0. Areas in squares are zoomed in. **a.** P45 - an overlay of 6 stages of HSQC titration (protein:peptide ratios of 1:0, 1:1, , 1:3, , 1:5). **b.** P4 - an overlay of 6 stages of HSQC titration (protein:peptide ratios of 1:0, 1:1, , 1:4, , 1:8).

Identification of ^{4}F1 and ^{5}F1 binding motifs in other FnBPs (*S. pyogenes* SfbII and *S. dysgalactiae* FnbB)

A2 VIIGGQGQVIDFTEDTQSG

PII45 DVLVGGQSDPIDITEDTQPG

The A2 peptide from *S. dysgalactiae* FnbB was selected for binding studies as it was reported to have a very high affinity for ^{4}F1^{5}F1 (K_D in sub-μM range – M. Höök – pers. com.) and contains the EDT sequence, which is not present in ^{4}F1^{5}F1-binding motifs in *S. pyogenes* SfbI (Fig.7.1). Due to poor solubility of the A2 peptide, the ITC experiment with ^{4}F1^{5}F1 was performed in a different way to the rest of experiments (as described above). The K_D of 0.8 μM, obtained at pH 5.0 and 25°C (Fig.7.18a), is the lowest (i.e. highest affinity) of those measured for interactions with ^{4}F1^{5}F1 reported here. On the basis of the A2 sequence, the PII45 motif was identified in *S. pyogenes* FnBP SfbII and synthesised for binding studies.

The ITC experiment performed for ^{4}F1^{5}F1 and PII45 revealed the possibility that two binding sites exist for this ligand (Fig.7.18c). Although it was possible to get a relatively small error on a fit with a single binding site resulting in a K_D of ~33 μM for 25°C and pH 5.0, the fitted curve did not align correctly to the data points at the beginning of titration (Fig.7.18b). For a two-site model the K_D values are respectively: 25 μM and 23 μM, but a comparison of errors for single-site and two-site gave a probability Q of 0.86, indicating that the implementation of the two-site model cannot be justified on the basis of this data. Despite this, there is other evidence

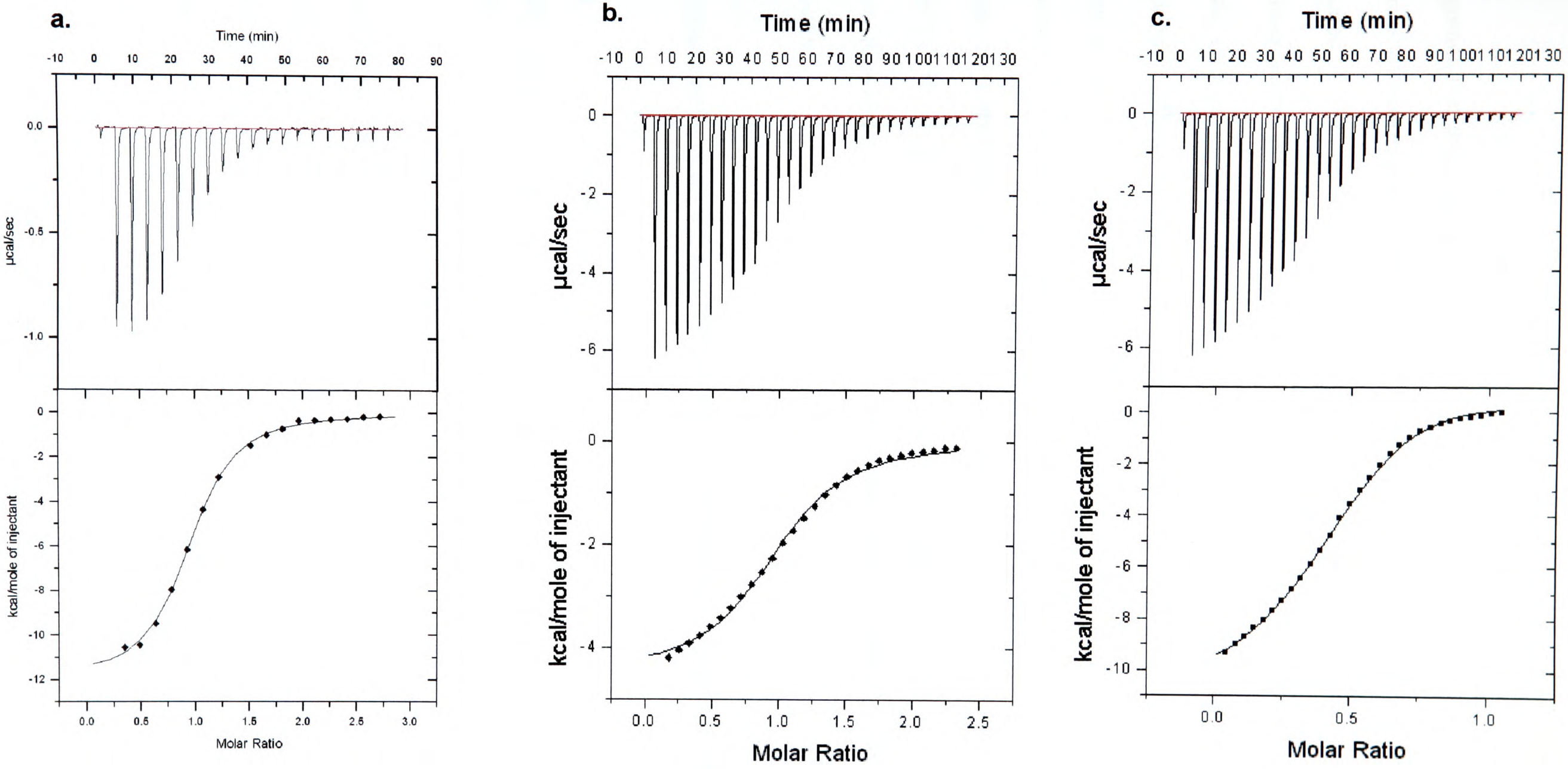


Fig.7.18. ITC experiments of ^{4}F1^{5}F1 with A2/PII45. The solid lines describe the best fit of experimental points. **a.** A2 - 25°C. Heat of interaction obtained for 20 injections. Data were fitted using a one-site model resulting in: stoichiometry n=0.93 ± 0.008; K_D=0.8 ± 0.05 µM; ΔH=-11980 cal/mol; ΔS=-12.31 cal/mol·K. **b.** PII45 - 25°C. Heat of interaction obtained for 30 injections. Data were fitted using a one-site model resulting in: stoichiometry n=1.00 ± 0.009; K_D=33 ± 2.09 µM; ΔH=-4514 cal/mol; ΔS=5.341 cal/mol·K. **c.** PII45 - 25°C. The same experiment as previously, with a fit to a two-sites model. Respective K_Ds: K_{D1}=25 µM and K_{D2}=23 µM.

suggesting that this is not a simple 1:1 interaction. NMR suggests that more than one process is occurring in a solution of the ^{4}F1^{5}F1/PII45 complex. An HSQC spectrum acquired at 25°C and at the ^{4}F1^{5}F1:PII45 ratio of 1:3 is shown in Fig.7.19. Many residues underwent big chemical shift changes with the peaks in intermediate stages of the titration too broad to be observed (Fig.7.19b). The identification of the new positions of these residues in the HSQC spectrum required sequential assignment using a 3D TOCSY-HSQC and NOESY-HSQC spectra. Unfortunately, the 3D spectra of ^{4}F1^{5}F1/PII45 acquired at 25°C and pH of 4.5 could not be assigned. A range of temperatures was then tested in attempt to improve the quality of 2D TOCSY. However, in all the spectra acquired, a significant proportion of the peaks were broadened indicating intermediate exchange between unbound and bound species.

Cross-reactivity of ^{2}F1 and ^{4}F1 binding motifs from *S. pyogenes* SfbI

P2 ETVTIVEDTRP

P23 TGMSGFSETVTIVEDTRP

P4 ESVEFTKDTQTG

Experiments were conducted (with U. Schwarz-Linek) to test the specificity of binding of ^{4}F1- (P4) and ^{2}F1-binding (P2) motifs from *S. pyogenes* SfbI to F1 module pairs from the N-terminal domain of Fn. No detectable binding (i.e. no significant chemical shift changes) was detected with ^{1}F1, ^{3}F1 and ^{5}F1 (eg. in P4 binding to ^{4}F1^{5}F1, Fig.7.16b). However, P4 and P2 have caused chemical shift changes of

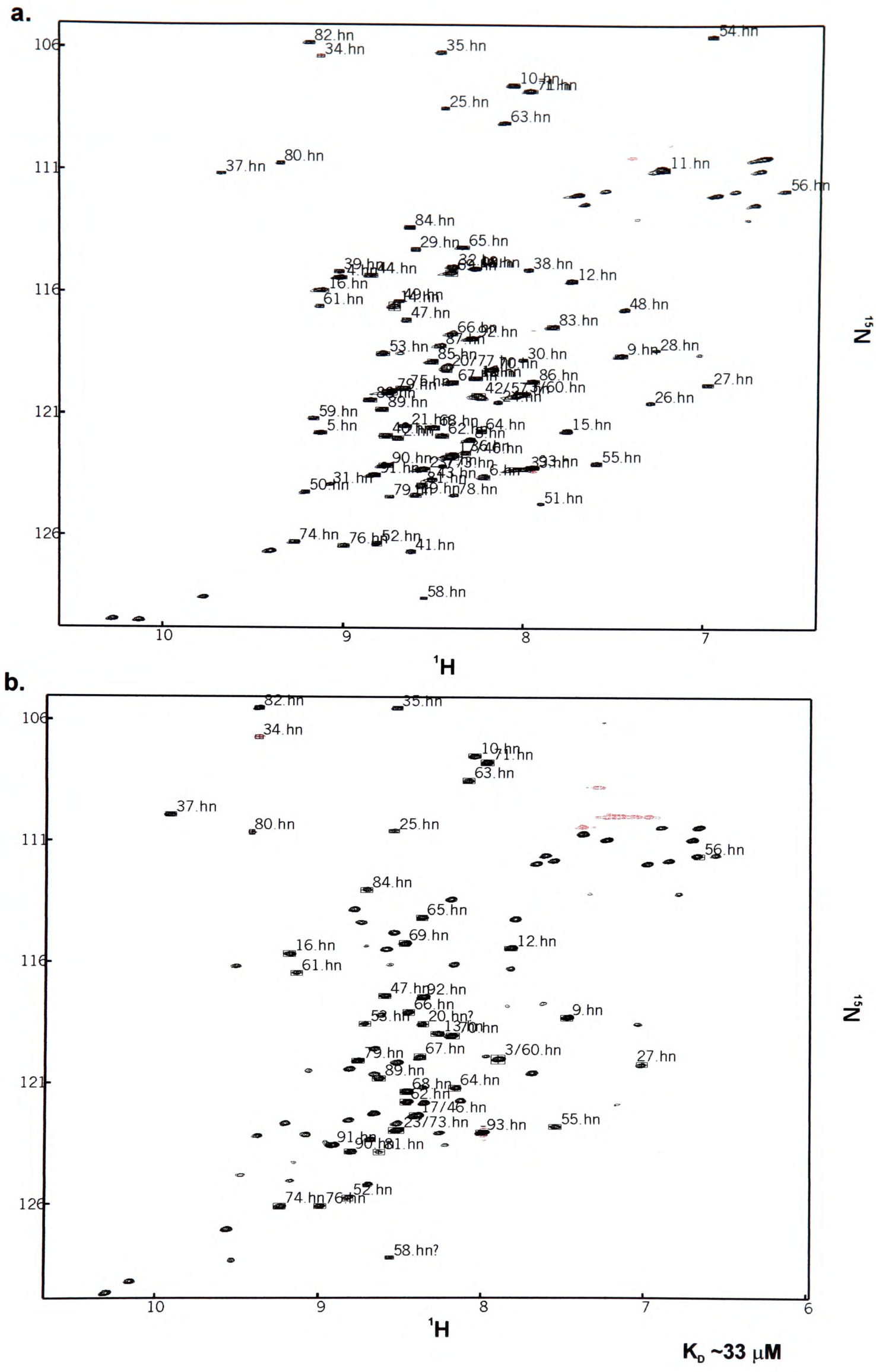


Fig.7.19. a. HSQC spectrum of 0.1 mM ^{15}N-^{4}F1^{5}F1 at 25°C, pH 4.5. **b.** HSQC spectrum of 0.1 mM ^{15}N-^{4}F1^{5}F1 with PII45 in a ratio of 1:3 at 25°C, pH 4.5. Only the residues indicated were assigned unambigously.

similar magnitude in both ^{2}F1 and ^{4}F1. Table.7.2. shows the K_D values determined for P2 and P4 binding to three module pairs.

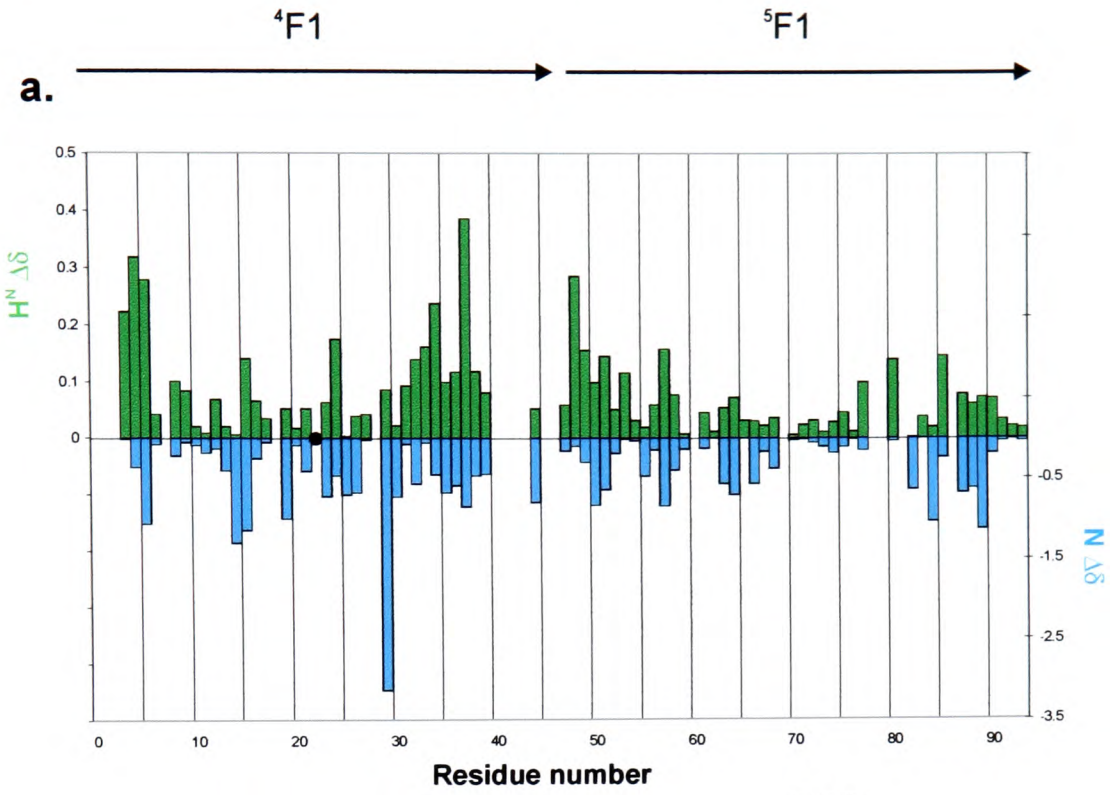
Table.7.2. K_D values for module pair/ligand binding*

	^{1}F1^{2}F1	^{2}F1^{3}F1	^{4}F1^{5}F1
P2	>1 mM	960 μM	>>1 mM
P4	>>1.8 mM	1.8 mM	900 μM

*The affinity in all cases was determined from a series of HSQC experiments as described above

The peptide P23 from SfbI-5 (Fig.7.1) was also tested with the ^{4}F1^{5}F1 module pair. Surprisingly, the saturation of ^{4}F1^{5}F1 was reached sooner with P23 than with P45 (Fig.7.20). Also, P23 induced much larger chemical shift changes, the largest among all the *S. pyogenes* ligands used. The pattern of chemical shift changes resembles that caused by N-STAB (Fig.7.13). From U. Schwarz-Linek's studies it is apparent though, that P23 binds stronger to ^{2}F1^{3}F1 than to ^{4}F1^{5}F1 showing therefore the specificity predicted by the model from Fig.7.1. However, the affinity of P23 to ^{4}F1^{5}F1 seems high enough to be measured by ITC. This experiment will be carried out in the future.

Both P4 and P2 interact with ^{2}F1 and ^{4}F1 but not with the other three F1 modules within the N-terminal domain of Fn. In addition, P4 binds ^{4}F1 with higher affinity than P2 and P2 binds ^{2}F1 with higher affinity than P4. That confirms a certain level of specificity of these peptides. However, the P45 affinity for ^{4}F1^{5}F1 is lower than that of a unique ^{2}F1^{3}F1 binding motif from SfbI-5, which directly follows the ^{1}F1^{2}F1 binding sequence. These results indicate that as P2 and P4 seem to be much weaker



P23 TGMSGFSETVTIVEDTRP

P45 EPGVLMGGQSESVEFTKDTQTG

Fig.7.20. The magnitude of backbone $^1H^N$ and ^{15}N chemical shift changes in $^4F1^5F1$ upon P23 (acquired at 37°C), and P45 (acquired at 25°C) binding ($\Delta\delta = \delta^{bound} - \delta^{free}$). **a.** P23 - $\Delta\delta$ at the protein-peptide ratio of 1:3 (saturation), **b.** P45 - $\Delta\delta$ at the protein-peptide ratio of 1:5 (saturation). • - proline residue.

than ^{4}F1-binding motif Df4 from *S. aureus*, they might recognise and bind with higher affinity to the F1 modules from the gelatin-binding domain and such hypothesis should be tested.

UR and SUR and their interactions with the N-terminal domain of Fn

UR KDQSPLAGESGETEYITEVYGNQQNPVDIDKKLPNETGFSGNMVETEDT

SUR KDQSPLAGESGETEYITEVYGNQQNPVDIDKKLPNETGFSGNM

The UR domain is situated N-terminal to the Fn-binding repeats in *S. pyogenes* SfbI. It was shown to bind the 70 kDa domain of Fn (Jaffe *et al.*, 1996) and its deletion significantly reduced the uptake of *S. pyogenes* by respiratory epithelial cells, although the adherence rate did not change (Talay *et al.*, 2000). Recently it has also been suggested that its main affinity is towards $^{1\text{-}5}$F1 modules (Ensenberg *et al.*, 2001). According to Ensenberg *et al.* (2001) the ^{3}F1 module is the most important for the high-affinity binding of UR, which suggests the existence of a specific ^{3}F1-binding sequence within the domain.

Due to limitations in the available amount of peptides the HSQC experiments were carried out to a protein:peptide ratio of 1:3 for ^{15}N-^{1}F1^{2}F1, ^{15}N-^{2}F1^{3}F1 and ^{15}N-^{4}F1^{5}F1 (with the exception of the titration of ^{15}N-^{1}F1^{2}F1 and UR, which was continued up to a ratio of 1:5). None of the module pairs were saturated at the final point. Significant differences in chemical shifts were observed in ^{1}F1^{2}F1 on the addition of UR compared with the addition of SUR (25°C, pH 5.0). In the case of UR,

many $^{1}H^{15}N$ peaks disappeared directly from their original positions after adding the first aliquot. These peaks mainly corresponded to residues of the $^{2}F1$ module. By the $^{1}F1^{2}F1$:UR ratio of 1:5 the line-broadening caused majority of $^{1}H^{15}N$ peaks to disappear, without reappearing in new positions. On the addition of SUR aliquots to $^{1}F1^{2}F1$ however, it was possible to 'track' the migration of $^{1}H^{15}N$ peaks. Almost identical chemical shift changes were observed in $^{4}F1^{5}F1$ on addition of either UR or SUR (37°C, pH 5.0 – Fig.7.21). This suggests that the incomplete $^{2}F1$-binding motif at the C-terminus of UR (the sequence VETEDT, missing in SUR) results in a higher affinity of UR for $^{1}F1^{2}F1$ compared with SUR.

The results obtained here suggest, that SUR contains a weak $^{4}F1^{5}F1$-binding motif. As SUR bound to $^{1}F1^{2}F1$ causes chemical shift changes very different to the ones caused by UR, the conclusion is that $^{2}F1$ binding activity depends on C-terminal residues of UR. This is consistent with the prediction from the model presented in Fig.7.1 that these residues are part of the $^{2}F1$-binding motif in SfbI. Recently, Ensenberg *et al.* (2001) suggested that the C-terminus of UR is responsible for specific recognition of the $^{4}F1^{5}F1$ module pair, while the N-terminus binds $^{1}F1$ and/or $^{2}F1$. The difference between this study and the results presented here may arise in the use by Ensenberg of deletion mutants of the whole 70 kDa domain of Fn, while here only F1 module pairs from the 29 kDa domain were used. UR interacts also with both modules in the $^{2}F1^{3}F1$ module pair. The interaction with this module pair is stronger than with $^{4}F1^{5}F1$. Therefore the following conclusions can be drawn: UR as defined by Ensenberg *et al.* (2001) contains a $^{2\text{-}5}F1$-binding sequence, starting from the C-terminus of the peptide. That is in agreement with the alignment of the UR sequence,

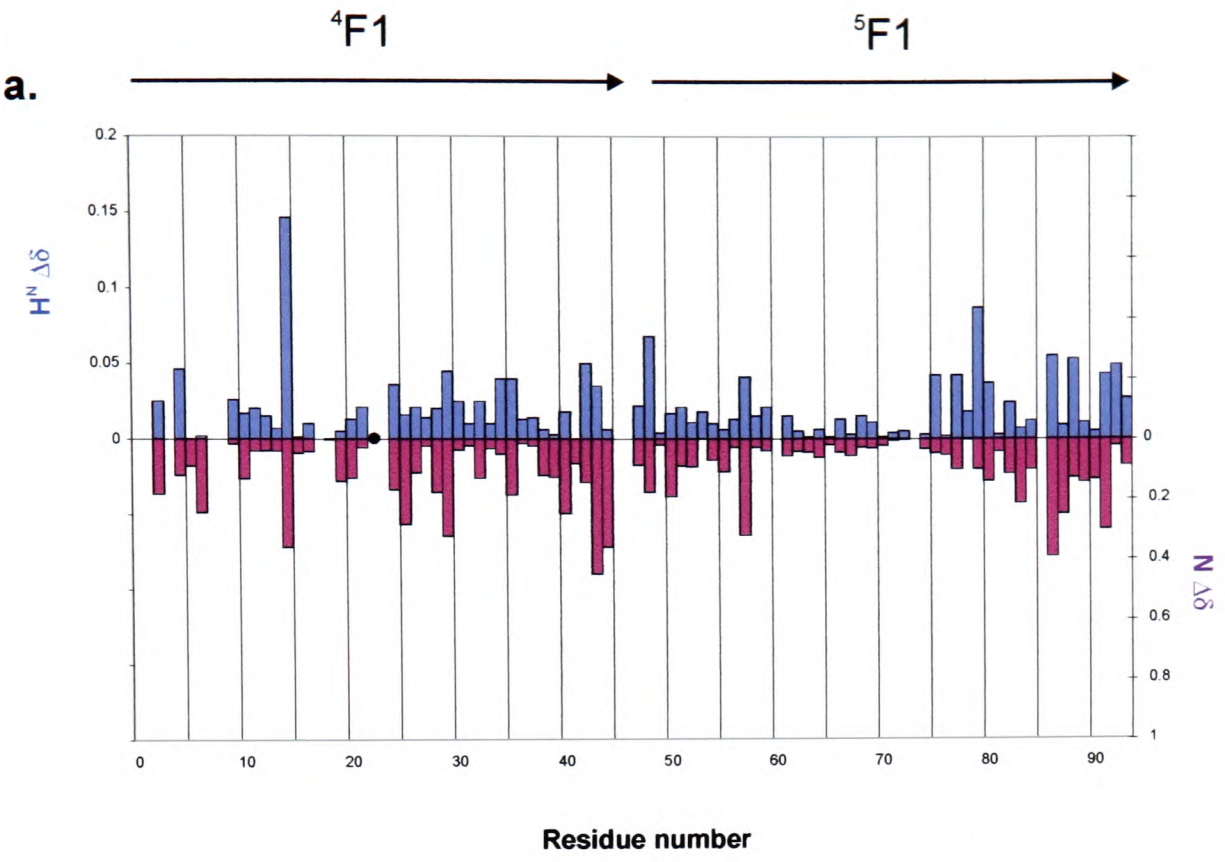


Fig.7.21. The magnitude of backbone $^1H^N$ and ^{15}N chemical shift changes in $^4F1^5F1$ upon UR and SUR binding ($\Delta\delta = \delta^{bound} - \delta^{free}$), at 37°C. No saturation was reached. **a.** UR - $\Delta\delta$ at the protein-peptide ratio of 1:3. **b.** SUR - $\Delta\delta$ at the protein-peptide ratio of 1:3. • - proline residue.

presented in Fig.7.1. The results presented here did not indicate that UR binds to the N-terminal domain with high, nM affinity, as suggested by Ensenberg *et al.* (2001). Thus, in the near future the affinity of the UR domain for a 29 kDa fragment of Fn will be tested using ITC.

8. BINDING STUDIES - FINAL REMARKS

A new alignment of Fn-binding repeats (Fig.7.1) suggests the presence of conserved short motifs that bind specifically to individual F1 modules from the N-terminal domain of Fn ($^{1\text{-}5}$F1). According to this alignment, the consensus sequence of the ^{4}F1-binding motif in *S. aureus* and *S. pyogenes* FnBPs is:

hahxxap

where

h = F, I, V, Y (hydrophobic)

a = D, E (acidic)

p = S, T (polar)

The ^{5}F1-binding sequence in *S. aureus* FnbpA has been difficult to identify so far, but seems to be well conserved in *S. pyogenes* FnBPs, with the following consensus sequence:

hhhGGb

where

h = I, L, M, V (hydrophobic)

b = Q, K

ITC and NMR results suggest that both D3 and P45 interact with ^{4}F1^{5}F1 with a stoichiometry of 1:1. In each case both F1 modules appear to be involved in the interaction. Among all the ligands studied, D3 induced the most significant changes in

$^{1}H^{N}$ and ^{15}N chemical shifts in both $^{4}F1$ and $^{5}F1$ modules (Fig.7.6a). McGavin *et al.* (1991), through mutation studies have shown that the acidic residues in D3 are crucial for Fn binding. However, according to Huff *et al.* (1994) the EED cluster is neither necessary (when 'deactivated' by methylation) nor sufficient for the binding of the N-terminal domain of Fn. Nevertheless, alteration of EDT to FDT seems to reduce the peptide affinity (Fn-binding repeats D1 and D2 - McGavin *et al.*, 1991).

The reason for the low affinity of the P45 peptide from *S. pyogenes* SfbI towards the $^{4}F1^{5}F1$ module pair (~100 μM, Fig.7.17a), which is comparable with the single F1-binding motif Df4 from *S. aureus* FnbpA, may lie in the substitution of EDT by KDT. The affinity of P45 for $^{4}F1^{5}F1$ is at least 3-fold lower than that of another motif from *S. pyogenes* FnBP, PII45, possessing the EDT sequence. Also, Penkett *et al.* (1998) showed the presence of medium-range interactions in the FEEDT region of D3, inducing a more ordered conformation in this unfolded protein region. It is possible that the homologous region of P45 is less ordered due to differences in the sequence. Therefore the energy penalty for adopting a regular structure would be higher for P45 than for D3t, explaining the lower affinity of the first ligand.

The comparison of chemical shift changes in $^{4}F1^{5}F1$ on the addition of D3 and Df4 suggests that Df4 contains a full-length $^{4}F1$-binding motif and that the part of D3 N-terminal to Df4 interacts with $^{5}F1$, indicating an antiparallel orientation of the ligand. As the D3t peptide, which contains both Df4 and Df5 sequences, shows much higher affinity towards $^{4}F1^{5}F1$ than Df4, but very similar $^{4}F1$ chemical shift changes, Df5 appears to interact directly with $^{5}F1$. It was not possible however, to confirm the

binding of Df5 to ^{4}F1^{5}F1 as an isolated motif (Fig.7.5c). Df5 is the only sequence in *S. aureus* FnbpA (hbhGGb) resembling the highly conserved putative ^{5}F1-binding motif from *S. pyogenes*: hhhGGb. This might be responsible for the exceptional strength of D3 binding to ^{4}F1^{5}F1 in comparison with other D repeats (McGavin *et al.*, 1991).

The studies presented here (carried out by H. Jenkins) confirmed the presence of a ^{4}F1-binding motif in N-STAB from the B region of *S. aureus* FnbpA, as predicted on the basis of the alignment in Fig.7.1 and suggested by other research (Massey *et al.*, 2001). Although the sequences of Df4 and N-STAB are quite different, they interact with the ^{4}F1^{5}F1 module pair in a very similar way. For both peptides, the residues affected in ^{4}F1 belong to the A and E β-strand, and the interface residues and the D-E loop in ^{5}F1 are also involved. Comparison of the Df4 and N-STAB sequences reveals the hahaxxT pattern, suggesting that this is the binding site of the peptides. The lower affinity of N-STAB for ^{4}F1^{5}F1 might be due to the presence of YH residues in N-STAB, replacing the ED residues, that are more common in other repeats, and probably interact with I41 and R40 in ^{4}F1, as shown on a model in Fig.7.12. That would change the interactions in this place from ionic to hydrophobic. The HSQC spectra are consistent with this hypothesis as the backbone ^{1}H^{15}N chemical shifts of R40, which undergo large changes on D3 binding, remained almost unchanged during N-STAB binding. The opposite direction of the chemical shift changes of ^{15}N of I41 on N-STAB binding compared with Df4, also suggests a different chemical environment for this residue in the two complexes.

The alignment of D3, N-STAB, P45 and PII45 suggests that the Pro residue on the C-terminus of F1-binding motif might be important – it is well conserved in FnbpA and SfbII (Fn-binding motifs of higher affinity), but replaced by Tyr in SfbI. The D3t peptide, when truncated of the flanking sequences KPSY and LPK, completely lost activity, although each of these sequences was able to restore a half of it (McGavin *et al.*, 1991). Pro residues often occur immediately after the C-terminus of β-strand and restrict the φ, ψ angles of the preceding residue in favour of the β conformation (MacArthur *et al.*, 1991). In the case of Fn-binding repeats the presence of Pro at the end of an F1-binding motif would then support the adoption of an extended structure by the unfolded ligand and reduce the energy penalty for the Fn/ligand interaction.

It is possible that the D3 repeat does not contain a specific ^{2}F1-binding sequence (contrary to the new model). Df2 has not shown any significant binding to neither ^{2}F1 nor ^{4}F1, while Df4 interacted with both modules, although with different affinities. The sequences of the E β-strand of ^{4}F1 and ^{2}F1 are very similar: RI(S/T)CT (Fig.8.1). That would indicate again, that this β-strand determines the specificity of the ligand binding to the large extent. As the ligand binds in an antiparallel orientation, this result indicates that the N-terminal part of Df4 is not specific for the ^{2}F1 module (I106 and A107 in comparison to S45 and R46 in ^{4}F1).

^{2}F1 possesses an RGR sequence in the D-E loop (Fig.8.1), interacting with the acidic residues at the C-terminus of B3 peptide (J. Potts – manuscript in preparation). From the alignment of the $^{1\text{-}5}$F1 modules of Fn (Potts *et al.*, 1999) it is apparent that all these modules possess a large basic residue at least in one of the positions identical to

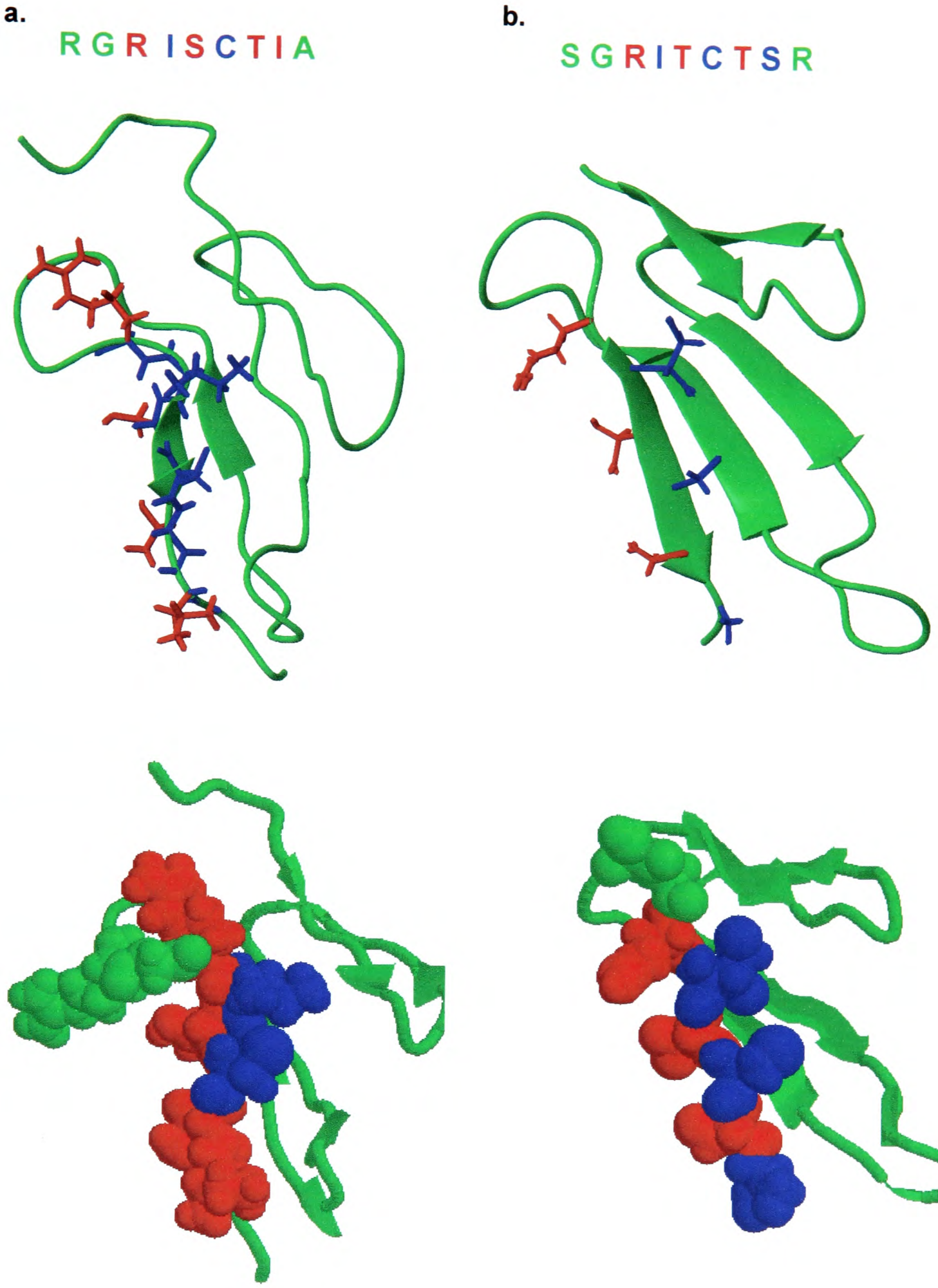


Fig.8.1. The structures of ^{2}F1 bound to B3 (**a**) and ^{4}F1 bound to D3 (**b**) with side-chains of C-terminal β-strands, presented in line and space-filling form (here also two additional N-terminal residues are shown). Solvent-exposed side-chains, likely to interact with the ligand, are coloured red, the residues whose backbone might interact, blue.

RGR motif in ^{2}F1. It can be speculated that these residues (Arg, except Lys in ^{3}F1) are essential for the bacterial peptide binding as well as important for the binding specificity.

In intact Fn, the interactions between ^{6}F1 and ^{7}F1 (Litvinovich *et al.*, 1991), as well as between the N-terminal domain (primarily ^{3}F1) and ^{2}F3 (Fig.1.2 - Sechler *et al.*, 2001), were reported. Pickford *et al.* (2001) suggested that the binding of collagen to Fn might induce a change in the ^{6}F1^{1}F2^{2}F2 hairpin conformation 'releasing' the N-terminal domain and thus facilitating matrix assembly. A similar model can be proposed for binding of SfbI to Fn where the UR domain, shown to have affinity to the collagen-binding domain, can interact with the newly freed binding site after the N-terminal domain is bound to the repeat region, altering the hairpin structure. This might explain the positioning of Fn-binding repeats directly after the UR domain.

To summarize, in *S. aureus* FnbpA the ^{4}F1- and ^{5}F1-binding motifs have been identified within the D repeats, consistent with the 'β-zipper' model for Fn/bacterial interactions, and an antiparallel orientation of binding was shown. While many aspects have been explained, the ^{2}F1-binding motif predicted by the model does not seem to bind ^{2}F1 with the expected affinity, and the identification of ^{4}F1- and ^{5}F1-binding motifs in *S. pyogenes* is not straightforward. It is expected that further studies of different peptides will resolve these uncertainties.

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APPENDIX 1

Fermentation protocols (A. Pickford)

Pichia pastoris fermentation (unlabelled, ^{15}N-labelled and $^{13}C^{15}$N-labelled protonated $^{4}F1^{5}F1$):

Stock solutions:

1 M potassium phosphate pH 6.0
118.1 g KH_2PO_4, 30.1 g K_2HPO_4 x 3 H_2O in a total of 1000 ml H_2O, pH adjusted to 6.0 with KOH, sterilised by autoclaving

10 x YNB
- unlabelled protein: 100 g $(NH_4)_2SO_4$, 34 g yeast nitrogen base (without amino acids and $(NH_4)_2SO_4$) in a total of 1000 ml H_2O, sterilised by autoclaving
- for ^{15}N labelling: YNB without $(NH_4)_2SO_4$, separately 5 g $(^{15}NH_4)_2SO_4$ in 25 ml H_2O, all sterilised by autoclaving

energy source:
- unlabelled and ^{15}N-labelled protein:
  10 x G
  100 ml glycerol and 900 ml H_2O, sterilised by autoclaving
- for ^{13}C labelling:
  2.5 g ^{13}C-glucose in 25 ml H_2O, filter sterilised

500 x B
20 mg D-biotin in a total of 100 ml H_2O, filter sterilised

Basal salt medium for fermentation (0.5 l)
13.4 ml 85% H_3PO_4
0.46 g $CaSO_4$ (anhydrous)
7.5 g $MgSO_4$ x 7 H_2O
400 ml H_2O
pH adjusted to ~3.5 with solid KOH

Starter culture:

To 35 ml of deionised H_2O, sterilised by autoclaving, through the syringe filter there were added: 5 ml of 1 M potassium phosphate pH 6.0, 5 ml of 10 x YNB (for ^{15}N labelling 4 ml 10 x YNB and 1 ml of $(^{15}NH_4)_2SO_4$ solution), 5 ml of 10 x G (for ^{13}C labelling 1 ml of ^{13}C-glucose with 4 ml H_2O instead) and 0.2 ml of 500 x B. Medium was inoculated with the glycerol stock of transformed *P. pastoris* or with a single colony from a fresh plate, and incubated overnight at 30°C, 250 rpm.

Fermentation:

400 ml of basal salts medium was poured to the vessel of the fermentor and autoclaved. After setting the fermentor up, when the system cooled down to ~30°C, the pH was adjusted to ~5.5. Through the syringe filter there were added: 5 g glucose in 20 ml H_2O (labelled stock solution for

^{13}C labelling), 5 g $(NH_4)_2SO_4$ in 20 ml H_2O (labelled stock solution for ^{15}N labelling), 2.5 ml PTM, 2.5 ml 500 x B. After equilibration the medium was inoculated with the starting culture through the syringe.

After ~24 h, when the oxygen level after dropping to ~20% went up to 50-80% (no carbon source left), MeOH (^{13}C-MeOH for ^{13}C labelling) was added to 1% concentration. After equilibration a drop-feeding was started at 0.05 ml/min, later increased to 0.1 ml/min. Fermentation finished ~120 h after induction or, for ^{13}C labelling, after feeding the culture with 50 g ^{13}C-MeOH. The cell pellet was centrifuged down (3000 rpm, 10 min) and the supernatant with the protein was recovered for the protein isolation.

***Pichia pastoris* fermentation (^{15}N-labelled fully deuterated ^{4}F1^{5}F1):**

Stock solutions:

1 M potassium phosphate pH 6.0

118.1 g KH_2PO_4, 30.1 g K_2HPO_4 x 3 H_2O in a total of 1000 ml H_2O, pH adjusted to 6.0 with KOH, sterilised by autoclaving
5 ml freeze-dried and resuspended in 5 ml of sterile D_2O

10 x YNB

- 34 g yeast nitrogen base (without amino acids and $(NH_4)_2SO_4$) in a total of 1000 ml H_2O, sterilised by autoclaving
- 5 ml of 10 x YNB in D_2O, sterilised by autoclaving
- separately 5 g $(^{15}NH_4)_2SO_4$ in 25 ml D_2O, all sterilised by autoclaving

10 x G

100ml glycerol and 900 ml H_2O, sterilised by autoclaving
1ml ^{2}H-glycerol and 24 ml D_2O, sterilised by autoclaving

500 x B

20 mg D-biotin in a total of 100 ml H_2O, filter sterilised

Basal salt medium for fermentation (0.5l)
27.2g KH_2PO_4 in 100 ml D_2O
1.47g $CaCl_2$ x 2 H_2O in 10 ml D_2O
12.4g $MgSO_4$ x 7 H_2O in 20 ml D_2O

Starter cultures (cell conditioning):

1. Protonated BMG (buffered minimal glycerol): to 10 ml of deionised H_2O, sterilised by autoclaving, through the syringe filter there were added: 5 ml of 1 M potassium phosphate pH 6.0, 5 ml of 10 x YNB, 1 ml of $(^{15}NH_4)_2SO_4$ in D_2O solution, 5 ml of 10 x G and 0.2 ml of 500 x B. Medium was inoculated with the glycerol stock of transformed *P. pastoris* or with a single colony from a fresh plate, and incubated overnight at 30°C, 250 rpm.

2. 70% deuterated BMG: to 10 ml of sterile D_2O through the syringe filter there were added: 5 ml of 1 M potassium phosphate pH 6.0, 5 ml of 10 x YNB, 1 ml of $(^{15}NH_4)_2SO_4$ in D_2O solution, 5 ml of 10 x G and 0.2 ml of 500 x B. The cell pellet from 1 ml of the overnight culture was resuspended in 1ml of prepared medium, inoculated and incubated for 48 h at 30°C, 250 rpm.
3. 100% deuterated BMG: to 10 ml of sterile D_2O through the syringe filter there were added: 5 ml of 1 M potassium phosphate pH 6.0 in D_2O, 5 ml of 10 x YNB in D_2O, 1 ml of $(^{15}NH_4)_2SO_4$ in D_2O solution, 2 ml of 10 x G in D_2O and 0.2 ml of 500 x B. The cell pellet from 1 ml of the overnight culture was resuspended in 1 ml of prepared medium, inoculated and incubated for 48 h at 30°C, 250 rpm.

Fermentation:

The closed empty vessel was autoclaved and dried in 50°C. KH_2PO_4, $CaCl_2$, $MgSO_4$, $(^{15}NH_4)_2SO_4$, freeze-dried 500 x B and PTM were dissolved in D_2O and filter sterilized, together with ^2H-glycerol in D_2O, into the vessel. A drying column with anhydrous $CaCl_2$ was attached to the air tubing. After setting the fermentor up, when the system cooled down to ~30°C, the pH was adjusted to ~5.5 with saturated solution of K_3PO_4 in D_2O. After equilibration the medium was inoculated with the starting culture through the syringe.

After 3-4 days, when the oxygen level after dropping to ~20% went up to 50-80% (no carbon source left), MeOD was added to 1% concentration. After equilibration a drop-feeding was started at 0.05 ml/min, later increased to 0.1 ml/min. Fermentation finished 10 days after induction. The cell pellet was centrifuged down (3000 rpm, 10 min) and the supernatant with the protein was recovered for the protein isolation.

APPENDIX 2A

Chemical shifts for the $^{4}F1^{5}F1$ module pair in free state and bound to D3 peptide (37°C, pH 5.0) – value with ? indicates peak overlap

Atom	$^{4}F1^{5}F1$	$^{4}F1^{5}F1$/D3
Ala 01.ha	4.152	4.176
01.hb#	1.553	1.571
01.hn	-	-
01.ca	-	-
01.cb	-	-
01.n	-	-
Glu 02.ha	4.36	4.35
02.hb#	2.02	2.049
02.hg1	2.25	2.31
02.hg2	2.33	2.31
02.hn	8.718	8.661
02.ca	52.724?	-
02.cb	30.498	-
02.n	121.86	121.854
Lys 03.ha	5.085	5.264
03.hb#	1.47	1.486
03.hg#	1.24	1.23
03.hd#	1.4	1.36
03.he#	2.795	2.819
03.hn	8.01	7.927
03.ca	54.935	-
03.cb	36.555	-
03.n	120.011	119.229
Cys 04.ha	4.827	4.562
04.hb1	2.66	2.521
04.hb2	3.131	3.064
04.hn	9.07	8.901
04.ca	56.644	-
04.cb	43.224	-
04.n	115.48	113.123
Phe 05.ha	4.76	4.787
05.hb#	2.68	2.555, 2.671
05.hd#	7.042	6.957
05.he#z	7.195	7.207
05.hn	9.119	8.785
05.ca	57.21	-
05.cb	41.27	-
05.n	121.926	121.394
Asp 06.ha	4.8	4.91
06.hb1	2.31	2.225
06.hb2	3.18	3.202
06.hn	8.247	8.239
06.ca	52.534?	-
06.cb	41.553?	-
06.n	123.778	122.452
His 07.ha	4.292	4.335
07.hb1	3.296	3.333

Atom	$^{4}F1^{5}F1$	$^{4}F1^{5}F1$/D3
Asn 50.ha	5.185	5.195
50.hb1	2.485	2.472
50.hb2	2.964	2.966
50.hd21	7.181	
50.hd22	7.85	
50.hn	9.212	9.069
50.ca	52.945	-
50.cb	39.253	-
50.n	124.262	124.565
50.nd2	116.473	-
Asp 51.ha	4.747	4.619
51.hb1	1.55	1.547
51.hb2	2.945	2.917
51.hn	7.95	7.937
51.ca	53.488	-
51.cb	41.694	-
51.n	124.716	124.425
Gln 52.ha	3.825	3.783
52.hb#	2.37	2.323
52.hg#	2.636	2.584, 2.675
52.he2#	-	
52.hn	8.832	8.885
52.ca	58.318	-
52.cb	28.466	-
52.n	126.339	126.496
Asp 53.ha	4.473	4.478
53.hb1	2.848	2.849
53.hb2	2.928	2.849
53.hn	8.799	8.799
53.ca	57.208	-
53.cb	40.877	-
53.n	118.528	-
Thr 54.hahb	4.346	4.333
54.hg2#	1.194	1.192
54.hn	7.013	6.986
54.ca	69.327	-
54.cb	60.175	-
54.n	105.572	105.413
Arg 55.ha	1.38	1.495
55.hb1	0.65	0.745
55.hb2	1.18	1.256
55.hg1	0.594	0.468
55.hg2	0.95	0.921
55.hd#	2.755	2.691
55.he	7.015	6.979
55.hn	7.624	7.708
55.ca	56.373	-

07.hb2	3.312	3.333
07.hd#	7.404	
07.he#	8.286	
07.hn	8.723	8.769
07.ca	47.26?	-
07.cb	28.623?	-
07.n	122.501	122.945
Ala 08.ha	4.24	4.272
08.hb#	1.51	1.558
08.hn	8.31	8.39
08.ca	54.425	-
08.cb	18.296	-
08.n	122.054	122.179
Ala 09.ha	4.376	4.405
09.hb#	1.38	1.412
09.hn	7.494	7.528
09.ca	51.899	-
09.cb	20.442	-
09.n	118.572	118.266
Gly 10.ha1	3.806	3.824
10.ha2	4	4.023
10.hn	8.104	8.115
10.ca	46.157	-
10.n	107.533	107.683
Thr 11.ha	4.35	4.363
11.hb	3.59	3.606
11.hg2#	0.79	0.817
11.hn	7.256	7.282
11.ca	70.914	-
11.cb	59.916	-
11.n	110.975	111.137
Ser 12.ha	4.886	4.764
12.hb1	3.25	3.322
12.hb2	3.467	3.497
12.hn	7.748	7.839
12.ca	66.26	-
12.cb	56.386	-
12.n	115.548	115.617
Tyr 13.ha	4.617	4.603
13.hb1	1.869	1.676
13.hb2	2.86	2.949
13.hd#	6.046	5.988
13.he#	5.795	5.834
13.hn	8.303	8.267
13.ca	56.536	-
13.cb	43.551	-
13.n	119.613	119.565
Val 14.ha	4.699	4.889
14.hb	2.24	2.271
14.hg1#	0.966	0.926
14.hg2#	1.027	0.976
14.hn	8.89	8.76
14.ca	60.357	-

55.cb	27.122	-
55.n	123.002	122.898
55.ne	110.109?	-
Thr 56.ha	4.254	4.34
56.hb	2.995	3.188
56.hg2#	0.206	0.407
56.hn	6.605	6.738
56.ca	-	-
56.cb	-	-
56.n	111.782	111.77
Ser 57.ha	4.933	4.954
57.hb1	3.706	3.64
57.hb2	3.876	3.836
57.hn	8.258	8.078
57.ca	63.898	-
57.cb	55.904	-
57.n	120.357	118.943
Tyr 58.ha	4.363	4.587
58.hb1	2.184	2.159
58.hb2	3.191	3.212?
58.hd#	6.201	6.195
58.he#	5.757	5.748
58.hn	8.569	8.548
58.ca	58.383	-
58.cb	40.292	-
58.n	128.514	126.963
Arg 59.ha	4.659	4.707
59.hb1	1.74	1.936
59.hb2	1.875	1.936
59.hg1	1.56	1.632
59.hg2	1.72	1.762
59.hd#	3.236	3.255
59.he	7.438	7.477
59.hn	9.19	9.213
59.ca	54.833?	-
59.cb	30.498	-
59.n	121.173	121.788
59.ne	110.559?	-
Ile 60.ha	3.444	3.395
60.hb	1.71	1.721
60.hg1#	0.993	0.951
60.hg2#d1#d2#	0.93	0.951
60.hn	8.018	8.392
60.ca	63.966	-
60.cb	37.463	-
60.n	120.152	120.297
Gly 61.ha1	3.604	3.603
61.ha2	4.52	4.47
61.hn	9.167	9.193
61.ca	44.64	-
61.n	116.541	116.515
Asp 62.ha	4.823	4.861
62.hb1	2.94	2.985

14.cb	33.676	-
14.n	116.789	115.239
Val 15.ha	3.59	3.45
15.hb	1.92	1.933
15.hg1#hg2#	0.966	0.981
15.hn	7.753	7.562
15.ca	65.287	-
15.cb	31.561	-
15.n	121.71	120.053
Gly 16.ha1	3.632	3.603
16.ha2	4.452	4.47
16.hn	9.137	9.191
16.ca	44.565	-
16.n	115.872	115.289
Glu 17.ha	4.461	4.51
17.hb1	2.4	2.379
17.hb2	2.5	2.535
17.hg#	2.68	2.653
17.hn	8.43	8.347
17.ca	57.715	-
17.cb	31	-
17.n	122.549	121.49
Thr 18.ha	5.615	5.744
18.hb	4.048	3.963
18.hg2#	1.132	1.104
18.hn	8.276	8.206
18.ca	71.945	-
18.cb	59.781	-
18.n	114.992	114.001
Trp 19.ha	4.978	4.928
19.hb1	3.191	3.05
19.hb2	3.318	3.154
19.hd1	6.712	6.815
19.he1	9.437	9.659
19.he3	6.443	6.45
19.hh2	6.176	6.344?
19.hn	8.603	8.722
19.hz2	6.79	6.904
19.hz3	6.372	6.356
19.ca	56.758	-
19.cb	31.387	-
19.n	124.303	124.395
19.ne1	126.703	-
Glu 20.ha	5.4	5.259
20.hb1	1.693	1.781
20.hb2	1.87	1.879
20.hg1	2.198	2.177
20.hg2	2.28	2.351
20.hn	8.41	8.25
20.ca	53.557	-
20.cb	32.276	-
20.n	118.997	118.994
Lys 21.ha	5.04	5.065

62.hb2	3.38	3.436
62.hn	8.491	8.478
62.ca	55.329	-
62.cb	41.742	-
62.n	121.892	121.85
Thr 63.ha	5.523	5.576
63.hb	4.136	4.163
63.hg2#	1.18	1.19
63.hn	8.127	8.144
63.ca	72.155?	-
63.cb	58.718?	-
63.n	109.068	108.702
Trp 64.ha	4.908	4.911
64.hb1	3.068	3.045
64.hb2	3.131	3.157
64.hd1	6.501	6.504
64.he1	9.781	9.616
64.he3	6.109	6.143
64.hh2	6.145	6.268
64.hn	8.283	8.27
64.hz2	6.328	6.385
64.hz3	5.922	5.94
64.ca	56.792	-
64.cb	31.798	-
64.n	121.72	121.289
64.ne1	128.57	-
Ser 65.ha	5.475	5.492
65.hb#	3.8	3.804
65.hn	8.362	8.366
65.ca	66.181	-
65.cb	56.072	-
65.n	114.169	114.127
Lys 66.ha	4.658	4.67
66.hb1	1.28	1.269
66.hb2	1.998	1.984
66.hg#	1.093	1.094
66.hd#	1.83	1.801
66.he1	2.744	3.404?
66.he2	2.939	3.404?
66.hn	8.41	8.496
66.ca	54.976	-
66.cb	34.932	-
66.n	117.766	118.011
Lys 67.ha	5.388	5.388
67.hb1	1.699	1.755
67.hb2	1.733	1.755
67.hg1	1.423	1.413
67.hg2	1.593	1.62
67.hd#	1.29	1.302
67.he#	2.932	2.947
67.hn	-	8.38
67.ca	54.867	-
67.cb	35.107	-

21.hb#	1.708	1.73
21.hg#	1.815	1.813
21.hd#	1.41	1.432
21.he#	3.025	2.833
21.hn	8.68	8.808
21.ca	-	-
21.cb	-	-
21.n	121.471	122.369
Pro 22.ha	5.227	5.243
22.hb1	1.78	1.784
22.hb2	2.265	2.286
22.hg1	1.942	1.942
22.hg2	2.09	2.098
22.hd1	3.715	3.607
22.hd2	3.83	3.778
22.hn	-	-
22.ca	62.38	-
22.cb	32.385	-
22.n	-	-
Tyr 23.ha	4.57	4.543
23.hb1	2.28	2.205
23.hb2	2.746	2.774
23.hd#	6.948	6.927
23.he#	6.708	6.439
23.hn	8.49	8.388
23.ca	56.786	-
23.cb	41.656	-
23.n	123.177	123.371
Gln 24.ha	3.638	3.491
24.hb1	1.435	1.342
24.hb2	1.5	1.398
24.hg1	1.606	1.443
24.hg2	1.88	1.797
24.he2#	-	
24.hn	8.13	8.212
24.ca	55.982	-
24.cb	27.084	-
24.n	120.449	121.487
Gly 25.ha1	3.43	3.385
25.ha2	4.306	4.385
25.hn	8.466	8.386
25.ca	47.22	-
25.n	108.541	107.388
Trp 26.ha	4.6	4.573
26.hb1	2.934	2.94
26.hb2	3.481	3.491
26.hd1	7.411	7.452
26.he1	10.292	10.303
26.he3	6.883	6.904
26.hh2	6.59	6.6
26.hn	7.317	7.425
26.hz2	7.426	7.447
26.hz3	6.394	6.401

67.n	119.759	119.934
Asp 68.ha	4.743	4.707
68.hb1	2.622	2.538
68.hb2	3.388	3.325
68.hn	8.518	8.446
68.ca	57.723	-
68.cb	31.014	-
68.n	121.576	121.505
Asn 69.ha	4.494	4.5
69.hb1	2.88	2.897
69.hb2	3.02	3.015
69.hd21	6.858	6.903?
69.hd22	7.561	7.606?
69.hn	8.42	8.389
69.ca	54.954	-
69.cb	37.453	-
69.n	115.148	114.934
60.nd2	111.794	-
Arg 70.ha	4.5	4.502
70.hb1	1.72	1.725
70.hb2	2.095	2.106
70.hg1	1.6	1.606
70.hg2	1.637	1.668
70.hd#	3.186	3.193
70.he	7.251	~7.169
70.hn	8.211	8.22
70.ca	54.94	-
70.cb	30.516	-
70.n	119.109	118.994
70.ne	110.018?	-
Gly 71.ha1	3.56	3.559
71.ha2	4.274	4.275
71.hn	8.004	7.971
71.ca	45.48	-
71.n	107.74	107.606
Asn 72.ha	4.652	4.723
72.hb1	2.385	2.314
72.hb2	2.852	2.814
72.hd21	7.041	7.122
72.hd22	8.719	8.864
72.hn	8.788	8.814
72.ca	-	-
72.cb	-	-
72.n	120.12	120.067
72.nd2	118.409	-
Leu 73.ha	4.486	4.499
73.hb#	1.736	1.73
73.hg	1.515	1.525
73.hd1#	0.808	0.819
73.hd2#	0.875	0.88
73.hn	8.559	8.542
73.ca	55.302	-
73.cb	42.918	-

26.ca	55.93	-
26.cb	30.347	-
26.n	120.49	120.108
26.ne1	129.526	-
Met 27.ha	3.717	3.673
27.hb1	1.33	1.202
27.hb2	1.645	1.918
27.hg#	0.475	0.39
27.he#	1.749	1.802
27.hn	7.022	6.931
27.ca	56.201	-
27.cb	33.288	-
27.n	119.834	119.021
Met 28.ha	5.14	5.169
28.hb#	1.575	1.527
28.hg1	2.358	2.283
28.hg2	2.508	2.499
28.he#	1.791	1.852
28.hn	7.29	7.342
28.ca	53.594	-
28.cb	34.251	-
28.n	118.384	117.451
Val 29.ha	4.34	4.268
29.hb	1.586	1.49
29.hg1#	0.423	0.39
29.hg2#	0.595	0.663
29.hn	8.627	8.471
29.ca	58.917	-
29.cb	34.716	-
29.n	114.057	111.209
Asp 30.ha	5.122	5.165
30.hb#	2.41	2.306
30.hn	8.008	8.201
30.ca	53.86	-
30.cb	43.512	-
30.n	118.72	118.099
Cys 31.ha	5.19	5.32
31.hb1	0.398	0.625
31.hb2	1.507	1.549
31.hn	9.086	9.114
31.ca	54.784?	-
31.cb	45.956?	-
31.n	123.788	123.384
Thr 32.ha	4.88	4.955
32.hb	3.59	3.582
32.hg2#	0.95	0.973
32.hn	8.427	8.779
32.ca	71.856	-
32.cb	60.382	-
32.n	114.915	115.785
Cys 33.ha	5.17	5.104
33.hb1	3.04	3.129
33.hb2	3.31	3.355

73.n	123.251	123.245
Leu 74.ha	4.849	4.898
74.hb1	1.08	1.07
74.hb2hg	1.609	1.604
74.hd1#	0.681	0.725
74.hd2#	0.798	0.814
74.hn	9.289	9.241
74.ca	53.173	-
74.cb	45.07	-
74.n	126.267	125.886
Gln 75.ha	4.75	4.768
75.hb1	1.739	1.795
75.hb2	1.952	1.98
75.hg1	2.133	2.132
75.hg2	2.133	2.188
75.he2#	-	
75.hn	8.695	8.642
75.ca	54.688	-
75.cb	30.652	-
75.n	119.991	120.136
Cys 76.ha	5.412	5.417
76.hb1	0.335	0.372
76.hb2	1.322	1.432
76.hn	9.021	9.076
76.ca	54.943	-
76.cb	46.528	-
76.n	126.383	126.119
Ile 77.ha	4.915	4.96
77.hb	1.59	1.602
77.hg11	0.793	0.814
77.hg12	0.865	0.863
77.hg2#	1.019	1.019
77.hd1#	1.178	1.472
77.hn	8.469	8.751
77.ca	58.739	-
77.cb	42.223	-
77.n	119.138	119.281
Cys 78.ha	5.365	5.383
78.hb1	3.224	3.245
78.hb2	3.61	3.727
78.hn	8.415	8.37
78.ca	54.514	-
78.cb	38.597	-
78.n	124.367	124.171
Thr 79.ha	4.536	4.598
79.hb	4.2	4.243
79.hg2#	1.206	1.217
79.hn	8.764	8.894
79.ca	68.914	-
79.cb	64.897	-
79.n	124.313	124.217
Gly 80.ha1	3.335	3.247
80.ha2	4.53	4.506

33.hn	8.045	8.502
33.ca	-	-
33.cb	-	-
33.n	123.219	123.048
Leu 34.ha	4.584	5.104
34.hb#g	1.545	1.541
34.hd1#hd2#	0.804	0.777
34.hn	9.157	9.465
34.ca	55.032	-
34.cb	43.171	-
34.n	106.325	106.808
Gly 35.ha1	3.423	3.221
35.ha2	4.01	4.151
35.hn	8.503	8.564
35.ca	47.264	-
35.n	106.324	105.798
Glu 36.ha	4.058	4.007
36.hb#g1	2.1	2.125
36.hg2	2.2	2.259
36.hn	8.293	8.756
36.ca	56.827	-
36.cb	27.978	-
36.n	122.512	123.645
Gly 37.ha1	3.738	3.8
37.ha2	4.233	4.303
37.hn	9.645	9.942
37.ca	45.996	-
37.n	111.222	109.347
Ser 38.ha	4.722	4.87
38.hb1	3.873	3.628
38.hb2	4.1	4.196
38.hn	8.035	7.85
38.ca	64.125	-
38.cb	57.592	-
38.n	115.241	113.936
Gly 39.ha1	3.905	4.017
39.ha2	4.22	4.421
39.hn	9.01	9.598
39.ca	47.093	-
39.n	114.92	116.684
Arg 40.ha	4.242	4.875
40.hb1	1.58	1.835
40.hb2	1.735	1.835
40.hg1	1.41	1.495
40.hg2	1.66	1.495
40.hd1	3.17	3.064
40.hd2	3.17	3.097
40.he	7.18	7.226
40.hn	8.778	9.611
40.ca	57.42	-
40.cb	31.73	-
40.n	122.013	124.339
40.ne	110.116?	-

80.hn	9.357	9.586
80.ca	53.109	-
80.n	110.69	111.465
Asn 81.ha	4.513	4.504
81.hb1	2.67	2.552
81.hb2	3.058	3.085
81.hd21	6.745	6.693
81.hd22	7.687	7.671
81.hn	8.622	8.635
81.ca	54.18	-
81.cb	37.541	-
81.n	123.972	124.818
81.nd2	112.293	-
Gly 82.ha1	3.664	3.663
82.ha2	4.153	4.102
82.hn	9.174	9.397
82.ca	45.976	-
82.n	105.864	105.56
Arg 83.ha	4.823	4.707
83.hb1	1.56	1.563
83.hb2	1.847	1.816
83.hg#	1.926	1.976
83.hd#	3.18	3.088
83.he	7.245	
83.hn	7.873	8.043
83.ca	55.103	-
83.cb	31.435	-
83.n	117.378	117.013
83.ne	109.185?	-
Gly 84.ha1	3.87	3.879
84.ha2	4.064	4.389?
84.hn	8.663	9.039
84.ca	47.913	-
84.n	113.247	113.362
Glu 85.ha	4.254	4.36
85.hb#	1.83	1.806
85.hg1	2.08	1.916
85.hg2	2.188	2.151
85.hn	8.537	8.667
85.ca	56.786	-
85.cb	31.77	-
85.n	118.896	118.653
Trp 86.ha	5.607	5.652
86.hb1	3.478	3.419
86.hb2	4.016	4.17
86.hd1	7.342	7.306
86.he1	10.153	10.278
86.he3	7.643	7.568
86.hh2	7.52	7.426
86.hn	7.957	8.705
86.hz2	7.753	7.652
86.hz3	7.326	7.193
86.ca	55.795	-

Ile 41.ha	4.908	5.409
41.hb	1.765	1.537
41.hg1#	1.077	1.331
41.hg2#	0.925	0.992
41.hd1#	2.029	1.71
41.hn	8.63	9.43
41.ca	60.483	-
41.cb	41.451	-
41.n	126.613	122.36
Thr 42.ha	4.737	5.098
42.hb	3.9	3.947
42.hg2#	1.107	1.179
42.hn	8.288	8.206
42.ca	70.609	-
42.cb	59.669	-
42.n	120.274	115.202
Cys 43.ha	5.555	5.404
43.hb1	2.29	2.616?
43.hb2	2.885	2.989?
43.hn	8.522	8.863
43.ca	54.951	-
43.cb	48.421	-
43.n	123.783	124.217
Thr 44.ha	4.739	5.611
44.hb	4.152	4.238
44.hg2#	1.097	1.121
44.hn	8.896	8.643
44.ca	-	-
44.cb	-	-
44.n	115.2	113.912
Ser 45.ha	4.714	4.662
45.hb1	3.799	3.678
45.hb2	4.026	4.423
45.hn	8.728	9.007
45.ca	63.779	-
45.cb	58.173	-
45.n	122.679	-
Arg 46.ha	4.054	3.874
46.hb1	1.633	1.68
46.hb2	1.686	1.68
46.hg1	1.505	1.483
46.hg2	1.581	1.571
46.hd#	3.136	3.088
46.he	7.123	7.132
46.hn	8.414	8.58
46.ca	58.607	-
46.cb	31.019	-
46.n	122.713	120.924
46.ne	109.617?	-
Asn 47.ha	5.01	5.165
47.hb1	2.677	2.601
47.hb2	3.068	3.107
47.hd21	6.783	6.851

86.cb	34.473	-
86.n	119.681	-
86.ne1	129.592	-
Lys 87.ha	4.76	4.749
87.hb1	1.746	1.698
87.hb2	1.825	1.827
87.hg#	1.36	1.493
87.hd#	1.64	1.568
87.he#	2.89	2.798
87.hn	8.48	8.663
87.ca	54.853	-
87.cb	36.024	-
87.n	118.2	118.582
Cys 88.ha	5.516	5.636
88.hb1	2.59	2.637
88.hb2	3.126	2.976
88.hn	8.846	8.855
88.ca	55.244	-
88.cb	48.808	-
88.n	120.403	123.503
Glu 89.ha	4.69	4.845
89.hb#	2.045	2.036
89.hg1	1.906	1.92
89.hg2	2.21	2.21
89.hn	8.82	8.885
89.ca	54.388	-
89.cb	33.458	-
89.n	120.853	120.067
Arg 90.ha	4.525	4.557
90.hb1	1.728	1.691
90.hb2	1.825	1.839
90.hg1	1.556	1.547
90.hg2	1.663	1.45
90.hd#	3.143	3.178
90.he	7.236	
90.hn	8.776	8.78
90.ca	56.438	-
90.cb	30.765	-
90.n	123.144	123.076
90.ne	109.137?	-
His 91.ha	4.802	4.826
91.hb1	2.996	3
91.hb2	3.197	3.221
91.hd#	7.183	
91.he#	8.475	
91.hn	8.852	8.868
91.ca	55.219	-
91.cb	30.113	-
91.n	123.512	123.866
Thr 92.ha	4.383	4.405
92.hb	4.246	4.262
92.hg2#	1.186	1.194
92.hn	8.285	8.308

47.hd22	7.392	7.56
47.hn	8.68	8.542
47.ca	52.11	-
47.cb	38.943	-
47.n	117.14	116.763
47.nd2	112.867	-
Arg 48.ha	5.087	4.796
48.hb#g1	1.582	1.483
48.hg2	1.76	1.731
48.hd#	2.973	3.027
48.he	7.18	
48.hn	7.473	8.071
48.ca	52.665	-
48.cb	32.843	-
48.n	116.702	117.287
48.ne	109.31?	-
Cys 49.ha	4.918	4.937?
49.hb1	2.747	3.05
49.hb2	3.22	3.157
49.hn	8.738	8.867
49.ca	55.832	-
49.cb	44.833	-
49.n	116.398	114.932

92.ca	69.946	-
92.cb	61.835	-
92.n	117.742	117.328
Ser 93.ha	4.252	4.254
93.hb#	3.855	3.864
93.hn	7.959	7.969
93.ca	-	-
93.cb	-	-
93.n	123.178	123.096

^{15}N relaxation data (ms)

^{4}F1^{5}F1					^{4}F1^{5}F1/D3				
Residue	**T1**	**error**	**T2**	**error**	**Residue**	**T1**	**error**	**T2**	**error**
2	438.60	22.12	85.90	3.99	2	355.2	56.66	167.11	11.3
3	581.73	21.66	130.34	4.18	3	610.8	86.95	109.04	5.6
4	440.72	36.32	100.09	7.75	4	502.0	110.13	103.26	8.9
5	460.62	25.67	146.43	7.10	5	460.8	114.68	126.92	12.2
6	447.63	22.64	130.45	5.87	6	467.5	89.39	112.54	8.3
7	371.33	14.06	114.32	4.01	7	658.7	129.32	117.01	6.8
9	487.33	15.20	152.37	4.13	8	508.1	53.45	132.93	5.7
10	490.20	20.18	143.58	5.09	9	498.7	56.47	128.22	5.6
11	473.71	13.24	153.26	3.99	10	491.8	70.17	126.89	7.2
12	425.89	13.60	139.88	3.97	11	514.1	60.53	120.29	5.4
13	465.12	23.80	132.29	6.13	12	482.6	53.57	131.51	5.8
14	488.76	25.08	108.53	4.92	13	543.7	85.45	120.74	7.3
15	612.00	24.35	96.06	3.38	15	649.7	112.73	90.08	6.0
16	457.25	34.08	112.78	7.00	16	495.2	133.45	104.10	10.3
18	449.03	21.98	117.05	4.96	17	557.1	54.93	86.84	5.5
21	414.42	19.58	119.92	5.15	18	528.8	100.67	107.41	7.1
24	547.95	30.02	100.01	4.66	19	364.7	67.17	104.11	7.3
25	642.67	38.00	104.82	4.66	21	479.3	48.95	96.45	6.7
26	521.92	25.88	141.06	5.85	23	534.4	156.83	94.47	10.2
27	413.39	18.80	113.51	4.50	25	558.6	118.29	86.36	6.9
28	455.79	30.33	99.53	5.85	26	508.6	120.31	125.16	11.1
29	441.11	29.58	113.90	6.82	27	485.2	93.70	96.69	6.8

30	424.63	27.41	109.11	5.85	28	494.3	165.91	81.42	10.2
31	408.83	28.58	110.95	6.59	29	546.7	156.34	91.35	9.7
32	492.85	17.00	141.00	4.51	30	534.1	160.94	101.62	11.2
34	397.61	32.25	118.54	9.06	31	479.6	106.04	109.85	9.7
35	496.03	41.09	106.06	7.14	32	464.2	104.10	122.23	11.2
37	500.25	49.55	139.86	12.38	33	634.9	56.84	108.75	6.8
38	464.90	28.10	85.77	4.74	34	634.9	56.84	108.75	6.8
39	490.68	26.24	125.52	5.78	35	512.3	119.94	105.44	9.0
41	429.55	26.57	103.49	5.63	36	508.1	65.58	116.51	6.8
44	424.09	21.04	125.27	5.62	37	480.3	104.50	111.31	9.6
47	438.02	24.56	102.85	4.90	38	496.7	83.91	96.33	6.1
48	400.96	17.20	110.56	4.09	39	472.3	166.23	92.38	12.8
49	406.83	30.12	128.97	8.03	40	709.7	569.19	92.40	26.2
50	403.39	25.71	135.06	7.15	41	530.7	431.90	56.15	20.4
51	411.02	30.75	113.10	6.69	42	455.5	137.82	80.89	9.7
52	436.30	18.66	131.49	4.88	43	389.8	60.04	117.90	11.1
53	408.16	16.16	116.65	4.01	44	500.5	125.75	99.85	9.3
54	398.41	21.27	129.35	6.49	46	456.0	141.60	81.31	9.4
55	440.72	26.03	108.37	5.26	47	575.0	157.07	78.77	7.3
56	430.29	20.18	103.61	3.99	50	458.0	154.44	126.23	16.1
58	397.30	28.41	107.15	6.26	52	459.9	90.98	117.00	8.4
59	489.48	36.90	120.51	7.51	54	382.5	75.96	119.99	9.2
60	569.15	32.07	96.52	4.59	55	407.6	113.84	102.35	9.9
61	484.26	41.51	109.99	7.16	56	450.6	71.49	102.66	6.3
62	459.35	21.52	99.52	3.74	57	405.6	125.57	88.35	9.9
63	402.41	22.51	116.09	5.59	58	436.6	147.59	110.96	14.5
64	399.52	19.15	115.70	4.93	59	471.7	127.94	121.91	13.3
65	479.39	26.20	109.47	4.91	60	566.2	151.98	120.32	12.5
66	419.29	17.05	113.28	4.11	61	527.7	166.53	107.27	12.2
67	508.91	21.75	128.02	4.74	62	599.8	51.82	110.61	6.2
68	532.48	23.53	127.02	5.00	63	473.2	91.83	112.65	8.5
69	502.26	16.15	140.98	4.00	65	516.2	91.69	107.15	7.3
70	522.19	22.09	133.37	4.86	66	503.2	84.34	94.09	6.1
71	581.06	24.98	119.96	4.30	67	574.3	86.77	119.40	7.3
72	465.77	15.62	141.42	4.02	68	558.9	94.98	86.34	5.4
73	598.09	22.18	124.33	4.05	69	434.2	61.47	132.75	7.44
74	479.62	32.43	135.50	8.15	71	631.3	95.25	111.92	5.9
75	439.37	21.62	125.25	5.48	73	425.8	76.54	109.57	7.3
76	426.08	29.95	115.58	6.76	74	486.1	104.70	117.14	10.2
77	444.64	25.90	126.15	6.35	75	536.1	110.11	111.92	8.7
78	445.83	36.18	130.07	9.15	76	457.8	115.10	106.68	10.1
79	434.40	33.59	111.86	6.88	77	469.4	136.22	97.82	10.8
80	477.10	37.10	126.69	8.67	78	524.1	243.93	105.94	16.0
82	493.34	29.69	141.32	7.73	80	424.9	146.30	105.54	14.4
83	529.38	23.82	114.36	4.38	81	453.3	81.17	107.10	6.7
84	436.49	23.43	114.72	5.47	82	458.7	87.96	120.60	9.5

86	455.58	20.55	115.98	4.43	84	514.4	200.57	102.24	15.2
87	438.40	26.33	117.14	5.80	85	424.6	171.83	72.22	10.6
88	462.53	22.04	137.36	5.55	87	438.9	111.96	92.05	8.9
89	468.60	23.50	118.08	4.94	88	349.0	118.78	101.64	13.3
92	546.75	10.16	249.44	5.23	90	505.3	40.60	118.85	6.6
					91	414.2	56.29	142.76	7.4
					92	412.7	39.69	248.82	11.7
					93	805.1	39.54	513.87	17.1

N-H residual dipolar couplings (Hz)

Residue	$^{4}F1^{5}F1$	^{2}H-$^{4}F1^{5}F1$/D3	$^{4}F1^{5}F1$/D3
2	0	-5	-7
3	0	15	20
4	0	-4	-7
5	-10	-11	-14
6	-19	-10	-14
7	0	0	0
8	-8	-8	0
9	-6	-7	-19
10	-15	-14	-17
11	0	-3	0
12	-14	-10	-12
13	-5	-4	0
14	13	-23	0
15	23	26	36
16	-16	-15	-19
17	0	0	0
18	-18	-10	-11
19	-15	-12	0
20	0	0	0
21	-11	-9	0
22	0	0	0
23	0	25	10
24	21	23	0
25	23	21	27
26	-13	-11	-16
27	2	6	0
28	7	12	0
29	-6	-1	8
30	-12	0	0
31	-16	-12	0
32	0	-13	-20
33	0	-6	26
34	-11	0	-23
35	-1	0	-9
36	0	-12	0

37	-9	-9	-13
38	7	13	11
39	0	-4	0
40	0	0	10
41	-6	-13	0
42	0	-14	0
43	0	0	0
44	0	-11	-19
45	0	0	0
46	0	18	0
47	14	19	32
48	-9	0	-7
49	0	22	0
50	-14	-9	-7
51	-19	0	0
52	-14	-11	0
53	-6	0	0
54	2	0	0
55	-15	-14	0
56	0	0	0
57	0	0	0
58	-16	-10	-19
59	3	2	-5
60	0	0	0
61	-17	-10	-8
62	14	16	0
63	-18	-13	-20
64	-16	0	0
65	-9	-4	-4
66	2	0	0
67	4	9	12
68	13	18	0
69	0	-3	-8
70	0	-8	0
71	22	19	32
72	-6	0	0
73	19	22	31
74	-3	2	8
75	-9	-1	1
76	-16	-11	-16
77	0	-10	-17
78	0	-11	-25
79	-11	0	0
80	2	-6	-5
81	0	-9	0
82	-18	-13	-17

83	11	0	19
84	-1	-7	0
85	0	-13	0
86	-18	0	0
87	0	-12	-19
88	-19	-12	-20
89	0	0	0
90	-15	-9	0
91	-5	-3	-9
92	-4	-6	-7
93	-2	-2	-3

APPENDIX 2B

Chemical shifts for the unbound D3 peptide (37°C, pH 5.0)

Proton	Shift
Glu 01.ha	4.545
01.hb1	1.94
01.hb2	2.108
01.hg#	2.407
01.hn	-
Ile 02.ha	4.201
02.hb	1.859
02.hg1#	1.2
02.hg2#	0.888
02.hd1#	0.888
02.hn	8.211
Ile 03.ha	4.215
03.hb	1.867
03.hg1#	1.21
03.hg2#	0.9
03.hd1#	0.9
03.hn	8.241
Glu 04.ha	4.358
04.hb1	1.965
04.hb2	2.071
04.hg#	2.33
04.hn	8.451
Glu 05.ha	4.323
05.hb1	1.97
05.hb2	2.06
05.hg#	2.315
05.hn	8.418
Asp 06.ha	4.702
06.hb1	2.701
06.hb2	2.796
06.hn	8.451
Thr 07.ha	4.281
07.hb	
07.hg2#	1.211
07.hn	8.109
Asn 08.ha	4.723
08.hb1	2.819
08.hb2	2.877
08.hd21	6.887
08.hd22	7.653
08.hn	8.411
Lys 09.ha	4.224
09.hb#	1.788
09.hg1	1.398
09.hg2	1.448
09.hd#	1.694
09.he#	
09.hn	8.024

Proton	Shift
Gly 17.ha1	3.92
17.ha2	3.92
17.hn	8.284
Gly 18.ha1	3.902
18.ha2	3.902
18.hn	7.976
His 19.ha	4.713
19.hb1	3.15
19.hb2	3.274
19.hd#	
19.he#	
19.hn	8.338
Asn 20.ha	4.76
20.hb1	2.563
20.hb2	2.85
20.hd21	6.87
20.hd22	7.545
20.hn	8.528
Ser 21.ha	4.466
21.hb1	3.869
21.hb2	3.91
21.hn	8.278
Val 22.ha	4.157
22.hb	2.08
22.hg1#	0.889
22.hg2#	1.213
22.hn	8.062
Asp 23.ha	4.601
23.hb1	2.565
23.hb2	2.659
23.hn	8.191
Phe 24.ha	4.611
24.hb1	3.054
24.hb2	3.145
24.hd#	7.358
24.he#z	7.25
24.hn	8.006
Glu 25.ha	4.284
25.hb1	1.939
25.hb2	2.025
25.hg#	2.3
25.hn	
Glu 26.ha	4.261
26.hb1	1.94
26.hb2	2.091
26.hg#	2.3
26.hn	8.218

Proton	Shift
Asp 10.ha	4.618
10.hb1	2.652
10.hb2	2.73
10.hn	8.25
Lys 11.ha	4.613
11.hb#	1.817
11.hg#	1.444
11.hd#	1.72
11.he#	
11.hn	7.927
Pro 12.ha	4.423
12.hb1	1.776
12.hb2	2.258
12.hg1	2.017
12.hg2	2.017
12.hd1	3.636
12.hd2	3.799
12.hn	-
Ser 13.ha	4.374
13.hb1	3.832
13.hb2	3.866
13.hn	8.27
Tyr 14.ha	4.552
14.hb1	2.971
14.hb2	2.971
14.hd#	6.813
14.he#	7.088
14.hn	8.021
Gln 15.ha	4.252
15.hb1	1.815
15.hb2	1.909
15.hg1	2.149
15.hg2	2.149
15.he21	6.772
15.he22	7.048
15.hn	8.077
Phe 16.ha	4.63
16.hb1	3.039
16.hb2	3.197
16.hd#	7.382
16.he#z	7.289
16.hn	8.081

Proton	Shift
Asp 27.ha	4.679
27.hb1	2.722
27.hb2	2.78
27.hn	8.382
Thr 28.ha	4.328
28.hb	4.285
28.hg21	1.183
28.hg22	1.244
28.hn	8.014
Leu 29.ha	4.617
29.hb#	1.691
29.hg	1.589
29.hd1#	0.94
29.hd2#	0.974
29.hn	7.982
Pro 30.ha	4.419
30.hb1	1.888
30.hb2	2.308
30.hg1	2.036
30.hg2	2.064
30.hd1	3.632
30.hd2	3.87
30.hn	-
Lys 31.ha	4.372
31.hb1	1.777
31.hb2	1.855
31.hg1	1.453
31.hg2	1.509
31.hd#	1.71
31.he#	3.032
31.hn	8.361
Val 32.ha	4.132
32.hb	2.077
32.hg1#	0.973
32.hg2#	1.35
32.hn	8.034

APPENDIX 3A

Restraints for a free ^{4}F1^{5}F1 module pair structure calculations

Distance restraints

number of intraresidue NOEs: 441
number of sequential NOEs: 298
number of medium range NOEs: 115
number of long range NOEs: 457

INTRARESIDUE NOES

```
assign ( resid    2 and name ha   )( resid    2 and name hg2  ) 0.0 0.0 3.5
assign ( resid    2 and name ha   )( resid    2 and name hg1  ) 0.0 0.0 5.0
assign ( resid    2 and name ha   )( resid    2 and name hn   ) 0.0 0.0 5.0
assign ( resid    2 and name hb#  )( resid    2 and name hn   ) 0.0 0.0 3.5
assign ( resid    3 and name ha   )( resid    3 and name hg#  ) 0.0 0.0 3.5
assign ( resid    3 and name he#  )( resid    3 and name hb#  ) 0.0 0.0 3.5
assign ( resid    3 and name he#  )( resid    3 and name hg#  ) 0.0 0.0 5.0
assign ( resid    3 and name ha   )( resid    3 and name hd#  ) 0.0 0.0 3.5
assign ( resid    3 and name ha   )( resid    3 and name hn   ) 0.0 0.0 3.5
assign ( resid    3 and name hb#  )( resid    3 and name hn   ) 0.0 0.0 3.5
assign ( resid    3 and name hd#  )( resid    3 and name hn   ) 0.0 0.0 5.0
assign ( resid    4 and name ha   )( resid    4 and name hn   ) 0.0 0.0 5.0
assign ( resid    4 and name hb2  )( resid    4 and name hn   ) 0.0 0.0 5.0
assign ( resid    4 and name hb1  )( resid    4 and name hn   ) 0.0 0.0 3.5
assign ( resid    5 and name ha   )( resid    5 and name hd#  ) 0.0 0.0 3.5
assign ( resid    5 and name ha   )( resid    5 and name hn   ) 0.0 0.0 5.0
assign ( resid    5 and name hb#  )( resid    5 and name hn   ) 0.0 0.0 3.5
assign ( resid    5 and name hd#  )( resid    5 and name hn   ) 0.0 0.0 5.0
assign ( resid    5 and name hb#  )( resid    5 and name he#  ) 0.0 0.0 5.0
assign ( resid    5 and name hb#  )( resid    5 and name hd#  ) 0.0 0.0 2.8
assign ( resid    6 and name hb2  )( resid    6 and name hn   ) 0.0 0.0 3.5
assign ( resid    5 and name hb#  )( resid    5 and name hd#  ) 0.0 0.0 2.8
assign ( resid    6 and name hb2  )( resid    6 and name hn   ) 0.0 0.0 3.5
assign ( resid    6 and name hb1  )( resid    6 and name hn   ) 0.0 0.0 3.5
assign ( resid    7 and name ha   )( resid    7 and name hn   ) 0.0 0.0 5.0
assign ( resid    7 and name hb1  )( resid    7 and name hn   ) 0.0 0.0 5.0
assign ( resid    7 and name hb2  )( resid    7 and name hn   ) 0.0 0.0 5.0
assign ( resid    8 and name ha   )( resid    8 and name hn   ) 0.0 0.0 3.5
assign ( resid    8 and name hb#  )( resid    8 and name hn   ) 0.0 0.0 2.8
assign ( resid    9 and name ha   )( resid    9 and name hn   ) 0.0 0.0 3.5
assign ( resid    9 and name hb#  )( resid    9 and name hn   ) 0.0 0.0 2.8
assign ( resid   10 and name ha2  )( resid   10 and name hn   ) 0.0 0.0 3.5
assign ( resid   10 and name ha1  )( resid   10 and name hn   ) 0.0 0.0 3.5
assign ( resid   11 and name ha   )( resid   11 and name hg2# ) 0.0 0.0 2.8
assign ( resid   11 and name ha   )( resid   11 and name hn   ) 0.0 0.0 3.5
assign ( resid   11 and name hb   )( resid   11 and name hn   ) 0.0 0.0 5.0
assign ( resid   11 and name hg2# )( resid   11 and name hn   ) 0.0 0.0 3.5
assign ( resid   12 and name ha   )( resid   12 and name hn   ) 0.0 0.0 3.5
assign ( resid   12 and name hb2  )( resid   12 and name hn   ) 0.0 0.0 5.0
assign ( resid   12 and name hb1  )( resid   12 and name hn   ) 0.0 0.0 5.0
assign ( resid   13 and name hb2  )( resid   13 and name hd#  ) 0.0 0.0 2.8
assign ( resid   13 and name hb1  )( resid   13 and name hd#  ) 0.0 0.0 3.5
assign ( resid   13 and name ha   )( resid   13 and name hd#  ) 0.0 0.0 5.0
assign ( resid   13 and name hb2  )( resid   13 and name he#  ) 0.0 0.0 5.0
assign ( resid   13 and name hb1  )( resid   13 and name he#  ) 0.0 0.0 5.0
assign ( resid   13 and name ha   )( resid   13 and name he#  ) 0.0 0.0 6.0
assign ( resid   13 and name ha   )( resid   13 and name hn   ) 0.0 0.0 3.5
assign ( resid   13 and name hb2  )( resid   13 and name hn   ) 0.0 0.0 5.0
assign ( resid   13 and name hb1  )( resid   13 and name hn   ) 0.0 0.0 5.0
assign ( resid   13 and name hd#  )( resid   13 and name hn   ) 0.0 0.0 3.5
assign ( resid   14 and name ha   )( resid   14 and name hg2# ) 0.0 0.0 3.5
assign ( resid   14 and name ha   )( resid   14 and name hg1# ) 0.0 0.0 5.0
assign ( resid   14 and name hb   )( resid   14 and name hn   ) 0.0 0.0 3.5
assign ( resid   14 and name hg2# )( resid   14 and name hn   ) 0.0 0.0 3.5
assign ( resid   15 and name ha   )( resid   15 and name hn   ) 0.0 0.0 2.8
assign ( resid   15 and name hb   )( resid   15 and name hn   ) 0.0 0.0 3.5
assign ( resid   16 and name ha2  )( resid   16 and name hn   ) 0.0 0.0 5.0
assign ( resid   16 and name ha1  )( resid   16 and name hn   ) 0.0 0.0 3.5
assign ( resid   17 and name ha   )( resid   17 and name hg#  ) 0.0 0.0 3.5
assign ( resid   17 and name hg#  )( resid   17 and name hn   ) 0.0 0.0 5.0
assign ( resid   17 and name hb2  )( resid   17 and name hn   ) 0.0 0.0 3.5
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assign ( resid   17 and name hb1  )( resid   17 and name hn   ) 0.0 0.0 3.5
assign ( resid   17 and name ha   )( resid   17 and name hn   ) 0.0 0.0 5.0
assign ( resid   18 and name ha   )( resid   18 and name hg2# ) 0.0 0.0 2.8
assign ( resid   18 and name ha   )( resid   18 and name hn   ) 0.0 0.0 5.0
assign ( resid   18 and name hb   )( resid   18 and name hn   ) 0.0 0.0 3.5
assign ( resid   18 and name hg2# )( resid   18 and name hn   ) 0.0 0.0 3.5
assign ( resid   19 and name ha   )( resid   19 and name hd1  ) 0.0 0.0 5.0
assign ( resid   19 and name hb2  )( resid   19 and name hd1  ) 0.0 0.0 5.0
assign ( resid   19 and name hb1  )( resid   19 and name hd1  ) 0.0 0.0 3.5
assign ( resid   19 and name hb1  )( resid   19 and name he3  ) 0.0 0.0 5.0
assign ( resid   19 and name hz2  )( resid   19 and name hz3  ) 0.0 0.0 5.0
assign ( resid   19 and name he3  )( resid   19 and name hh2  ) 0.0 0.0 5.0
assign ( resid   19 and name ha   )( resid   19 and name he3  ) 0.0 0.0 5.0
assign ( resid   19 and name ha   )( resid   19 and name hn   ) 0.0 0.0 3.5
assign ( resid   19 and name hb2  )( resid   19 and name he3  ) 0.0 0.0 3.5
assign ( resid   19 and name he3  )( resid   19 and name hn   ) 0.0 0.0 3.5
assign ( resid   19 and name hz2  )( resid   19 and name he1  ) 0.0 0.0 3.5
assign ( resid   19 and name hz3  )( resid   19 and name hn   ) 0.0 0.0 5.0
assign ( resid   20 and name ha   )( resid   20 and name hg2  ) 0.0 0.0 5.0
assign ( resid   20 and name ha   )( resid   20 and name hg1  ) 0.0 0.0 5.0
assign ( resid   20 and name ha   )( resid   20 and name hn   ) 0.0 0.0 3.5
assign ( resid   20 and name hg1  )( resid   20 and name hn   ) 0.0 0.0 5.0
assign ( resid   20 and name hg2  )( resid   20 and name hn   ) 0.0 0.0 3.5
assign ( resid   20 and name hb2  )( resid   20 and name hn   ) 0.0 0.0 3.5
assign ( resid   21 and name ha   )( resid   21 and name hg#  ) 0.0 0.0 3.5
assign ( resid   21 and name ha   )( resid   21 and name hd#  ) 0.0 0.0 5.0
assign ( resid   21 and name hd#  )( resid   21 and name hn   ) 0.0 0.0 5.0
assign ( resid   21 and name ha   )( resid   21 and name hn   ) 0.0 0.0 3.5
assign ( resid   22 and name ha   )( resid   22 and name hg2  ) 0.0 0.0 5.0
assign ( resid   22 and name ha   )( resid   22 and name hg1  ) 0.0 0.0 5.0
assign ( resid   22 and name hd1  )( resid   22 and name hb2  ) 0.0 0.0 5.0
assign ( resid   22 and name hd2  )( resid   22 and name hb2  ) 0.0 0.0 5.0
assign ( resid   22 and name hd1  )( resid   22 and name hb1  ) 0.0 0.0 3.5
assign ( resid   22 and name hd2  )( resid   22 and name hb1  ) 0.0 0.0 5.0
assign ( resid   22 and name ha   )( resid   22 and name hd1  ) 0.0 0.0 5.0
assign ( resid   23 and name ha   )( resid   23 and name hd#  ) 0.0 0.0 3.5
assign ( resid   23 and name hb2  )( resid   23 and name hd#  ) 0.0 0.0 3.5
assign ( resid   23 and name hb1  )( resid   23 and name hd#  ) 0.0 0.0 3.5
assign ( resid   23 and name hb1  )( resid   23 and name he#  ) 0.0 0.0 5.0
assign ( resid   23 and name ha   )( resid   23 and name he#  ) 0.0 0.0 5.0
assign ( resid   23 and name hb2  )( resid   23 and name hn   ) 0.0 0.0 5.0
assign ( resid   23 and name hb1  )( resid   23 and name hn   ) 0.0 0.0 5.0
assign ( resid   23 and name hd#  )( resid   23 and name hn   ) 0.0 0.0 5.0
assign ( resid   23 and name hb2  )( resid   23 and name he#  ) 0.0 0.0 5.0
assign ( resid   24 and name ha   )( resid   24 and name hg2  ) 0.0 0.0 3.5
assign ( resid   24 and name ha   )( resid   24 and name hg1  ) 0.0 0.0 3.5
assign ( resid   24 and name ha   )( resid   24 and name hn   ) 0.0 0.0 2.8
assign ( resid   24 and name hg1  )( resid   24 and name hn   ) 0.0 0.0 5.0
assign ( resid   24 and name hg2  )( resid   24 and name hn   ) 0.0 0.0 5.0
assign ( resid   24 and name hb1  )( resid   24 and name hn   ) 0.0 0.0 5.0
assign ( resid   24 and name hb2  )( resid   24 and name hn   ) 0.0 0.0 5.0
assign ( resid   25 and name ha2  )( resid   25 and name hn   ) 0.0 0.0 3.5
assign ( resid   25 and name ha1  )( resid   25 and name hn   ) 0.0 0.0 3.5
assign ( resid   26 and name hz2  )( resid   26 and name hz3  ) 0.0 0.0 5.0
assign ( resid   26 and name he3  )( resid   26 and name hh2  ) 0.0 0.0 5.0
assign ( resid   26 and name hb2  )( resid   26 and name he3  ) 0.0 0.0 3.5
assign ( resid   26 and name hb2  )( resid   26 and name hd1  ) 0.0 0.0 5.0
assign ( resid   26 and name hb1  )( resid   26 and name hd1  ) 0.0 0.0 3.5
assign ( resid   26 and name ha   )( resid   26 and name hd1  ) 0.0 0.0 5.0
assign ( resid   26 and name ha   )( resid   26 and name hn   ) 0.0 0.0 3.5
assign ( resid   26 and name ha   )( resid   26 and name he3  ) 0.0 0.0 5.0
assign ( resid   26 and name hb1  )( resid   26 and name hn   ) 0.0 0.0 5.0
assign ( resid   26 and name hz2  )( resid   26 and name he1  ) 0.0 0.0 5.0
assign ( resid   27 and name ha   )( resid   27 and name hg#  ) 0.0 0.0 3.5
assign ( resid   27 and name ha   )( resid   27 and name hn   ) 0.0 0.0 5.0
assign ( resid   27 and name hg#  )( resid   27 and name hn   ) 0.0 0.0 3.5
assign ( resid   27 and name hb2  )( resid   27 and name hn   ) 0.0 0.0 3.5
assign ( resid   27 and name he#  )( resid   27 and name hn   ) 0.0 0.0 5.0
assign ( resid   27 and name hb1  )( resid   27 and name hn   ) 0.0 0.0 5.0
assign ( resid   28 and name ha   )( resid   28 and name he#  ) 0.0 0.0 5.0
assign ( resid   28 and name ha   )( resid   28 and name hg1  ) 0.0 0.0 5.0
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assign ( resid   28 and name ha   )( resid   28 and name hg2  ) 0.0 0.0 5.0
assign ( resid   28 and name hg2  )( resid   28 and name he#  ) 0.0 0.0 3.5
assign ( resid   28 and name ha   )( resid   28 and name hn   ) 0.0 0.0 5.0
assign ( resid   28 and name hg1  )( resid   28 and name hn   ) 0.0 0.0 5.0
assign ( resid   28 and name he#  )( resid   28 and name hn   ) 0.0 0.0 5.0
assign ( resid   28 and name hb#  )( resid   28 and name hn   ) 0.0 0.0 5.0
assign ( resid   28 and name hg2  )( resid   28 and name hn   ) 0.0 0.0 5.0
assign ( resid   29 and name ha   )( resid   29 and name hg2# ) 0.0 0.0 3.5
assign ( resid   29 and name ha   )( resid   29 and name hg1# ) 0.0 0.0 2.8
assign ( resid   29 and name ha   )( resid   29 and name hn   ) 0.0 0.0 5.0
assign ( resid   29 and name hg2# )( resid   29 and name hn   ) 0.0 0.0 3.5
assign ( resid   29 and name hg1# )( resid   29 and name hn   ) 0.0 0.0 5.0
assign ( resid   29 and name hb   )( resid   29 and name hn   ) 0.0 0.0 5.0
assign ( resid   30 and name hb#  )( resid   30 and name hn   ) 0.0 0.0 3.5
assign ( resid   30 and name ha   )( resid   30 and name hn   ) 0.0 0.0 5.0
assign ( resid   31 and name ha   )( resid   31 and name hn   ) 0.0 0.0 5.0
assign ( resid   31 and name hb2  )( resid   31 and name hn   ) 0.0 0.0 5.0
assign ( resid   31 and name hb1  )( resid   31 and name hn   ) 0.0 0.0 5.0
assign ( resid   32 and name ha   )( resid   32 and name hg2# ) 0.0 0.0 3.5
assign ( resid   32 and name ha   )( resid   32 and name hn   ) 0.0 0.0 2.8
assign ( resid   32 and name hb   )( resid   32 and name hn   ) 0.0 0.0 3.5
assign ( resid   32 and name hg2# )( resid   32 and name hn   ) 0.0 0.0 5.0
assign ( resid   33 and name ha   )( resid   33 and name hn   ) 0.0 0.0 5.0
assign ( resid   33 and name hb2  )( resid   33 and name hn   ) 0.0 0.0 3.5
assign ( resid   33 and name hb1  )( resid   33 and name hn   ) 0.0 0.0 5.0
assign ( resid   34 and name ha   )( resid   34 and name hn   ) 0.0 0.0 3.5
assign ( resid   34 and name ha   )( resid   34 and name hd1# ) 0.0 0.0 2.8
assign ( resid   34 and name ha   )( resid   34 and name hg   ) 0.0 0.0 3.5
assign ( resid   34 and name hn   )( resid   34 and name hd1# ) 0.0 0.0 5.0
assign ( resid   35 and name ha2  )( resid   35 and name hn   ) 0.0 0.0 3.5
assign ( resid   36 and name ha   )( resid   36 and name hg2  ) 0.0 0.0 2.8
assign ( resid   36 and name hg2  )( resid   36 and name hn   ) 0.0 0.0 5.0
assign ( resid   36 and name hb#  )( resid   36 and name hn   ) 0.0 0.0 5.0
assign ( resid   36 and name hg1  )( resid   36 and name hn   ) 0.0 0.0 5.0
assign ( resid   36 and name ha   )( resid   36 and name hn   ) 0.0 0.0 3.5
assign ( resid   37 and name ha2  )( resid   37 and name hn   ) 0.0 0.0 5.0
assign ( resid   37 and name ha1  )( resid   37 and name hn   ) 0.0 0.0 3.5
assign ( resid   38 and name hb1  )( resid   38 and name hn   ) 0.0 0.0 5.0
assign ( resid   38 and name hb2  )( resid   38 and name hn   ) 0.0 0.0 5.0
assign ( resid   39 and name ha2  )( resid   39 and name hn   ) 0.0 0.0 3.5
assign ( resid   39 and name ha1  )( resid   39 and name hn   ) 0.0 0.0 3.5
assign ( resid   40 and name ha   )( resid   40 and name hg2  ) 0.0 0.0 3.5
assign ( resid   40 and name ha   )( resid   40 and name hg1  ) 0.0 0.0 3.5
assign ( resid   40 and name ha   )( resid   40 and name hn   ) 0.0 0.0 3.5
assign ( resid   40 and name hb2  )( resid   40 and name hn   ) 0.0 0.0 3.5
assign ( resid   40 and name hd#  )( resid   40 and name hn   ) 0.0 0.0 5.0
assign ( resid   40 and name hb1  )( resid   40 and name hn   ) 0.0 0.0 2.8
assign ( resid   41 and name ha   )( resid   41 and name hd1# ) 0.0 0.0 3.5
assign ( resid   41 and name ha   )( resid   41 and name hg1# ) 0.0 0.0 3.5
assign ( resid   41 and name ha   )( resid   41 and name hg2# ) 0.0 0.0 3.5
assign ( resid   41 and name ha   )( resid   41 and name hn   ) 0.0 0.0 5.0
assign ( resid   41 and name hd1# )( resid   41 and name hn   ) 0.0 0.0 5.0
assign ( resid   41 and name hb   )( resid   41 and name hn   ) 0.0 0.0 3.5
assign ( resid   41 and name hg1# )( resid   41 and name hn   ) 0.0 0.0 5.0
assign ( resid   41 and name hg2# )( resid   41 and name hn   ) 0.0 0.0 5.0
assign ( resid   42 and name ha   )( resid   42 and name hg2# ) 0.0 0.0 3.5
assign ( resid   42 and name hb   )( resid   42 and name hn   ) 0.0 0.0 5.0
assign ( resid   42 and name ha   )( resid   42 and name hn   ) 0.0 0.0 3.5
assign ( resid   42 and name hg2# )( resid   42 and name hn   ) 0.0 0.0 5.0
assign ( resid   43 and name ha   )( resid   43 and name hn   ) 0.0 0.0 5.0
assign ( resid   43 and name hb2  )( resid   43 and name hn   ) 0.0 0.0 5.0
assign ( resid   43 and name hb1  )( resid   43 and name hn   ) 0.0 0.0 5.0
assign ( resid   44 and name ha   )( resid   44 and name hn   ) 0.0 0.0 5.0
assign ( resid   44 and name hb   )( resid   44 and name hn   ) 0.0 0.0 5.0
assign ( resid   44 and name hg2# )( resid   44 and name hn   ) 0.0 0.0 3.5
assign ( resid   46 and name ha   )( resid   46 and name hd#  ) 0.0 0.0 5.0
assign ( resid   46 and name ha   )( resid   46 and name hg1  ) 0.0 0.0 3.5
assign ( resid   46 and name ha   )( resid   46 and name hg2  ) 0.0 0.0 3.5
assign ( resid   46 and name ha   )( resid   46 and name hn   ) 0.0 0.0 3.5
assign ( resid   46 and name hg2  )( resid   46 and name hn   ) 0.0 0.0 3.5
assign ( resid   47 and name ha   )( resid   47 and name hn   ) 0.0 0.0 3.5
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assign ( resid   47 and name hb2  )( resid   47 and name hd21 ) 0.0 0.0 5.0
assign ( resid   47 and name hb2  )( resid   47 and name hd22 ) 0.0 0.0 5.0
assign ( resid   47 and name hb2  )( resid   47 and name hn   ) 0.0 0.0 5.0
assign ( resid   47 and name hb1  )( resid   47 and name hn   ) 0.0 0.0 3.5
assign ( resid   47 and name hb1  )( resid   47 and name hd21 ) 0.0 0.0 5.0
assign ( resid   47 and name hb1  )( resid   47 and name hd22 ) 0.0 0.0 3.5
assign ( resid   47 and name hd21 )( resid   47 and name hn   ) 0.0 0.0 5.0
assign ( resid   47 and name hd22 )( resid   47 and name hn   ) 0.0 0.0 5.0
assign ( resid   48 and name ha   )( resid   48 and name hd1  ) 0.0 0.0 3.5
assign ( resid   48 and name ha   )( resid   48 and name hd2  ) 0.0 0.0 3.5
assign ( resid   48 and name ha   )( resid   48 and name hg2  ) 0.0 0.0 5.0
assign ( resid   48 and name ha   )( resid   48 and name hn   ) 0.0 0.0 5.0
assign ( resid   48 and name hg#  )( resid   48 and name hn   ) 0.0 0.0 5.0
assign ( resid   49 and name ha   )( resid   49 and name hn   ) 0.0 0.0 5.0
assign ( resid   49 and name hb2  )( resid   49 and name hn   ) 0.0 0.0 5.0
assign ( resid   49 and name hb1  )( resid   49 and name hn   ) 0.0 0.0 3.5
assign ( resid   50 and name ha   )( resid   50 and name hn   ) 0.0 0.0 5.0
assign ( resid   50 and name hb2  )( resid   50 and name hd21 ) 0.0 0.0 5.0
assign ( resid   50 and name hb2  )( resid   50 and name hd22 ) 0.0 0.0 3.5
assign ( resid   50 and name hb2  )( resid   50 and name hn   ) 0.0 0.0 3.5
assign ( resid   50 and name hb1  )( resid   50 and name hn   ) 0.0 0.0 5.0
assign ( resid   50 and name hb1  )( resid   50 and name hd22 ) 0.0 0.0 5.0
assign ( resid   50 and name hb1  )( resid   50 and name hd21 ) 0.0 0.0 5.0
assign ( resid   50 and name hd22 )( resid   50 and name hn   ) 0.0 0.0 5.0
assign ( resid   50 and name hd21 )( resid   50 and name hn   ) 0.0 0.0 5.0
assign ( resid   51 and name ha   )( resid   51 and name hn   ) 0.0 0.0 5.0
assign ( resid   51 and name hb1  )( resid   51 and name hn   ) 0.0 0.0 5.0
assign ( resid   51 and name hb2  )( resid   51 and name hn   ) 0.0 0.0 5.0
assign ( resid   52 and name ha   )( resid   52 and name hg#  ) 0.0 0.0 5.0
assign ( resid   52 and name ha   )( resid   52 and name hn   ) 0.0 0.0 3.5
assign ( resid   52 and name hb2  )( resid   52 and name hn   ) 0.0 0.0 3.5
assign ( resid   52 and name hb1  )( resid   52 and name hn   ) 0.0 0.0 3.5
assign ( resid   52 and name hg#  )( resid   52 and name hn   ) 0.0 0.0 5.0
assign ( resid   53 and name ha   )( resid   53 and name hn   ) 0.0 0.0 3.5
assign ( resid   53 and name hb#  )( resid   53 and name hn   ) 0.0 0.0 3.5
assign ( resid   54 and name hg2# )( resid   54 and name hn   ) 0.0 0.0 3.5
assign ( resid   54 and name ha   )( resid   54 and name hg2# ) 0.0 0.0 5.0
assign ( resid   54 and name ha   )( resid   54 and name hn   ) 0.0 0.0 5.0
assign ( resid   54 and name hb   )( resid   54 and name hn   ) 0.0 0.0 5.0
assign ( resid   55 and name hd#  )( resid   55 and name hb2  ) 0.0 0.0 3.5
assign ( resid   55 and name hd#  )( resid   55 and name ha   ) 0.0 0.0 5.0
assign ( resid   55 and name ha   )( resid   55 and name hg2  ) 0.0 0.0 3.5
assign ( resid   55 and name hd#  )( resid   55 and name hn   ) 0.0 0.0 5.0
assign ( resid   55 and name ha   )( resid   55 and name hn   ) 0.0 0.0 2.8
assign ( resid   55 and name hb2  )( resid   55 and name hn   ) 0.0 0.0 5.0
assign ( resid   55 and name hg2  )( resid   55 and name hn   ) 0.0 0.0 5.0
assign ( resid   55 and name hg1  )( resid   55 and name hn   ) 0.0 0.0 5.0
assign ( resid   55 and name hg2  )( resid   55 and name he   ) 0.0 0.0 5.0
assign ( resid   56 and name ha   )( resid   56 and name hg2# ) 0.0 0.0 2.8
assign ( resid   56 and name ha   )( resid   56 and name hn   ) 0.0 0.0 5.0
assign ( resid   56 and name hb   )( resid   56 and name hn   ) 0.0 0.0 3.5
assign ( resid   56 and name hg2# )( resid   56 and name hn   ) 0.0 0.0 5.0
assign ( resid   57 and name hb2  )( resid   57 and name hn   ) 0.0 0.0 3.5
assign ( resid   57 and name hb1  )( resid   57 and name hn   ) 0.0 0.0 3.5
assign ( resid   58 and name hb1  )( resid   58 and name hd#  ) 0.0 0.0 3.5
assign ( resid   58 and name ha   )( resid   58 and name hd#  ) 0.0 0.0 3.5
assign ( resid   58 and name ha   )( resid   58 and name he#  ) 0.0 0.0 5.0
assign ( resid   58 and name ha   )( resid   58 and name hn   ) 0.0 0.0 5.0
assign ( resid   58 and name hb2  )( resid   58 and name hn   ) 0.0 0.0 5.0
assign ( resid   58 and name hb1  )( resid   58 and name hn   ) 0.0 0.0 5.0
assign ( resid   58 and name hd#  )( resid   58 and name hn   ) 0.0 0.0 3.5
assign ( resid   58 and name hb2  )( resid   58 and name hd#  ) 0.0 0.0 3.5
assign ( resid   59 and name hd#  )( resid   59 and name hb2  ) 0.0 0.0 3.5
assign ( resid   59 and name ha   )( resid   59 and name hg1  ) 0.0 0.0 5.0
assign ( resid   59 and name ha   )( resid   59 and name hn   ) 0.0 0.0 5.0
assign ( resid   59 and name hd#  )( resid   59 and name hn   ) 0.0 0.0 5.0
assign ( resid   59 and name hd#  )( resid   59 and name he   ) 0.0 0.0 5.0
assign ( resid   59 and name hb2  )( resid   59 and name hn   ) 0.0 0.0 5.0
assign ( resid   59 and name hb1  )( resid   59 and name hn   ) 0.0 0.0 5.0
assign ( resid   59 and name hg2  )( resid   59 and name hn   ) 0.0 0.0 3.5
assign ( resid   60 and name ha   )( resid   60 and name hg1# ) 0.0 0.0 3.5
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assign ( resid   60 and name ha   )( resid   60 and name hn   ) 0.0 0.0 3.5
assign ( resid   60 and name hb   )( resid   60 and name hn   ) 0.0 0.0 2.8
assign ( resid   60 and name hg1# )( resid   60 and name hn   ) 0.0 0.0 5.0
assign ( resid   62 and name ha   )( resid   62 and name hn   ) 0.0 0.0 3.5
assign ( resid   62 and name hb1  )( resid   62 and name hn   ) 0.0 0.0 5.0
assign ( resid   62 and name hb2  )( resid   62 and name hn   ) 0.0 0.0 3.5
assign ( resid   63 and name ha   )( resid   63 and name hg2# ) 0.0 0.0 2.8
assign ( resid   63 and name ha   )( resid   63 and name hn   ) 0.0 0.0 5.0
assign ( resid   63 and name hb   )( resid   63 and name hn   ) 0.0 0.0 5.0
assign ( resid   63 and name hg2# )( resid   63 and name hn   ) 0.0 0.0 3.5
assign ( resid   64 and name hb2  )( resid   64 and name hd1  ) 0.0 0.0 5.0
assign ( resid   64 and name hb1  )( resid   64 and name hd1  ) 0.0 0.0 3.5
assign ( resid   64 and name hz3  )( resid   64 and name hz2  ) 0.0 0.0 5.0
assign ( resid   64 and name ha   )( resid   64 and name hd1  ) 0.0 0.0 5.0
assign ( resid   64 and name hb1  )( resid   64 and name he3  ) 0.0 0.0 5.0
assign ( resid   64 and name hb2  )( resid   64 and name he3  ) 0.0 0.0 5.0
assign ( resid   64 and name hz2  )( resid   64 and name he1  ) 0.0 0.0 3.5
assign ( resid   64 and name hb2  )( resid   64 and name hn   ) 0.0 0.0 5.0
assign ( resid   64 and name hb#  )( resid   64 and name he1  ) 0.0 0.0 5.0
assign ( resid   64 and name he3  )( resid   64 and name hn   ) 0.0 0.0 3.5
assign ( resid   64 and name ha   )( resid   64 and name hn   ) 0.0 0.0 2.8
assign ( resid   64 and name hb1  )( resid   64 and name hn   ) 0.0 0.0 5.0
assign ( resid   65 and name ha   )( resid   65 and name hn   ) 0.0 0.0 3.5
assign ( resid   65 and name hb#  )( resid   65 and name hn   ) 0.0 0.0 3.5
assign ( resid   66 and name he#  )( resid   66 and name hb1  ) 0.0 0.0 5.0
assign ( resid   66 and name hd#  )( resid   66 and name hb1  ) 0.0 0.0 3.5
assign ( resid   66 and name hb1  )( resid   66 and name hg#  ) 0.0 0.0 2.8
assign ( resid   66 and name ha   )( resid   66 and name hn   ) 0.0 0.0 3.5
assign ( resid   66 and name hb1  )( resid   66 and name hn   ) 0.0 0.0 5.0
assign ( resid   67 and name ha   )( resid   67 and name hg2  ) 0.0 0.0 3.5
assign ( resid   67 and name ha   )( resid   67 and name hg1  ) 0.0 0.0 5.0
assign ( resid   67 and name ha   )( resid   67 and name hd#  ) 0.0 0.0 3.5
assign ( resid   67 and name he#  )( resid   67 and name hg1  ) 0.0 0.0 3.5
assign ( resid   67 and name hb1  )( resid   67 and name hn   ) 0.0 0.0 3.5
assign ( resid   67 and name hd#  )( resid   67 and name hn   ) 0.0 0.0 5.0
assign ( resid   67 and name hg1  )( resid   67 and name hn   ) 0.0 0.0 5.0
assign ( resid   68 and name hb1  )( resid   68 and name hn   ) 0.0 0.0 5.0
assign ( resid   69 and name ha   )( resid   69 and name hn   ) 0.0 0.0 3.5
assign ( resid   69 and name hb1  )( resid   69 and name hd22 ) 0.0 0.0 5.0
assign ( resid   69 and name hb2  )( resid   69 and name hd22 ) 0.0 0.0 5.0
assign ( resid   69 and name hb2  )( resid   69 and name hn   ) 0.0 0.0 5.0
assign ( resid   69 and name hb1  )( resid   69 and name hn   ) 0.0 0.0 5.0
assign ( resid   69 and name hb2  )( resid   69 and name hd21 ) 0.0 0.0 5.0
assign ( resid   69 and name hb1  )( resid   69 and name hd21 ) 0.0 0.0 5.0
assign ( resid   70 and name ha   )( resid   70 and name hd#  ) 0.0 0.0 5.0
assign ( resid   70 and name hd#  )( resid   70 and name hb2  ) 0.0 0.0 3.5
assign ( resid   70 and name ha   )( resid   70 and name hg#  ) 0.0 0.0 3.5
assign ( resid   70 and name hd#  )( resid   70 and name hb1  ) 0.0 0.0 3.5
assign ( resid   70 and name hd#  )( resid   70 and name he   ) 0.0 0.0 5.0
assign ( resid   70 and name hb2  )( resid   70 and name hn   ) 0.0 0.0 5.0
assign ( resid   70 and name hg2  )( resid   70 and name hn   ) 0.0 0.0 5.0
assign ( resid   70 and name hb1  )( resid   70 and name hn   ) 0.0 0.0 3.5
assign ( resid   70 and name hg1  )( resid   70 and name hn   ) 0.0 0.0 5.0
assign ( resid   70 and name hd#  )( resid   70 and name hn   ) 0.0 0.0 5.0
assign ( resid   70 and name ha   )( resid   70 and name hn   ) 0.0 0.0 3.5
assign ( resid   71 and name ha1  )( resid   71 and name hn   ) 0.0 0.0 3.5
assign ( resid   71 and name ha2  )( resid   71 and name hn   ) 0.0 0.0 3.5
assign ( resid   72 and name ha   )( resid   72 and name hd22 ) 0.0 0.0 3.5
assign ( resid   72 and name hb2  )( resid   72 and name hn   ) 0.0 0.0 3.5
assign ( resid   72 and name hb2  )( resid   72 and name hd22 ) 0.0 0.0 3.5
assign ( resid   72 and name hb1  )( resid   72 and name hd22 ) 0.0 0.0 5.0
assign ( resid   72 and name hb1  )( resid   72 and name hn   ) 0.0 0.0 3.5
assign ( resid   72 and name hb1  )( resid   72 and name hd21 ) 0.0 0.0 5.0
assign ( resid   72 and name hb2  )( resid   72 and name hd21 ) 0.0 0.0 5.0
assign ( resid   73 and name ha   )( resid   73 and name hd2# ) 0.0 0.0 2.8
assign ( resid   73 and name ha   )( resid   73 and name hd1# ) 0.0 0.0 5.0
assign ( resid   73 and name hb#  )( resid   73 and name hd1# ) 0.0 0.0 2.8
assign ( resid   73 and name hb#  )( resid   73 and name hd2# ) 0.0 0.0 3.5
assign ( resid   73 and name ha   )( resid   73 and name hg   ) 0.0 0.0 3.5
assign ( resid   73 and name ha   )( resid   73 and name hn   ) 0.0 0.0 3.5
assign ( resid   73 and name hg   )( resid   73 and name hn   ) 0.0 0.0 5.0
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assign ( resid   73 and name hb#  )( resid   73 and name hn   ) 0.0 0.0 3.5
assign ( resid   73 and name hd2# )( resid   73 and name hn   ) 0.0 0.0 5.0
assign ( resid   74 and name ha   )( resid   74 and name hd2# ) 0.0 0.0 5.0
assign ( resid   74 and name hb1  )( resid   74 and name hd1# ) 0.0 0.0 3.5
assign ( resid   74 and name ha   )( resid   74 and name hn   ) 0.0 0.0 5.0
assign ( resid   74 and name hb1  )( resid   74 and name hn   ) 0.0 0.0 5.0
assign ( resid   74 and name hd2# )( resid   74 and name hn   ) 0.0 0.0 5.0
assign ( resid   74 and name hd1# )( resid   74 and name hn   ) 0.0 0.0 5.0
assign ( resid   74 and name hb2  )( resid   74 and name hd1# ) 0.0 0.0 3.5
assign ( resid   74 and name ha   )( resid   74 and name hd1# ) 0.0 0.0 5.0
assign ( resid   74 and name hg   )( resid   74 and name hb2  ) 0.0 0.0 3.5
assign ( resid   74 and name hb2  )( resid   74 and name hn   ) 0.0 0.0 3.5
assign ( resid   74 and name hg   )( resid   74 and name hn   ) 0.0 0.0 3.5
assign ( resid   75 and name ha   )( resid   75 and name hg#  ) 0.0 0.0 5.0
assign ( resid   75 and name ha   )( resid   75 and name hn   ) 0.0 0.0 3.5
assign ( resid   75 and name hb2  )( resid   75 and name hn   ) 0.0 0.0 5.0
assign ( resid   75 and name hb1  )( resid   75 and name hn   ) 0.0 0.0 3.5
assign ( resid   75 and name hg#  )( resid   75 and name hn   ) 0.0 0.0 5.0
assign ( resid   76 and name ha   )( resid   76 and name hn   ) 0.0 0.0 5.0
assign ( resid   76 and name hb2  )( resid   76 and name hn   ) 0.0 0.0 5.0
assign ( resid   76 and name hb1  )( resid   76 and name hn   ) 0.0 0.0 5.0
assign ( resid   77 and name ha   )( resid   77 and name hg11 ) 0.0 0.0 5.0
assign ( resid   77 and name ha   )( resid   77 and name hg12 ) 0.0 0.0 3.5
assign ( resid   77 and name ha   )( resid   77 and name hd1# ) 0.0 0.0 3.5
assign ( resid   77 and name ha   )( resid   77 and name hg2# ) 0.0 0.0 3.5
assign ( resid   77 and name ha   )( resid   77 and name hn   ) 0.0 0.0 5.0
assign ( resid   77 and name hd1# )( resid   77 and name hn   ) 0.0 0.0 5.0
assign ( resid   77 and name hb   )( resid   77 and name hn   ) 0.0 0.0 5.0
assign ( resid   77 and name hg12 )( resid   77 and name hn   ) 0.0 0.0 5.0
assign ( resid   78 and name ha   )( resid   78 and name hn   ) 0.0 0.0 3.5
assign ( resid   78 and name hb1  )( resid   78 and name hn   ) 0.0 0.0 5.0
assign ( resid   78 and name hb2  )( resid   78 and name hn   ) 0.0 0.0 3.5
assign ( resid   79 and name ha   )( resid   79 and name hg2# ) 0.0 0.0 2.8
assign ( resid   79 and name ha   )( resid   79 and name hn   ) 0.0 0.0 3.5
assign ( resid   79 and name hg2# )( resid   79 and name hn   ) 0.0 0.0 3.5
assign ( resid   79 and name hb   )( resid   79 and name hn   ) 0.0 0.0 5.0
assign ( resid   80 and name ha1  )( resid   80 and name hn   ) 0.0 0.0 3.5
assign ( resid   80 and name ha2  )( resid   80 and name hn   ) 0.0 0.0 3.5
assign ( resid   81 and name hb2  )( resid   81 and name hd21 ) 0.0 0.0 5.0
assign ( resid   81 and name hb2  )( resid   81 and name hd22 ) 0.0 0.0 5.0
assign ( resid   81 and name hb2  )( resid   81 and name hn   ) 0.0 0.0 5.0
assign ( resid   81 and name hb1  )( resid   81 and name hn   ) 0.0 0.0 5.0
assign ( resid   81 and name hb1  )( resid   81 and name hd22 ) 0.0 0.0 3.5
assign ( resid   81 and name ha   )( resid   81 and name hn   ) 0.0 0.0 2.8
assign ( resid   82 and name ha2  )( resid   82 and name hn   ) 0.0 0.0 3.5
assign ( resid   82 and name ha1  )( resid   82 and name hn   ) 0.0 0.0 3.5
assign ( resid   83 and name ha   )( resid   83 and name hg#  ) 0.0 0.0 3.5
assign ( resid   83 and name ha   )( resid   83 and name hn   ) 0.0 0.0 3.5
assign ( resid   83 and name hg#  )( resid   83 and name hn   ) 0.0 0.0 5.0
assign ( resid   83 and name hb2  )( resid   83 and name hn   ) 0.0 0.0 5.0
assign ( resid   83 and name hb1  )( resid   83 and name hn   ) 0.0 0.0 3.5
assign ( resid   84 and name ha1  )( resid   84 and name hn   ) 0.0 0.0 3.5
assign ( resid   84 and name ha2  )( resid   84 and name hn   ) 0.0 0.0 2.8
assign ( resid   85 and name ha   )( resid   85 and name hg2  ) 0.0 0.0 5.0
assign ( resid   85 and name ha   )( resid   85 and name hg1  ) 0.0 0.0 5.0
assign ( resid   85 and name ha   )( resid   85 and name hn   ) 0.0 0.0 3.5
assign ( resid   85 and name hg2  )( resid   85 and name hn   ) 0.0 0.0 5.0
assign ( resid   85 and name hg1  )( resid   85 and name hn   ) 0.0 0.0 5.0
assign ( resid   85 and name hb#  )( resid   85 and name hn   ) 0.0 0.0 3.5
assign ( resid   86 and name hz2  )( resid   86 and name hz3  ) 0.0 0.0 5.0
assign ( resid   86 and name he3  )( resid   86 and name hh2  ) 0.0 0.0 5.0
assign ( resid   86 and name hb2  )( resid   86 and name hd1  ) 0.0 0.0 5.0
assign ( resid   86 and name hb2  )( resid   86 and name he3  ) 0.0 0.0 3.5
assign ( resid   86 and name hb1  )( resid   86 and name he3  ) 0.0 0.0 5.0
assign ( resid   86 and name hb1  )( resid   86 and name hd1  ) 0.0 0.0 3.5
assign ( resid   86 and name ha   )( resid   86 and name hd1  ) 0.0 0.0 5.0
assign ( resid   86 and name ha   )( resid   86 and name he3  ) 0.0 0.0 5.0
assign ( resid   86 and name hd1  )( resid   86 and name ha   ) 0.0 0.0 5.0
assign ( resid   86 and name he3  )( resid   86 and name ha   ) 0.0 0.0 5.0
assign ( resid   86 and name ha   )( resid   86 and name hn   ) 0.0 0.0 5.0
assign ( resid   86 and name hb1  )( resid   86 and name hn   ) 0.0 0.0 5.0
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assign ( resid   86 and name hb2  )( resid   86 and name hn   ) 0.0 0.0 5.0
assign ( resid   86 and name hz2  )( resid   86 and name he1  ) 0.0 0.0 3.5
assign ( resid   86 and name hd1  )( resid   86 and name hn   ) 0.0 0.0 5.0
assign ( resid   87 and name ha   )( resid   87 and name hg#  ) 0.0 0.0 5.0
assign ( resid   87 and name hd#  )( resid   87 and name hn   ) 0.0 0.0 5.0
assign ( resid   87 and name ha   )( resid   87 and name hn   ) 0.0 0.0 3.5
assign ( resid   87 and name hb1  )( resid   87 and name hn   ) 0.0 0.0 3.5
assign ( resid   87 and name hb2  )( resid   87 and name hn   ) 0.0 0.0 5.0
assign ( resid   87 and name hg#  )( resid   87 and name hn   ) 0.0 0.0 5.0
assign ( resid   88 and name hb2  )( resid   88 and name hn   ) 0.0 0.0 3.5
assign ( resid   89 and name ha   )( resid   89 and name hg1  ) 0.0 0.0 5.0
assign ( resid   89 and name ha   )( resid   89 and name hn   ) 0.0 0.0 3.5
assign ( resid   89 and name hg2  )( resid   89 and name hn   ) 0.0 0.0 5.0
assign ( resid   89 and name hb#  )( resid   89 and name hn   ) 0.0 0.0 5.0
assign ( resid   89 and name hg1  )( resid   89 and name hn   ) 0.0 0.0 5.0
assign ( resid   90 and name ha   )( resid   90 and name hd#  ) 0.0 0.0 5.0
assign ( resid   90 and name hd#  )( resid   90 and name hb2  ) 0.0 0.0 3.5
assign ( resid   90 and name hd#  )( resid   90 and name hb1  ) 0.0 0.0 3.5
assign ( resid   90 and name hd#  )( resid   90 and name hn   ) 0.0 0.0 5.0
assign ( resid   90 and name hb2  )( resid   90 and name hn   ) 0.0 0.0 3.5
assign ( resid   90 and name ha   )( resid   90 and name hn   ) 0.0 0.0 3.5
assign ( resid   90 and name hd#  )( resid   90 and name he   ) 0.0 0.0 5.0
assign ( resid   91 and name hb1  )( resid   91 and name hn   ) 0.0 0.0 5.0
assign ( resid   91 and name hb2  )( resid   91 and name hn   ) 0.0 0.0 5.0
assign ( resid   92 and name ha   )( resid   92 and name hn   ) 0.0 0.0 5.0
assign ( resid   92 and name hg2# )( resid   92 and name hn   ) 0.0 0.0 5.0
assign ( resid   93 and name hb#  )( resid   93 and name hn   ) 0.0 0.0 5.0

SEQUENTIAL NOES

assign ( resid    1 and name ha   )( resid    2 and name hb#  ) 0.0 0.0 5.0
assign ( resid    1 and name ha   )( resid    2 and name hn   ) 0.0 0.0 3.5
assign ( resid    1 and name hb#  )( resid    2 and name hn   ) 0.0 0.0 5.0
assign ( resid    2 and name ha   )( resid    3 and name ha   ) 0.0 0.0 5.0
assign ( resid    2 and name hb#  )( resid    3 and name ha   ) 0.0 0.0 5.0
assign ( resid    2 and name ha   )( resid    3 and name hn   ) 0.0 0.0 2.8
assign ( resid    2 and name hg2  )( resid    3 and name hn   ) 0.0 0.0 5.0
assign ( resid    2 and name hg1  )( resid    3 and name hn   ) 0.0 0.0 5.0
assign ( resid    2 and name hb#  )( resid    3 and name hn   ) 0.0 0.0 5.0
assign ( resid    3 and name ha   )( resid    4 and name hn   ) 0.0 0.0 2.8
assign ( resid    3 and name hb#  )( resid    4 and name hn   ) 0.0 0.0 3.5
assign ( resid    3 and name hd#  )( resid    4 and name hn   ) 0.0 0.0 5.0
assign ( resid    3 and name hg#  )( resid    4 and name hn   ) 0.0 0.0 5.0
assign ( resid    4 and name ha   )( resid    5 and name hd#  ) 0.0 0.0 5.0
assign ( resid    4 and name hb2  )( resid    5 and name hn   ) 0.0 0.0 5.0
assign ( resid    4 and name ha   )( resid    5 and name he#  ) 0.0 0.0 6.0
assign ( resid    4 and name ha   )( resid    5 and name hn   ) 0.0 0.0 3.5
assign ( resid    5 and name ha   )( resid    6 and name hn   ) 0.0 0.0 2.8
assign ( resid    5 and name hb#  )( resid    6 and name hn   ) 0.0 0.0 3.5
assign ( resid    5 and name hd#  )( resid    6 and name hn   ) 0.0 0.0 5.0
assign ( resid    7 and name ha   )( resid    8 and name hn   ) 0.0 0.0 5.0
assign ( resid    7 and name hb2  )( resid    8 and name hn   ) 0.0 0.0 5.0
assign ( resid    7 and name hd#  )( resid    8 and name hb#  ) 0.0 0.0 5.0
assign ( resid    8 and name ha   )( resid    9 and name hn   ) 0.0 0.0 5.0
assign ( resid    8 and name hn   )( resid    9 and name hb#  ) 0.0 0.0 5.0
assign ( resid    8 and name hb#  )( resid    9 and name hn   ) 0.0 0.0 3.5
assign ( resid    9 and name hb#  )( resid   10 and name ha2  ) 0.0 0.0 5.0
assign ( resid    9 and name ha   )( resid   10 and name hn   ) 0.0 0.0 5.0
assign ( resid    9 and name hb#  )( resid   10 and name hn   ) 0.0 0.0 3.5
assign ( resid   10 and name ha1  )( resid   11 and name hn   ) 0.0 0.0 5.0
assign ( resid   10 and name ha2  )( resid   11 and name hn   ) 0.0 0.0 5.0
assign ( resid   11 and name ha   )( resid   12 and name hn   ) 0.0 0.0 2.8
assign ( resid   11 and name hg2# )( resid   12 and name hn   ) 0.0 0.0 3.5
assign ( resid   12 and name ha   )( resid   13 and name he#  ) 0.0 0.0 5.0
assign ( resid   12 and name hb2  )( resid   13 and name hd#  ) 0.0 0.0 5.0
assign ( resid   12 and name hb1  )( resid   13 and name hd#  ) 0.0 0.0 5.0
assign ( resid   12 and name hb2  )( resid   13 and name he#  ) 0.0 0.0 6.0
assign ( resid   12 and name hb1  )( resid   13 and name he#  ) 0.0 0.0 6.0
assign ( resid   12 and name hb2  )( resid   13 and name hn   ) 0.0 0.0 5.0
assign ( resid   12 and name hb1  )( resid   13 and name hn   ) 0.0 0.0 3.5
assign ( resid   12 and name ha   )( resid   13 and name hn   ) 0.0 0.0 2.8
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assign ( resid   12 and name ha   )( resid   13 and name hd#  ) 0.0 0.0 3.5
assign ( resid   13 and name hd#  )( resid   14 and name ha   ) 0.0 0.0 6.0
assign ( resid   13 and name ha   )( resid   14 and name hn   ) 0.0 0.0 2.8
assign ( resid   13 and name hn   )( resid   14 and name hb   ) 0.0 0.0 6.0
assign ( resid   13 and name ha   )( resid   14 and name hg2# ) 0.0 0.0 5.0
assign ( resid   13 and name ha   )( resid   14 and name hg1# ) 0.0 0.0 6.0
assign ( resid   14 and name ha   )( resid   15 and name hn   ) 0.0 0.0 2.8
assign ( resid   14 and name hb   )( resid   15 and name hn   ) 0.0 0.0 5.0
assign ( resid   14 and name hg2# )( resid   15 and name hn   ) 0.0 0.0 3.5
assign ( resid   15 and name ha   )( resid   16 and name hn   ) 0.0 0.0 2.8
assign ( resid   15 and name hb   )( resid   16 and name hn   ) 0.0 0.0 5.0
assign ( resid   15 and name hn   )( resid   16 and name hn   ) 0.0 0.0 5.0
assign ( resid   15 and name hg1# )( resid   16 and name ha2  ) 0.0 0.0 5.0
assign ( resid   15 and name hg2# )( resid   16 and name hn   ) 0.0 0.0 5.0
assign ( resid   16 and name ha2  )( resid   17 and name hn   ) 0.0 0.0 5.0
assign ( resid   17 and name ha   )( resid   18 and name hg2# ) 0.0 0.0 5.0
assign ( resid   17 and name ha   )( resid   18 and name ha   ) 0.0 0.0 5.0
assign ( resid   17 and name hb1  )( resid   18 and name ha   ) 0.0 0.0 5.0
assign ( resid   17 and name ha   )( resid   18 and name hn   ) 0.0 0.0 2.8
assign ( resid   17 and name hb2  )( resid   18 and name hn   ) 0.0 0.0 5.0
assign ( resid   17 and name hg#  )( resid   18 and name hn   ) 0.0 0.0 5.0
assign ( resid   17 and name hb1  )( resid   18 and name hn   ) 0.0 0.0 5.0
assign ( resid   18 and name ha   )( resid   19 and name hz3  ) 0.0 0.0 3.5
assign ( resid   18 and name ha   )( resid   19 and name he3  ) 0.0 0.0 2.8
assign ( resid   18 and name hg2# )( resid   19 and name he3  ) 0.0 0.0 5.0
assign ( resid   18 and name hb   )( resid   19 and name he3  ) 0.0 0.0 5.0
assign ( resid   18 and name hb   )( resid   19 and name ha   ) 0.0 0.0 6.0
assign ( resid   18 and name hg2# )( resid   19 and name ha   ) 0.0 0.0 5.0
assign ( resid   18 and name ha   )( resid   19 and name hn   ) 0.0 0.0 2.8
assign ( resid   18 and name hb   )( resid   19 and name hn   ) 0.0 0.0 3.5
assign ( resid   18 and name hg2# )( resid   19 and name hn   ) 0.0 0.0 3.5
assign ( resid   19 and name hd1  )( resid   20 and name ha   ) 0.0 0.0 5.0
assign ( resid   19 and name ha   )( resid   20 and name hg2  ) 0.0 0.0 5.0
assign ( resid   19 and name ha   )( resid   20 and name hn   ) 0.0 0.0 2.8
assign ( resid   19 and name hb1  )( resid   20 and name hn   ) 0.0 0.0 3.5
assign ( resid   19 and name hd1  )( resid   20 and name hn   ) 0.0 0.0 5.0
assign ( resid   20 and name ha   )( resid   21 and name hn   ) 0.0 0.0 2.8
assign ( resid   20 and name hb2  )( resid   21 and name hn   ) 0.0 0.0 3.5
assign ( resid   21 and name ha   )( resid   22 and name hd1  ) 0.0 0.0 2.8
assign ( resid   21 and name ha   )( resid   22 and name hd2  ) 0.0 0.0 2.8
assign ( resid   21 and name hd#  )( resid   22 and name hd2  ) 0.0 0.0 5.0
assign ( resid   21 and name ha   )( resid   22 and name hg1  ) 0.0 0.0 5.0
assign ( resid   21 and name ha   )( resid   22 and name hg2  ) 0.0 0.0 5.0
assign ( resid   21 and name hg#  )( resid   22 and name hd2  ) 0.0 0.0 5.0
assign ( resid   21 and name hd#  )( resid   22 and name hd1  ) 0.0 0.0 5.0
assign ( resid   21 and name ha   )( resid   22 and name ha   ) 0.0 0.0 5.0
assign ( resid   21 and name hg#  )( resid   22 and name hd1  ) 0.0 0.0 5.0
assign ( resid   21 and name hb#  )( resid   22 and name hd2  ) 0.0 0.0 5.0
assign ( resid   21 and name hb#  )( resid   22 and name hd1  ) 0.0 0.0 5.0
assign ( resid   22 and name ha   )( resid   23 and name hn   ) 0.0 0.0 2.8
assign ( resid   23 and name hd#  )( resid   24 and name hg2  ) 0.0 0.0 5.0
assign ( resid   23 and name hd#  )( resid   24 and name hb2  ) 0.0 0.0 5.0
assign ( resid   23 and name hd#  )( resid   24 and name hb1  ) 0.0 0.0 5.0
assign ( resid   23 and name he#  )( resid   24 and name hb2  ) 0.0 0.0 5.0
assign ( resid   23 and name he#  )( resid   24 and name hb1  ) 0.0 0.0 5.0
assign ( resid   23 and name ha   )( resid   24 and name hn   ) 0.0 0.0 2.8
assign ( resid   23 and name hb1  )( resid   24 and name hn   ) 0.0 0.0 5.0
assign ( resid   23 and name hb2  )( resid   24 and name hn   ) 0.0 0.0 5.0
assign ( resid   23 and name hd#  )( resid   24 and name hn   ) 0.0 0.0 5.0
assign ( resid   23 and name hb2  )( resid   24 and name hg2  ) 0.0 0.0 6.0
assign ( resid   23 and name he#  )( resid   24 and name hg1  ) 0.0 0.0 5.0
assign ( resid   24 and name ha   )( resid   25 and name hn   ) 0.0 0.0 3.5
assign ( resid   25 and name ha2  )( resid   26 and name hd1  ) 0.0 0.0 5.0
assign ( resid   25 and name ha1  )( resid   26 and name hd1  ) 0.0 0.0 5.0
assign ( resid   25 and name ha2  )( resid   26 and name hn   ) 0.0 0.0 3.5
assign ( resid   25 and name ha1  )( resid   26 and name hn   ) 0.0 0.0 5.0
assign ( resid   25 and name hn   )( resid   26 and name hn   ) 0.0 0.0 5.0
assign ( resid   26 and name he3  )( resid   27 and name hg#  ) 0.0 0.0 5.0
assign ( resid   26 and name hz2  )( resid   27 and name hg#  ) 0.0 0.0 5.0
assign ( resid   26 and name hh2  )( resid   27 and name hg#  ) 0.0 0.0 5.0
assign ( resid   26 and name hz2  )( resid   27 and name he#  ) 0.0 0.0 3.5
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assign ( resid   26 and name hh2  )( resid   27 and name he#  ) 0.0 0.0 5.0
assign ( resid   26 and name hz3  )( resid   27 and name hg#  ) 0.0 0.0 5.0
assign ( resid   26 and name hz3  )( resid   27 and name he#  ) 0.0 0.0 5.0
assign ( resid   26 and name ha   )( resid   27 and name hn   ) 0.0 0.0 5.0
assign ( resid   27 and name ha   )( resid   28 and name hn   ) 0.0 0.0 3.5
assign ( resid   27 and name hg#  )( resid   28 and name hn   ) 0.0 0.0 5.0
assign ( resid   27 and name hb1  )( resid   28 and name hn   ) 0.0 0.0 5.0
assign ( resid   28 and name ha   )( resid   29 and name hg1# ) 0.0 0.0 5.0
assign ( resid   28 and name ha   )( resid   29 and name hg2# ) 0.0 0.0 5.0
assign ( resid   28 and name ha   )( resid   29 and name hn   ) 0.0 0.0 2.8
assign ( resid   28 and name hb#  )( resid   29 and name hn   ) 0.0 0.0 5.0
assign ( resid   29 and name ha   )( resid   30 and name ha   ) 0.0 0.0 5.0
assign ( resid   29 and name hg2# )( resid   30 and name hn   ) 0.0 0.0 5.0
assign ( resid   29 and name hg1# )( resid   30 and name hn   ) 0.0 0.0 5.0
assign ( resid   29 and name ha   )( resid   30 and name hn   ) 0.0 0.0 2.8
assign ( resid   30 and name ha   )( resid   31 and name hn   ) 0.0 0.0 2.8
assign ( resid   30 and name hb#  )( resid   31 and name hn   ) 0.0 0.0 5.0
assign ( resid   31 and name ha   )( resid   32 and name hn   ) 0.0 0.0 2.8
assign ( resid   31 and name hb1  )( resid   32 and name hn   ) 0.0 0.0 5.0
assign ( resid   32 and name ha   )( resid   33 and name ha   ) 0.0 0.0 5.0
assign ( resid   32 and name ha   )( resid   33 and name hn   ) 0.0 0.0 2.8
assign ( resid   32 and name hg2# )( resid   33 and name hn   ) 0.0 0.0 3.5
assign ( resid   33 and name ha   )( resid   34 and name hn   ) 0.0 0.0 2.8
assign ( resid   34 and name ha   )( resid   35 and name hn   ) 0.0 0.0 3.5
assign ( resid   34 and name hd1# )( resid   35 and name hn   ) 0.0 0.0 6.0
assign ( resid   34 and name hd2# )( resid   35 and name hn   ) 0.0 0.0 6.0
assign ( resid   34 and name hb#  )( resid   35 and name hn   ) 0.0 0.0 3.5
assign ( resid   35 and name ha1  )( resid   36 and name hn   ) 0.0 0.0 5.0
assign ( resid   35 and name ha2  )( resid   36 and name hn   ) 0.0 0.0 3.5
assign ( resid   36 and name ha   )( resid   37 and name hn   ) 0.0 0.0 3.5
assign ( resid   36 and name hn   )( resid   37 and name hn   ) 0.0 0.0 5.0
assign ( resid   36 and name hg2  )( resid   37 and name hn   ) 0.0 0.0 5.0
assign ( resid   36 and name hb#  )( resid   37 and name hn   ) 0.0 0.0 5.0
assign ( resid   37 and name ha2  )( resid   38 and name hn   ) 0.0 0.0 5.0
assign ( resid   37 and name ha1  )( resid   38 and name hn   ) 0.0 0.0 5.0
assign ( resid   37 and name hn   )( resid   38 and name hn   ) 0.0 0.0 5.0
assign ( resid   38 and name ha   )( resid   39 and name hn   ) 0.0 0.0 2.8
assign ( resid   39 and name ha1  )( resid   40 and name hn   ) 0.0 0.0 3.5
assign ( resid   40 and name ha   )( resid   41 and name hn   ) 0.0 0.0 2.8
assign ( resid   41 and name ha   )( resid   42 and name hn   ) 0.0 0.0 2.8
assign ( resid   41 and name hg2# )( resid   42 and name hn   ) 0.0 0.0 3.5
assign ( resid   41 and name hg1# )( resid   42 and name hn   ) 0.0 0.0 5.0
assign ( resid   41 and name hg2# )( resid   42 and name ha   ) 0.0 0.0 5.0
assign ( resid   42 and name ha   )( resid   43 and name hn   ) 0.0 0.0 2.8
assign ( resid   42 and name hb   )( resid   43 and name hn   ) 0.0 0.0 5.0
assign ( resid   42 and name hg2# )( resid   43 and name hn   ) 0.0 0.0 5.0
assign ( resid   43 and name ha   )( resid   44 and name hn   ) 0.0 0.0 2.8
assign ( resid   43 and name hb2  )( resid   44 and name hn   ) 0.0 0.0 5.0
assign ( resid   43 and name hb1  )( resid   44 and name hn   ) 0.0 0.0 5.0
assign ( resid   43 and name ha   )( resid   44 and name hg2# ) 0.0 0.0 5.0
assign ( resid   45 and name hn   )( resid   46 and name hn   ) 0.0 0.0 5.0
assign ( resid   46 and name hb2  )( resid   47 and name hn   ) 0.0 0.0 5.0
assign ( resid   46 and name hg1  )( resid   47 and name hn   ) 0.0 0.0 5.0
assign ( resid   47 and name ha   )( resid   48 and name hn   ) 0.0 0.0 5.0
assign ( resid   48 and name ha   )( resid   49 and name hn   ) 0.0 0.0 3.5
assign ( resid   48 and name hn   )( resid   49 and name hn   ) 0.0 0.0 5.0
assign ( resid   49 and name ha   )( resid   50 and name hn   ) 0.0 0.0 3.5
assign ( resid   49 and name hb2  )( resid   50 and name hn   ) 0.0 0.0 5.0
assign ( resid   50 and name ha   )( resid   51 and name hn   ) 0.0 0.0 3.5
assign ( resid   51 and name ha   )( resid   52 and name hn   ) 0.0 0.0 2.8
assign ( resid   52 and name ha   )( resid   53 and name hn   ) 0.0 0.0 5.0
assign ( resid   52 and name hb1  )( resid   53 and name hn   ) 0.0 0.0 5.0
assign ( resid   53 and name hb2  )( resid   54 and name hg2# ) 0.0 0.0 3.5
assign ( resid   53 and name hb1  )( resid   54 and name hg2# ) 0.0 0.0 5.0
assign ( resid   53 and name ha   )( resid   54 and name hg2# ) 0.0 0.0 5.0
assign ( resid   53 and name ha   )( resid   54 and name hn   ) 0.0 0.0 5.0
assign ( resid   53 and name hb#  )( resid   54 and name hn   ) 0.0 0.0 5.0
assign ( resid   53 and name hn   )( resid   54 and name hn   ) 0.0 0.0 3.5
assign ( resid   54 and name hn   )( resid   55 and name ha   ) 0.0 0.0 5.0
assign ( resid   54 and name ha   )( resid   55 and name hn   ) 0.0 0.0 5.0
assign ( resid   54 and name hb   )( resid   55 and name hn   ) 0.0 0.0 5.0
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assign ( resid   55 and name ha   )( resid   56 and name hn   ) 0.0 0.0 3.5
assign ( resid   56 and name hg2# )( resid   57 and name ha   ) 0.0 0.0 5.0
assign ( resid   56 and name ha   )( resid   57 and name hn   ) 0.0 0.0 2.8
assign ( resid   56 and name hg2# )( resid   57 and name hn   ) 0.0 0.0 3.5
assign ( resid   57 and name ha   )( resid   58 and name hn   ) 0.0 0.0 3.5
assign ( resid   58 and name ha   )( resid   59 and name hn   ) 0.0 0.0 3.5
assign ( resid   58 and name hb2  )( resid   59 and name hn   ) 0.0 0.0 3.5
assign ( resid   58 and name hb1  )( resid   59 and name hn   ) 0.0 0.0 5.0
assign ( resid   58 and name hd#  )( resid   59 and name hn   ) 0.0 0.0 5.0
assign ( resid   59 and name ha   )( resid   60 and name hn   ) 0.0 0.0 3.5
assign ( resid   59 and name hb2  )( resid   60 and name hn   ) 0.0 0.0 3.5
assign ( resid   59 and name hb1  )( resid   60 and name hn   ) 0.0 0.0 5.0
assign ( resid   60 and name ha   )( resid   61 and name hn   ) 0.0 0.0 2.8
assign ( resid   60 and name hb   )( resid   61 and name hn   ) 0.0 0.0 5.0
assign ( resid   60 and name hn   )( resid   61 and name hn   ) 0.0 0.0 5.0
assign ( resid   61 and name ha1  )( resid   62 and name hn   ) 0.0 0.0 3.5
assign ( resid   62 and name ha   )( resid   63 and name hg2# ) 0.0 0.0 5.0
assign ( resid   62 and name ha   )( resid   63 and name hn   ) 0.0 0.0 2.8
assign ( resid   62 and name hb2  )( resid   63 and name hn   ) 0.0 0.0 5.0
assign ( resid   62 and name hb1  )( resid   63 and name hn   ) 0.0 0.0 5.0
assign ( resid   63 and name ha   )( resid   64 and name he3  ) 0.0 0.0 2.8
assign ( resid   63 and name ha   )( resid   64 and name hz3  ) 0.0 0.0 5.0
assign ( resid   63 and name hb   )( resid   64 and name he3  ) 0.0 0.0 5.0
assign ( resid   63 and name hg2# )( resid   64 and name he3  ) 0.0 0.0 5.0
assign ( resid   63 and name hg2# )( resid   64 and name hz3  ) 0.0 0.0 5.0
assign ( resid   63 and name ha   )( resid   64 and name hn   ) 0.0 0.0 2.8
assign ( resid   63 and name hg2# )( resid   64 and name hn   ) 0.0 0.0 3.5
assign ( resid   63 and name hb   )( resid   64 and name hn   ) 0.0 0.0 3.5
assign ( resid   63 and name hn   )( resid   64 and name he3  ) 0.0 0.0 5.0
assign ( resid   64 and name hd1  )( resid   65 and name ha   ) 0.0 0.0 5.0
assign ( resid   64 and name hd1  )( resid   65 and name hb#  ) 0.0 0.0 5.0
assign ( resid   64 and name ha   )( resid   65 and name hn   ) 0.0 0.0 2.8
assign ( resid   64 and name hb2  )( resid   65 and name hn   ) 0.0 0.0 3.5
assign ( resid   64 and name hb1  )( resid   65 and name hn   ) 0.0 0.0 3.5
assign ( resid   64 and name hd1  )( resid   65 and name hn   ) 0.0 0.0 5.0
assign ( resid   65 and name hb#  )( resid   66 and name ha   ) 0.0 0.0 5.0
assign ( resid   65 and name ha   )( resid   66 and name hn   ) 0.0 0.0 2.8
assign ( resid   65 and name hb#  )( resid   66 and name hn   ) 0.0 0.0 3.5
assign ( resid   66 and name hb2  )( resid   67 and name hn   ) 0.0 0.0 5.0
assign ( resid   66 and name hd#  )( resid   67 and name hn   ) 0.0 0.0 3.5
assign ( resid   66 and name ha   )( resid   67 and name hn   ) 0.0 0.0 2.8
assign ( resid   67 and name hg2  )( resid   68 and name hb2  ) 0.0 0.0 5.0
assign ( resid   67 and name ha   )( resid   68 and name hn   ) 0.0 0.0 2.8
assign ( resid   67 and name hg2  )( resid   68 and name hn   ) 0.0 0.0 3.5
assign ( resid   68 and name hb2  )( resid   69 and name hn   ) 0.0 0.0 3.5
assign ( resid   68 and name hb1  )( resid   69 and name hn   ) 0.0 0.0 5.0
assign ( resid   68 and name ha   )( resid   69 and name hn   ) 0.0 0.0 3.5
assign ( resid   69 and name hn   )( resid   70 and name hn   ) 0.0 0.0 5.0
assign ( resid   69 and name hb2  )( resid   70 and name hn   ) 0.0 0.0 5.0
assign ( resid   69 and name ha   )( resid   70 and name hn   ) 0.0 0.0 3.5
assign ( resid   70 and name ha   )( resid   71 and name hn   ) 0.0 0.0 3.5
assign ( resid   70 and name hb2  )( resid   71 and name hn   ) 0.0 0.0 5.0
assign ( resid   71 and name ha1  )( resid   72 and name hn   ) 0.0 0.0 5.0
assign ( resid   71 and name ha2  )( resid   72 and name hn   ) 0.0 0.0 5.0
assign ( resid   72 and name ha   )( resid   73 and name hn   ) 0.0 0.0 2.8
assign ( resid   72 and name hb1  )( resid   73 and name hn   ) 0.0 0.0 5.0
assign ( resid   73 and name hb#  )( resid   74 and name hn   ) 0.0 0.0 5.0
assign ( resid   73 and name hg   )( resid   74 and name hn   ) 0.0 0.0 5.0
assign ( resid   73 and name hd2# )( resid   74 and name hn   ) 0.0 0.0 3.5
assign ( resid   73 and name hn   )( resid   74 and name hn   ) 0.0 0.0 5.0
assign ( resid   73 and name ha   )( resid   74 and name hn   ) 0.0 0.0 2.8
assign ( resid   74 and name ha   )( resid   75 and name hn   ) 0.0 0.0 2.8
assign ( resid   74 and name hb1  )( resid   75 and name hn   ) 0.0 0.0 5.0
assign ( resid   74 and name hd1# )( resid   75 and name hn   ) 0.0 0.0 5.0
assign ( resid   74 and name hd2# )( resid   75 and name hn   ) 0.0 0.0 5.0
assign ( resid   74 and name hb2  )( resid   75 and name hn   ) 0.0 0.0 5.0
assign ( resid   75 and name hb2  )( resid   76 and name hn   ) 0.0 0.0 5.0
assign ( resid   75 and name hb1  )( resid   76 and name hn   ) 0.0 0.0 5.0
assign ( resid   75 and name ha   )( resid   76 and name hn   ) 0.0 0.0 2.8
assign ( resid   75 and name hg#  )( resid   76 and name hn   ) 0.0 0.0 5.0
assign ( resid   76 and name ha   )( resid   77 and name hn   ) 0.0 0.0 2.8
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assign ( resid   76 and name hb1  )( resid   77 and name hn   ) 0.0 0.0 5.0
assign ( resid   76 and name hb2  )( resid   77 and name hn   ) 0.0 0.0 5.0
assign ( resid   77 and name ha   )( resid   78 and name hb1  ) 0.0 0.0 5.0
assign ( resid   77 and name hg11 )( resid   78 and name hn   ) 0.0 0.0 5.0
assign ( resid   77 and name ha   )( resid   78 and name hn   ) 0.0 0.0 2.8
assign ( resid   78 and name ha   )( resid   79 and name hg2# ) 0.0 0.0 5.0
assign ( resid   78 and name ha   )( resid   79 and name hn   ) 0.0 0.0 3.5
assign ( resid   79 and name hb   )( resid   80 and name hn   ) 0.0 0.0 5.0
assign ( resid   79 and name hg2# )( resid   80 and name hn   ) 0.0 0.0 5.0
assign ( resid   79 and name ha   )( resid   80 and name hn   ) 0.0 0.0 3.5
assign ( resid   80 and name ha1  )( resid   81 and name hn   ) 0.0 0.0 3.5
assign ( resid   80 and name hn   )( resid   81 and name hn   ) 0.0 0.0 5.0
assign ( resid   80 and name ha2  )( resid   81 and name hn   ) 0.0 0.0 2.8
assign ( resid   81 and name ha   )( resid   82 and name hn   ) 0.0 0.0 3.5
assign ( resid   82 and name ha2  )( resid   83 and name hn   ) 0.0 0.0 5.0
assign ( resid   82 and name ha1  )( resid   83 and name hn   ) 0.0 0.0 5.0
assign ( resid   82 and name ha1  )( resid   83 and name hb1  ) 0.0 0.0 5.0
assign ( resid   83 and name ha   )( resid   84 and name hn   ) 0.0 0.0 3.5
assign ( resid   83 and name hb1  )( resid   84 and name hn   ) 0.0 0.0 5.0
assign ( resid   83 and name hn   )( resid   84 and name ha2  ) 0.0 0.0 5.0
assign ( resid   83 and name hn   )( resid   84 and name ha1  ) 0.0 0.0 5.0
assign ( resid   84 and name ha1  )( resid   85 and name hn   ) 0.0 0.0 5.0
assign ( resid   84 and name ha2  )( resid   85 and name hn   ) 0.0 0.0 5.0
assign ( resid   85 and name hb#  )( resid   86 and name ha   ) 0.0 0.0 5.0
assign ( resid   85 and name hg2  )( resid   86 and name hn   ) 0.0 0.0 5.0
assign ( resid   85 and name hg1  )( resid   86 and name hn   ) 0.0 0.0 5.0
assign ( resid   85 and name hb#  )( resid   86 and name hn   ) 0.0 0.0 5.0
assign ( resid   85 and name ha   )( resid   86 and name he3  ) 0.0 0.0 5.0
assign ( resid   86 and name hd1  )( resid   87 and name ha   ) 0.0 0.0 5.0
assign ( resid   86 and name ha   )( resid   87 and name hn   ) 0.0 0.0 3.5
assign ( resid   86 and name hd1  )( resid   87 and name hn   ) 0.0 0.0 5.0
assign ( resid   87 and name ha   )( resid   88 and name hn   ) 0.0 0.0 2.8
assign ( resid   87 and name hg#  )( resid   88 and name hn   ) 0.0 0.0 5.0
assign ( resid   88 and name ha   )( resid   89 and name hn   ) 0.0 0.0 2.8
assign ( resid   88 and name hb2  )( resid   89 and name hn   ) 0.0 0.0 5.0
assign ( resid   88 and name hb1  )( resid   89 and name hn   ) 0.0 0.0 5.0
assign ( resid   89 and name ha   )( resid   90 and name hn   ) 0.0 0.0 2.8
assign ( resid   89 and name hb#  )( resid   90 and name hn   ) 0.0 0.0 5.0
assign ( resid   89 and name hg1  )( resid   90 and name hn   ) 0.0 0.0 3.5
assign ( resid   89 and name hg2  )( resid   90 and name hn   ) 0.0 0.0 5.0
assign ( resid   90 and name ha   )( resid   91 and name hn   ) 0.0 0.0 2.8
assign ( resid   90 and name hg1  )( resid   91 and name hn   ) 0.0 0.0 5.0
assign ( resid   91 and name ha   )( resid   92 and name hn   ) 0.0 0.0 3.5
assign ( resid   92 and name ha   )( resid   93 and name hn   ) 0.0 0.0 3.5

MEDIUM RANGE NOES

assign ( resid    3 and name hb#  )( resid    5 and name he#  ) 0.0 0.0 3.5
assign ( resid    3 and name hb#  )( resid    5 and name hd#  ) 0.0 0.0 5.0
assign ( resid    3 and name he#  )( resid    5 and name hd#  ) 0.0 0.0 6.0
assign ( resid    3 and name he#  )( resid    5 and name he#  ) 0.0 0.0 5.0
assign ( resid    3 and name hg#  )( resid    5 and name he#  ) 0.0 0.0 6.0
assign ( resid    3 and name hd#  )( resid    5 and name he#  ) 0.0 0.0 5.0
assign ( resid    5 and name hd#  )( resid    7 and name hd#  ) 0.0 0.0 5.0
assign ( resid    5 and name he#  )( resid    7 and name ha   ) 0.0 0.0 5.0
assign ( resid    5 and name hd#  )( resid    7 and name ha   ) 0.0 0.0 5.0
assign ( resid    5 and name hb#  )( resid    7 and name ha   ) 0.0 0.0 5.0
assign ( resid    6 and name hb2  )( resid    9 and name hb#  ) 0.0 0.0 3.5
assign ( resid    6 and name hb1  )( resid    9 and name hb#  ) 0.0 0.0 5.0
assign ( resid    7 and name ha   )( resid   10 and name ha1  ) 0.0 0.0 5.0
assign ( resid    7 and name ha   )( resid   10 and name ha2  ) 0.0 0.0 5.0
assign ( resid    7 and name ha   )( resid   10 and name hn   ) 0.0 0.0 5.0
assign ( resid    9 and name hb#  )( resid   11 and name hb   ) 0.0 0.0 5.0
assign ( resid    9 and name hb#  )( resid   13 and name he#  ) 0.0 0.0 5.0
assign ( resid    9 and name hb#  )( resid   13 and name hd#  ) 0.0 0.0 6.0
assign ( resid    9 and name hb#  )( resid   11 and name hn   ) 0.0 0.0 3.5
assign ( resid   11 and name hb   )( resid   13 and name he#  ) 0.0 0.0 3.5
assign ( resid   11 and name hb   )( resid   13 and name hd#  ) 0.0 0.0 5.0
assign ( resid   11 and name hg2# )( resid   13 and name hd#  ) 0.0 0.0 5.0
assign ( resid   11 and name ha   )( resid   13 and name he#  ) 0.0 0.0 5.0
assign ( resid   13 and name hd#  )( resid   17 and name hb2  ) 0.0 0.0 5.0
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assign ( resid   13 and name hd#  )( resid   17 and name hb1  ) 0.0 0.0 5.0
assign ( resid   13 and name hb2  )( resid   17 and name hb2  ) 0.0 0.0 5.0
assign ( resid   13 and name he#  )( resid   17 and name hb2  ) 0.0 0.0 6.0
assign ( resid   13 and name he#  )( resid   17 and name hb1  ) 0.0 0.0 5.0
assign ( resid   14 and name hn   )( resid   17 and name hb2  ) 0.0 0.0 5.0
assign ( resid   14 and name hn   )( resid   17 and name hg#  ) 0.0 0.0 5.0
assign ( resid   17 and name hb1  )( resid   19 and name hz3  ) 0.0 0.0 5.0
assign ( resid   17 and name hb1  )( resid   19 and name he3  ) 0.0 0.0 5.0
assign ( resid   17 and name hb2  )( resid   19 and name he3  ) 0.0 0.0 5.0
assign ( resid   17 and name ha   )( resid   19 and name he3  ) 0.0 0.0 5.0
assign ( resid   17 and name hg#  )( resid   19 and name he3  ) 0.0 0.0 3.5
assign ( resid   19 and name hd1  )( resid   21 and name hd#  ) 0.0 0.0 3.5
assign ( resid   19 and name hd1  )( resid   21 and name ha   ) 0.0 0.0 5.0
assign ( resid   19 and name hd1  )( resid   21 and name hb#  ) 0.0 0.0 3.5
assign ( resid   19 and name hd1  )( resid   21 and name hg#  ) 0.0 0.0 3.5
assign ( resid   19 and name he1  )( resid   21 and name hg#  ) 0.0 0.0 5.0
assign ( resid   19 and name he1  )( resid   21 and name hd#  ) 0.0 0.0 5.0
assign ( resid   20 and name hb2  )( resid   22 and name hd2  ) 0.0 0.0 3.5
assign ( resid   20 and name hb2  )( resid   22 and name hd1  ) 0.0 0.0 5.0
assign ( resid   23 and name hd#  )( resid   27 and name he#  ) 0.0 0.0 3.5
assign ( resid   23 and name hd#  )( resid   27 and name hb1  ) 0.0 0.0 5.0
assign ( resid   23 and name hd#  )( resid   27 and name hb2  ) 0.0 0.0 5.0
assign ( resid   23 and name hb2  )( resid   27 and name hb2  ) 0.0 0.0 5.0
assign ( resid   23 and name hb2  )( resid   27 and name hb1  ) 0.0 0.0 5.0
assign ( resid   23 and name hb1  )( resid   27 and name hb1  ) 0.0 0.0 5.0
assign ( resid   23 and name hb1  )( resid   27 and name hb2  ) 0.0 0.0 3.5
assign ( resid   23 and name he#  )( resid   27 and name he#  ) 0.0 0.0 5.0
assign ( resid   23 and name hd#  )( resid   27 and name hg#  ) 0.0 0.0 5.0
assign ( resid   23 and name hn   )( resid   27 and name hn   ) 0.0 0.0 5.0
assign ( resid   23 and name he#  )( resid   27 and name hg#  ) 0.0 0.0 5.0
assign ( resid   23 and name he#  )( resid   27 and name hb2  ) 0.0 0.0 5.0
assign ( resid   24 and name hb1  )( resid   26 and name hd1  ) 0.0 0.0 5.0
assign ( resid   32 and name hb   )( resid   34 and name hd1# ) 0.0 0.0 3.5
assign ( resid   34 and name hd2# )( resid   36 and name hg2  ) 0.0 0.0 5.0
assign ( resid   35 and name ha1  )( resid   39 and name ha2  ) 0.0 0.0 5.0
assign ( resid   35 and name ha1  )( resid   39 and name hn   ) 0.0 0.0 5.0
assign ( resid   35 and name ha1  )( resid   39 and name ha1  ) 0.0 0.0 5.0
assign ( resid   41 and name hg2# )( resid   43 and name ha   ) 0.0 0.0 5.0
assign ( resid   44 and name hb   )( resid   46 and name hn   ) 0.0 0.0 5.0
assign ( resid   46 and name ha   )( resid   48 and name hn   ) 0.0 0.0 5.0
assign ( resid   52 and name ha   )( resid   55 and name ha   ) 0.0 0.0 3.5
assign ( resid   52 and name ha   )( resid   55 and name hg1  ) 0.0 0.0 5.0
assign ( resid   52 and name ha   )( resid   55 and name hn   ) 0.0 0.0 5.0
assign ( resid   52 and name ha   )( resid   54 and name hn   ) 0.0 0.0 5.0
assign ( resid   53 and name hn   )( resid   55 and name hn   ) 0.0 0.0 5.0
assign ( resid   54 and name hg2# )( resid   58 and name he#  ) 0.0 0.0 5.0
assign ( resid   54 and name hg2# )( resid   56 and name hg2# ) 0.0 0.0 5.0
assign ( resid   54 and name ha   )( resid   56 and name hn   ) 0.0 0.0 5.0
assign ( resid   54 and name hb   )( resid   56 and name hn   ) 0.0 0.0 5.0
assign ( resid   54 and name hb   )( resid   56 and name hb   ) 0.0 0.0 5.0
assign ( resid   56 and name hg2# )( resid   58 and name hd#  ) 0.0 0.0 5.0
assign ( resid   56 and name hg2# )( resid   58 and name he#  ) 0.0 0.0 3.5
assign ( resid   56 and name hg2# )( resid   58 and name ha   ) 0.0 0.0 5.0
assign ( resid   56 and name ha   )( resid   58 and name he#  ) 0.0 0.0 5.0
assign ( resid   56 and name ha   )( resid   58 and name hd#  ) 0.0 0.0 6.0
assign ( resid   56 and name hb   )( resid   58 and name he#  ) 0.0 0.0 3.5
assign ( resid   56 and name hb   )( resid   58 and name hd#  ) 0.0 0.0 5.0
assign ( resid   58 and name hd#  )( resid   62 and name hb2  ) 0.0 0.0 5.0
assign ( resid   58 and name ha   )( resid   62 and name hb2  ) 0.0 0.0 5.0
assign ( resid   59 and name hn   )( resid   62 and name hb2  ) 0.0 0.0 5.0
assign ( resid   59 and name hn   )( resid   62 and name hb1  ) 0.0 0.0 5.0
assign ( resid   60 and name ha   )( resid   64 and name hz3  ) 0.0 0.0 6.0
assign ( resid   62 and name hb2  )( resid   64 and name he3  ) 0.0 0.0 5.0
assign ( resid   62 and name ha   )( resid   64 and name he3  ) 0.0 0.0 5.0
assign ( resid   62 and name hb2  )( resid   64 and name hz3  ) 0.0 0.0 5.0
assign ( resid   62 and name hb1  )( resid   64 and name he3  ) 0.0 0.0 5.0
assign ( resid   64 and name hd1  )( resid   66 and name hb2  ) 0.0 0.0 3.5
assign ( resid   64 and name hd1  )( resid   66 and name hg#  ) 0.0 0.0 5.0
assign ( resid   64 and name he1  )( resid   66 and name hd#  ) 0.0 0.0 3.5
assign ( resid   64 and name hd1  )( resid   66 and name hb1  ) 0.0 0.0 5.0
assign ( resid   64 and name hd1  )( resid   66 and name hd#  ) 0.0 0.0 3.5
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assign ( resid   67 and name hb#  )( resid   71 and name ha1  ) 0.0 0.0 5.0
assign ( resid   67 and name hg1  )( resid   71 and name ha1  ) 0.0 0.0 5.0
assign ( resid   68 and name hn   )( resid   72 and name hn   ) 0.0 0.0 3.5
assign ( resid   68 and name hb1  )( resid   70 and name hn   ) 0.0 0.0 6.0
assign ( resid   68 and name hb2  )( resid   70 and name hn   ) 0.0 0.0 5.0
assign ( resid   69 and name hn   )( resid   73 and name hd2# ) 0.0 0.0 5.0
assign ( resid   72 and name hb2  )( resid   74 and name hd1# ) 0.0 0.0 5.0
assign ( resid   72 and name hb1  )( resid   74 and name hd1# ) 0.0 0.0 5.0
assign ( resid   72 and name hb2  )( resid   74 and name hd2# ) 0.0 0.0 5.0
assign ( resid   73 and name hd2# )( resid   75 and name ha   ) 0.0 0.0 5.0
assign ( resid   77 and name hg11 )( resid   79 and name hb   ) 0.0 0.0 5.0
assign ( resid   77 and name hg12 )( resid   79 and name ha   ) 0.0 0.0 5.0
assign ( resid   77 and name hg12 )( resid   79 and name hb   ) 0.0 0.0 5.0
assign ( resid   78 and name ha   )( resid   80 and name hn   ) 0.0 0.0 5.0
assign ( resid   79 and name hb   )( resid   81 and name hb1  ) 0.0 0.0 5.0
assign ( resid   80 and name ha1  )( resid   84 and name ha1  ) 0.0 0.0 5.0
assign ( resid   80 and name ha1  )( resid   84 and name ha2  ) 0.0 0.0 5.0
assign ( resid   81 and name hn   )( resid   83 and name hn   ) 0.0 0.0 3.5
assign ( resid   82 and name ha1  )( resid   84 and name hn   ) 0.0 0.0 5.0
assign ( resid   86 and name hd1  )( resid   88 and name ha   ) 0.0 0.0 5.0

LONG RANGE NOES

assign ( resid    2 and name ha   )( resid   15 and name hb   ) 0.0 0.0 5.0
assign ( resid    2 and name hb#  )( resid   35 and name ha2  ) 0.0 0.0 5.0
assign ( resid    2 and name hb#  )( resid   35 and name ha1  ) 0.0 0.0 6.0
assign ( resid    2 and name hn   )( resid   14 and name ha   ) 0.0 0.0 5.0
assign ( resid    2 and name hb#  )( resid   15 and name hg2# ) 0.0 0.0 2.8
assign ( resid    2 and name ha   )( resid   15 and name hg2# ) 0.0 0.0 5.0
assign ( resid    2 and name hg2  )( resid   15 and name hg2# ) 0.0 0.0 5.0
assign ( resid    3 and name ha   )( resid   14 and name ha   ) 0.0 0.0 2.8
assign ( resid    3 and name ha   )( resid   14 and name hg1# ) 0.0 0.0 5.0
assign ( resid    3 and name he#  )( resid   14 and name hg2# ) 0.0 0.0 3.5
assign ( resid    3 and name ha   )( resid   14 and name hg2# ) 0.0 0.0 5.0
assign ( resid    3 and name ha   )( resid   14 and name hb   ) 0.0 0.0 5.0
assign ( resid    3 and name hb#  )( resid   14 and name ha   ) 0.0 0.0 5.0
assign ( resid    3 and name hg#  )( resid   14 and name ha   ) 0.0 0.0 5.0
assign ( resid    3 and name hb#  )( resid   12 and name hb2  ) 0.0 0.0 5.0
assign ( resid    3 and name hb#  )( resid   12 and name hb1  ) 0.0 0.0 3.5
assign ( resid    3 and name hb#  )( resid   12 and name ha   ) 0.0 0.0 5.0
assign ( resid    3 and name he#  )( resid   12 and name hb2  ) 0.0 0.0 5.0
assign ( resid    3 and name he#  )( resid   14 and name hg1# ) 0.0 0.0 5.0
assign ( resid    3 and name hg#  )( resid   12 and name hb1  ) 0.0 0.0 5.0
assign ( resid    3 and name hg#  )( resid   12 and name hb2  ) 0.0 0.0 6.0
assign ( resid    3 and name hd#  )( resid   12 and name hb2  ) 0.0 0.0 5.0
assign ( resid    3 and name hd#  )( resid   12 and name hb1  ) 0.0 0.0 5.0
assign ( resid    3 and name hd#  )( resid   14 and name ha   ) 0.0 0.0 5.0
assign ( resid    3 and name ha   )( resid   15 and name hn   ) 0.0 0.0 5.0
assign ( resid    3 and name hb#  )( resid   13 and name hn   ) 0.0 0.0 5.0
assign ( resid    3 and name hd#  )( resid   13 and name hn   ) 0.0 0.0 5.0
assign ( resid    4 and name hb1  )( resid   19 and name hh2  ) 0.0 0.0 5.0
assign ( resid    4 and name hb1  )( resid   19 and name hz3  ) 0.0 0.0 5.0
assign ( resid    4 and name ha   )( resid   39 and name ha2  ) 0.0 0.0 5.0
assign ( resid    4 and name ha   )( resid   39 and name ha1  ) 0.0 0.0 5.0
assign ( resid    4 and name hb2  )( resid   41 and name hd1# ) 0.0 0.0 5.0
assign ( resid    4 and name hb2  )( resid   13 and name hb2  ) 0.0 0.0 3.5
assign ( resid    4 and name hb1  )( resid   41 and name hg1# ) 0.0 0.0 3.5
assign ( resid    4 and name hb1  )( resid   41 and name hd1# ) 0.0 0.0 5.0
assign ( resid    4 and name hb1  )( resid   33 and name ha   ) 0.0 0.0 5.0
assign ( resid    4 and name hb2  )( resid   33 and name ha   ) 0.0 0.0 5.0
assign ( resid    4 and name hb2  )( resid   19 and name hh2  ) 0.0 0.0 5.0
assign ( resid    4 and name hb2  )( resid   19 and name hz3  ) 0.0 0.0 6.0
assign ( resid    4 and name hn   )( resid   14 and name ha   ) 0.0 0.0 5.0
assign ( resid    4 and name hn   )( resid   13 and name hn   ) 0.0 0.0 5.0
assign ( resid    4 and name hn   )( resid   13 and name hd#  ) 0.0 0.0 5.0
assign ( resid    4 and name hb2  )( resid   13 and name hd#  ) 0.0 0.0 5.0
assign ( resid    4 and name hb1  )( resid   13 and name hd#  ) 0.0 0.0 5.0
assign ( resid    4 and name hb2  )( resid   41 and name hg1# ) 0.0 0.0 2.8
assign ( resid    4 and name hb2  )( resid   41 and name hg2# ) 0.0 0.0 5.0
assign ( resid    5 and name ha   )( resid   12 and name ha   ) 0.0 0.0 2.8
assign ( resid    5 and name hd#  )( resid   12 and name ha   ) 0.0 0.0 5.0
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assign ( resid    5 and name he# )( resid   12 and name hb2  ) 0.0 0.0 5.0
assign ( resid    5 and name hd# )( resid   12 and name hb2  ) 0.0 0.0 3.5
assign ( resid    5 and name he# )( resid   12 and name hb1  ) 0.0 0.0 5.0
assign ( resid    5 and name ha  )( resid   13 and name hd#  ) 0.0 0.0 5.0
assign ( resid    5 and name he# )( resid   10 and name ha1  ) 0.0 0.0 5.0
assign ( resid    5 and name hd# )( resid   12 and name hb1  ) 0.0 0.0 5.0
assign ( resid    5 and name hb# )( resid   12 and name ha   ) 0.0 0.0 5.0
assign ( resid    5 and name ha  )( resid   12 and name hb1  ) 0.0 0.0 5.0
assign ( resid    5 and name ha  )( resid   12 and name hb2  ) 0.0 0.0 5.0
assign ( resid    5 and name hb# )( resid   12 and name hb2  ) 0.0 0.0 5.0
assign ( resid    5 and name hb# )( resid   12 and name hb1  ) 0.0 0.0 5.0
assign ( resid    5 and name he# )( resid   10 and name ha2  ) 0.0 0.0 5.0
assign ( resid    5 and name he# )( resid   11 and name ha   ) 0.0 0.0 5.0
assign ( resid    5 and name he# )( resid   12 and name ha   ) 0.0 0.0 5.0
assign ( resid    5 and name hb# )( resid   13 and name he#  ) 0.0 0.0 6.0
assign ( resid    5 and name hd# )( resid   13 and name hd#  ) 0.0 0.0 5.0
assign ( resid    5 and name he# )( resid   13 and name hd#  ) 0.0 0.0 6.0
assign ( resid    5 and name hn  )( resid   41 and name hg1# ) 0.0 0.0 5.0
assign ( resid    5 and name hz  )( resid   10 and name ha1  ) 0.0 0.0 6.0
assign ( resid    5 and name ha  )( resid   12 and name hn   ) 0.0 0.0 6.0
assign ( resid    6 and name hb1 )( resid   13 and name he#  ) 0.0 0.0 3.5
assign ( resid    6 and name hb1 )( resid   13 and name hd#  ) 0.0 0.0 5.0
assign ( resid    6 and name hb1 )( resid   19 and name hz2  ) 0.0 0.0 5.0
assign ( resid    6 and name hb2 )( resid   19 and name hz2  ) 0.0 0.0 5.0
assign ( resid    6 and name ha  )( resid   19 and name hz2  ) 0.0 0.0 6.0
assign ( resid    6 and name hn  )( resid   13 and name he#  ) 0.0 0.0 5.0
assign ( resid    6 and name hb1 )( resid   11 and name hn   ) 0.0 0.0 5.0
assign ( resid    6 and name hb2 )( resid   13 and name he#  ) 0.0 0.0 5.0
assign ( resid    6 and name hn  )( resid   11 and name hn   ) 0.0 0.0 5.0
assign ( resid    6 and name hn  )( resid   13 and name hd#  ) 0.0 0.0 5.0
assign ( resid    6 and name hb2 )( resid   13 and name hd#  ) 0.0 0.0 5.0
assign ( resid   13 and name he# )( resid   19 and name hz2  ) 0.0 0.0 5.0
assign ( resid   13 and name he# )( resid   19 and name he3  ) 0.0 0.0 5.0
assign ( resid   13 and name hd# )( resid   19 and name hz3  ) 0.0 0.0 5.0
assign ( resid   13 and name hd# )( resid   19 and name hz2  ) 0.0 0.0 5.0
assign ( resid   13 and name hd# )( resid   41 and name hg1# ) 0.0 0.0 5.0
assign ( resid   13 and name he# )( resid   19 and name hd1  ) 0.0 0.0 5.0
assign ( resid   13 and name hd# )( resid   33 and name hb1  ) 0.0 0.0 5.0
assign ( resid   13 and name hb1 )( resid   19 and name hz3  ) 0.0 0.0 5.0
assign ( resid   13 and name hb1 )( resid   19 and name he3  ) 0.0 0.0 5.0
assign ( resid   13 and name he# )( resid   41 and name hg1# ) 0.0 0.0 5.0
assign ( resid   13 and name he# )( resid   19 and name ha   ) 0.0 0.0 5.0
assign ( resid   13 and name ha  )( resid   19 and name he3  ) 0.0 0.0 6.0
assign ( resid   13 and name hb1 )( resid   19 and name hh2  ) 0.0 0.0 5.0
assign ( resid   13 and name hb2 )( resid   19 and name hh2  ) 0.0 0.0 5.0
assign ( resid   13 and name hd# )( resid   19 and name hh2  ) 0.0 0.0 5.0
assign ( resid   13 and name hd# )( resid   41 and name hd1# ) 0.0 0.0 6.0
assign ( resid   13 and name he# )( resid   19 and name hz3  ) 0.0 0.0 5.0
assign ( resid   13 and name he# )( resid   41 and name hg2# ) 0.0 0.0 6.0
assign ( resid   13 and name he# )( resid   19 and name he1  ) 0.0 0.0 5.0
assign ( resid   13 and name hd# )( resid   19 and name hb2  ) 0.0 0.0 3.5
assign ( resid   13 and name he# )( resid   19 and name hb1  ) 0.0 0.0 3.5
assign ( resid   13 and name he# )( resid   19 and name hb2  ) 0.0 0.0 3.5
assign ( resid   13 and name hd# )( resid   19 and name he3  ) 0.0 0.0 5.0
assign ( resid   13 and name hb1 )( resid   33 and name hb2  ) 0.0 0.0 5.0
assign ( resid   13 and name hb2 )( resid   19 and name hz3  ) 0.0 0.0 5.0
assign ( resid   13 and name hb2 )( resid   19 and name he3  ) 0.0 0.0 5.0
assign ( resid   15 and name ha  )( resid   33 and name hb2  ) 0.0 0.0 3.5
assign ( resid   15 and name ha  )( resid   33 and name hb1  ) 0.0 0.0 5.0
assign ( resid   15 and name hg2# )( resid   35 and name ha1  ) 0.0 0.0 3.5
assign ( resid   15 and name hg1# )( resid   34 and name ha   ) 0.0 0.0 5.0
assign ( resid   15 and name hg2# )( resid   34 and name ha   ) 0.0 0.0 5.0
assign ( resid   15 and name hg2# )( resid   35 and name ha2  ) 0.0 0.0 3.5
assign ( resid   15 and name hg2# )( resid   35 and name hn   ) 0.0 0.0 5.0
assign ( resid   15 and name hg2# )( resid   36 and name hn   ) 0.0 0.0 6.0
assign ( resid   15 and name ha  )( resid   33 and name ha   ) 0.0 0.0 5.0
assign ( resid   16 and name hn  )( resid   33 and name hb2  ) 0.0 0.0 5.0
assign ( resid   17 and name hn  )( resid   33 and name hb1  ) 0.0 0.0 5.0
assign ( resid   18 and name ha  )( resid   32 and name ha   ) 0.0 0.0 2.8
assign ( resid   18 and name ha  )( resid   32 and name hb   ) 0.0 0.0 5.0
assign ( resid   18 and name ha  )( resid   32 and name hg2# ) 0.0 0.0 5.0
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assign ( resid   18 and name hb   )( resid   32 and name ha   ) 0.0 0.0 5.0
assign ( resid   18 and name hg2# )( resid   32 and name ha   ) 0.0 0.0 3.5
assign ( resid   18 and name hg2# )( resid   30 and name ha   ) 0.0 0.0 5.0
assign ( resid   18 and name hg2# )( resid   32 and name hg2# ) 0.0 0.0 2.8
assign ( resid   18 and name ha   )( resid   33 and name hn   ) 0.0 0.0 5.0
assign ( resid   18 and name hg2# )( resid   31 and name hn   ) 0.0 0.0 5.0
assign ( resid   18 and name ha   )( resid   31 and name hn   ) 0.0 0.0 5.0
assign ( resid   19 and name hz3  )( resid   32 and name ha   ) 0.0 0.0 3.5
assign ( resid   19 and name he3  )( resid   32 and name ha   ) 0.0 0.0 5.0
assign ( resid   19 and name hh2  )( resid   33 and name hb1  ) 0.0 0.0 3.5
assign ( resid   19 and name hz3  )( resid   33 and name hb1  ) 0.0 0.0 3.5
assign ( resid   19 and name hh2  )( resid   41 and name hg1# ) 0.0 0.0 3.5
assign ( resid   19 and name hh2  )( resid   41 and name hg2# ) 0.0 0.0 3.5
assign ( resid   19 and name hz2  )( resid   31 and name hb2  ) 0.0 0.0 5.0
assign ( resid   19 and name hz2  )( resid   41 and name hg1# ) 0.0 0.0 2.8
assign ( resid   19 and name hz2  )( resid   41 and name hg2# ) 0.0 0.0 2.8
assign ( resid   19 and name hz2  )( resid   31 and name hb1  ) 0.0 0.0 5.0
assign ( resid   19 and name hh2  )( resid   33 and name ha   ) 0.0 0.0 3.5
assign ( resid   19 and name he3  )( resid   33 and name hb1  ) 0.0 0.0 5.0
assign ( resid   19 and name hz2  )( resid   41 and name hb   ) 0.0 0.0 5.0
assign ( resid   19 and name hh2  )( resid   41 and name hd1# ) 0.0 0.0 5.0
assign ( resid   19 and name hz2  )( resid   41 and name hd1# ) 0.0 0.0 5.0
assign ( resid   19 and name hz3  )( resid   41 and name hg2# ) 0.0 0.0 5.0
assign ( resid   19 and name hd1  )( resid   31 and name hb1  ) 0.0 0.0 5.0
assign ( resid   19 and name he3  )( resid   32 and name hg2# ) 0.0 0.0 5.0
assign ( resid   19 and name hz3  )( resid   33 and name ha   ) 0.0 0.0 3.5
assign ( resid   19 and name hh2  )( resid   31 and name hb1  ) 0.0 0.0 5.0
assign ( resid   19 and name hh2  )( resid   31 and name hb2  ) 0.0 0.0 5.0
assign ( resid   19 and name hh2  )( resid   41 and name hb   ) 0.0 0.0 5.0
assign ( resid   19 and name hz3  )( resid   41 and name hg1# ) 0.0 0.0 5.0
assign ( resid   19 and name he1  )( resid   31 and name hb1  ) 0.0 0.0 5.0
assign ( resid   19 and name hn   )( resid   31 and name hn   ) 0.0 0.0 3.5
assign ( resid   19 and name hn   )( resid   32 and name ha   ) 0.0 0.0 5.0
assign ( resid   19 and name hz3  )( resid   33 and name hn   ) 0.0 0.0 3.5
assign ( resid   19 and name he1  )( resid   41 and name hg1# ) 0.0 0.0 5.0
assign ( resid   19 and name he1  )( resid   31 and name hb2  ) 0.0 0.0 5.0
assign ( resid   19 and name he1  )( resid   41 and name hg2# ) 0.0 0.0 5.0
assign ( resid   19 and name he3  )( resid   33 and name hn   ) 0.0 0.0 5.0
assign ( resid   19 and name hh2  )( resid   33 and name hn   ) 0.0 0.0 5.0
assign ( resid   19 and name he3  )( resid   31 and name hb1  ) 0.0 0.0 6.0
assign ( resid   19 and name hz2  )( resid   41 and name ha   ) 0.0 0.0 5.0
assign ( resid   19 and name hz3  )( resid   33 and name hb2  ) 0.0 0.0 3.5
assign ( resid   19 and name he3  )( resid   33 and name hb2  ) 0.0 0.0 5.0
assign ( resid   19 and name hh2  )( resid   32 and name ha   ) 0.0 0.0 5.0
assign ( resid   20 and name ha   )( resid   30 and name ha   ) 0.0 0.0 2.8
assign ( resid   20 and name ha   )( resid   30 and name hb#  ) 0.0 0.0 5.0
assign ( resid   20 and name ha   )( resid   31 and name hn   ) 0.0 0.0 5.0
assign ( resid   20 and name ha   )( resid   29 and name hn   ) 0.0 0.0 5.0
assign ( resid   21 and name hn   )( resid   30 and name ha   ) 0.0 0.0 5.0
assign ( resid   21 and name hn   )( resid   29 and name hg2# ) 0.0 0.0 5.0
assign ( resid   21 and name hg#  )( resid   29 and name hg2# ) 0.0 0.0 5.0
assign ( resid   21 and name hb#  )( resid   29 and name hg2# ) 0.0 0.0 3.5
assign ( resid   21 and name he#  )( resid   29 and name hg2# ) 0.0 0.0 6.0
assign ( resid   22 and name ha   )( resid   28 and name ha   ) 0.0 0.0 2.8
assign ( resid   22 and name hb2  )( resid   28 and name ha   ) 0.0 0.0 5.0
assign ( resid   22 and name ha   )( resid   28 and name hg2  ) 0.0 0.0 5.0
assign ( resid   22 and name hg2  )( resid   28 and name ha   ) 0.0 0.0 5.0
assign ( resid   22 and name ha   )( resid   28 and name he#  ) 0.0 0.0 5.0
assign ( resid   22 and name ha   )( resid   29 and name hn   ) 0.0 0.0 5.0
assign ( resid   22 and name hd2  )( resid   28 and name hg2  ) 0.0 0.0 5.0
assign ( resid   22 and name ha   )( resid   28 and name hb#  ) 0.0 0.0 5.0
assign ( resid   23 and name hd#  )( resid   29 and name hg2# ) 0.0 0.0 3.5
assign ( resid   23 and name hb2  )( resid   29 and name hg2# ) 0.0 0.0 3.5
assign ( resid   23 and name ha   )( resid   29 and name hg2# ) 0.0 0.0 5.0
assign ( resid   23 and name ha   )( resid   29 and name hg1# ) 0.0 0.0 5.0
assign ( resid   23 and name hd#  )( resid   28 and name ha   ) 0.0 0.0 5.0
assign ( resid   23 and name he#  )( resid   29 and name hg2# ) 0.0 0.0 5.0
assign ( resid   23 and name hn   )( resid   28 and name ha   ) 0.0 0.0 5.0
assign ( resid   23 and name hb2  )( resid   29 and name hg1# ) 0.0 0.0 3.5
assign ( resid   23 and name hb1  )( resid   29 and name hg2# ) 0.0 0.0 5.0
assign ( resid   26 and name he3  )( resid   57 and name hb1  ) 0.0 0.0 3.5
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assign ( resid   26 and name hh2  )( resid   52 and name hg#  ) 0.0 0.0 5.0
assign ( resid   26 and name hd1  )( resid   55 and name hb1  ) 0.0 0.0 5.0
assign ( resid   26 and name hz2  )( resid   52 and name ha   ) 0.0 0.0 2.8
assign ( resid   26 and name hh2  )( resid   52 and name ha   ) 0.0 0.0 2.8
assign ( resid   26 and name hz3  )( resid   50 and name hb1  ) 0.0 0.0 3.5
assign ( resid   26 and name hz2  )( resid   52 and name hg#  ) 0.0 0.0 5.0
assign ( resid   26 and name hz2  )( resid   52 and name hb1  ) 0.0 0.0 5.0
assign ( resid   26 and name he3  )( resid   56 and name ha   ) 0.0 0.0 3.5
assign ( resid   26 and name he3  )( resid   57 and name hb2  ) 0.0 0.0 5.0
assign ( resid   26 and name hz3  )( resid   52 and name ha   ) 0.0 0.0 5.0
assign ( resid   26 and name hz3  )( resid   50 and name hb2  ) 0.0 0.0 5.0
assign ( resid   26 and name hh2  )( resid   50 and name hb1  ) 0.0 0.0 3.5
assign ( resid   26 and name hd1  )( resid   55 and name hd#  ) 0.0 0.0 3.5
assign ( resid   26 and name hz2  )( resid   55 and name ha   ) 0.0 0.0 5.0
assign ( resid   26 and name hh2  )( resid   55 and name ha   ) 0.0 0.0 5.0
assign ( resid   26 and name he3  )( resid   55 and name ha   ) 0.0 0.0 3.5
assign ( resid   26 and name hh2  )( resid   52 and name hb#  ) 0.0 0.0 5.0
assign ( resid   26 and name hz2  )( resid   52 and name hb2  ) 0.0 0.0 5.0
assign ( resid   26 and name hd1  )( resid   55 and name hb2  ) 0.0 0.0 5.0
assign ( resid   26 and name he3  )( resid   50 and name hb1  ) 0.0 0.0 5.0
assign ( resid   26 and name he3  )( resid   55 and name hb2  ) 0.0 0.0 6.0
assign ( resid   26 and name he3  )( resid   55 and name hb1  ) 0.0 0.0 6.0
assign ( resid   26 and name he3  )( resid   56 and name hg2# ) 0.0 0.0 6.0
assign ( resid   26 and name hz2  )( resid   55 and name hg1  ) 0.0 0.0 5.0
assign ( resid   26 and name hz3  )( resid   56 and name ha   ) 0.0 0.0 5.0
assign ( resid   26 and name hz3  )( resid   57 and name ha   ) 0.0 0.0 5.0
assign ( resid   26 and name hz3  )( resid   57 and name hb2  ) 0.0 0.0 5.0
assign ( resid   26 and name hh2  )( resid   50 and name ha   ) 0.0 0.0 5.0
assign ( resid   26 and name hh2  )( resid   51 and name ha   ) 0.0 0.0 6.0
assign ( resid   26 and name he3  )( resid   57 and name ha   ) 0.0 0.0 6.0
assign ( resid   26 and name hz2  )( resid   50 and name hb1  ) 0.0 0.0 6.0
assign ( resid   26 and name hz2  )( resid   55 and name hg2  ) 0.0 0.0 5.0
assign ( resid   26 and name hh2  )( resid   52 and name hb2  ) 0.0 0.0 5.0
assign ( resid   26 and name hh2  )( resid   52 and name hn   ) 0.0 0.0 5.0
assign ( resid   26 and name hz3  )( resid   51 and name hn   ) 0.0 0.0 3.5
assign ( resid   26 and name he1  )( resid   55 and name hg1  ) 0.0 0.0 5.0
assign ( resid   26 and name he1  )( resid   55 and name hd#  ) 0.0 0.0 5.0
assign ( resid   26 and name he3  )( resid   57 and name hn   ) 0.0 0.0 5.0
assign ( resid   27 and name hg#  )( resid   50 and name hb1  ) 0.0 0.0 5.0
assign ( resid   27 and name hg#  )( resid   50 and name hb2  ) 0.0 0.0 5.0
assign ( resid   27 and name he#  )( resid   50 and name hb1  ) 0.0 0.0 5.0
assign ( resid   28 and name hg2  )( resid   57 and name hb1  ) 0.0 0.0 5.0
assign ( resid   28 and name hg2  )( resid   57 and name hb2  ) 0.0 0.0 6.0
assign ( resid   28 and name hg1  )( resid   57 and name hb2  ) 0.0 0.0 5.0
assign ( resid   28 and name hg1  )( resid   57 and name hb1  ) 0.0 0.0 5.0
assign ( resid   28 and name hn   )( resid   57 and name hb2  ) 0.0 0.0 5.0
assign ( resid   28 and name hb#  )( resid   48 and name hn   ) 0.0 0.0 5.0
assign ( resid   29 and name hg1# )( resid   45 and name ha   ) 0.0 0.0 5.0
assign ( resid   29 and name ha   )( resid   45 and name ha   ) 0.0 0.0 3.5
assign ( resid   29 and name hg1# )( resid   45 and name hb2  ) 0.0 0.0 5.0
assign ( resid   29 and name hg1# )( resid   45 and name hb1  ) 0.0 0.0 3.5
assign ( resid   29 and name hg2# )( resid   43 and name hb2  ) 0.0 0.0 3.5
assign ( resid   29 and name ha   )( resid   45 and name hb2  ) 0.0 0.0 5.0
assign ( resid   29 and name ha   )( resid   45 and name hb1  ) 0.0 0.0 5.0
assign ( resid   29 and name hg1# )( resid   43 and name hb2  ) 0.0 0.0 5.0
assign ( resid   29 and name hg2# )( resid   45 and name hb2  ) 0.0 0.0 5.0
assign ( resid   29 and name hg2# )( resid   45 and name ha   ) 0.0 0.0 6.0
assign ( resid   29 and name hb   )( resid   44 and name hn   ) 0.0 0.0 5.0
assign ( resid   29 and name hg2# )( resid   44 and name hn   ) 0.0 0.0 5.0
assign ( resid   29 and name hg1# )( resid   44 and name hn   ) 0.0 0.0 5.0
assign ( resid   30 and name hn   )( resid   44 and name hn   ) 0.0 0.0 5.0
assign ( resid   31 and name ha   )( resid   43 and name ha   ) 0.0 0.0 2.8
assign ( resid   31 and name hb2  )( resid   43 and name ha   ) 0.0 0.0 5.0
assign ( resid   31 and name ha   )( resid   43 and name hb2  ) 0.0 0.0 5.0
assign ( resid   31 and name ha   )( resid   43 and name hb1  ) 0.0 0.0 5.0
assign ( resid   31 and name hb1  )( resid   43 and name ha   ) 0.0 0.0 5.0
assign ( resid   31 and name ha   )( resid   44 and name hn   ) 0.0 0.0 5.0
assign ( resid   32 and name hg2# )( resid   42 and name hb   ) 0.0 0.0 5.0
assign ( resid   32 and name hb   )( resid   42 and name hg2# ) 0.0 0.0 3.5
assign ( resid   32 and name hn   )( resid   43 and name ha   ) 0.0 0.0 5.0
assign ( resid   32 and name hn   )( resid   42 and name hn   ) 0.0 0.0 3.5
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assign ( resid   32 and name hb   )( resid   42 and name hn   ) 0.0 0.0 5.0
assign ( resid   33 and name ha   )( resid   41 and name hd1# ) 0.0 0.0 5.0
assign ( resid   33 and name ha   )( resid   41 and name hg1# ) 0.0 0.0 5.0
assign ( resid   33 and name ha   )( resid   41 and name hg2# ) 0.0 0.0 3.5
assign ( resid   33 and name ha   )( resid   41 and name ha   ) 0.0 0.0 2.8
assign ( resid   33 and name hb2  )( resid   41 and name hg1# ) 0.0 0.0 5.0
assign ( resid   33 and name ha   )( resid   42 and name hn   ) 0.0 0.0 5.0
assign ( resid   34 and name hn   )( resid   41 and name ha   ) 0.0 0.0 5.0
assign ( resid   34 and name hn   )( resid   40 and name hn   ) 0.0 0.0 5.0
assign ( resid   34 and name hd1# )( resid   42 and name hb   ) 0.0 0.0 3.5
assign ( resid   34 and name hd2# )( resid   42 and name hb   ) 0.0 0.0 3.5
assign ( resid   34 and name hd2# )( resid   42 and name hg2# ) 0.0 0.0 2.8
assign ( resid   34 and name hb#  )( resid   42 and name hb   ) 0.0 0.0 5.0
assign ( resid   34 and name hd1# )( resid   42 and name hn   ) 0.0 0.0 5.0
assign ( resid   35 and name ha2  )( resid   40 and name hn   ) 0.0 0.0 5.0
assign ( resid   35 and name hn   )( resid   40 and name hb2  ) 0.0 0.0 3.5
assign ( resid   35 and name ha1  )( resid   40 and name hn   ) 0.0 0.0 5.0
assign ( resid   46 and name ha   )( resid   83 and name hg#  ) 0.0 0.0 5.0
assign ( resid   46 and name hd#  )( resid   82 and name ha2  ) 0.0 0.0 5.0
assign ( resid   46 and name ha   )( resid   83 and name ha   ) 0.0 0.0 3.5
assign ( resid   46 and name hb2  )( resid   83 and name ha   ) 0.0 0.0 5.0
assign ( resid   46 and name hd#  )( resid   82 and name ha1  ) 0.0 0.0 5.0
assign ( resid   47 and name ha   )( resid   60 and name hg1# ) 0.0 0.0 3.5
assign ( resid   47 and name ha   )( resid   80 and name ha2  ) 0.0 0.0 5.0
assign ( resid   47 and name hb2  )( resid   60 and name hg1# ) 0.0 0.0 3.5
assign ( resid   47 and name hb1  )( resid   60 and name hg1# ) 0.0 0.0 5.0
assign ( resid   47 and name ha   )( resid   60 and name hb   ) 0.0 0.0 5.0
assign ( resid   48 and name hd1  )( resid   59 and name ha   ) 0.0 0.0 5.0
assign ( resid   49 and name ha   )( resid   86 and name hz3  ) 0.0 0.0 5.0
assign ( resid   49 and name ha   )( resid   86 and name he3  ) 0.0 0.0 5.0
assign ( resid   49 and name hb1  )( resid   64 and name hh2  ) 0.0 0.0 5.0
assign ( resid   49 and name hb2  )( resid   86 and name hz3  ) 0.0 0.0 5.0
assign ( resid   49 and name hb1  )( resid   64 and name hh2  ) 0.0 0.0 5.0
assign ( resid   49 and name hb2  )( resid   86 and name hz3  ) 0.0 0.0 5.0
assign ( resid   49 and name hb2  )( resid   86 and name he3  ) 0.0 0.0 3.5
assign ( resid   49 and name ha   )( resid   64 and name hz2  ) 0.0 0.0 5.0
assign ( resid   49 and name hb1  )( resid   58 and name hd#  ) 0.0 0.0 5.0
assign ( resid   49 and name hb1  )( resid   64 and name hz3  ) 0.0 0.0 5.0
assign ( resid   49 and name hb1  )( resid   86 and name he3  ) 0.0 0.0 5.0
assign ( resid   49 and name hb1  )( resid   58 and name hb1  ) 0.0 0.0 3.5
assign ( resid   49 and name ha   )( resid   64 and name hh2  ) 0.0 0.0 5.0
assign ( resid   49 and name hb1  )( resid   86 and name hz3  ) 0.0 0.0 5.0
assign ( resid   49 and name hb1  )( resid   64 and name hz2  ) 0.0 0.0 6.0
assign ( resid   49 and name hn   )( resid   58 and name hb1  ) 0.0 0.0 5.0
assign ( resid   49 and name hn   )( resid   58 and name hn   ) 0.0 0.0 5.0
assign ( resid   49 and name hn   )( resid   59 and name ha   ) 0.0 0.0 3.5
assign ( resid   49 and name hb2  )( resid   64 and name hz2  ) 0.0 0.0 5.0
assign ( resid   50 and name ha   )( resid   57 and name ha   ) 0.0 0.0 2.8
assign ( resid   50 and name ha   )( resid   57 and name hb2  ) 0.0 0.0 5.0
assign ( resid   50 and name hb2  )( resid   57 and name ha   ) 0.0 0.0 5.0
assign ( resid   50 and name ha   )( resid   57 and name hb1  ) 0.0 0.0 5.0
assign ( resid   50 and name hb1  )( resid   57 and name ha   ) 0.0 0.0 5.0
assign ( resid   50 and name ha   )( resid   58 and name hn   ) 0.0 0.0 5.0
assign ( resid   50 and name hn   )( resid   86 and name hz3  ) 0.0 0.0 5.0
assign ( resid   51 and name ha   )( resid   86 and name hz2  ) 0.0 0.0 5.0
assign ( resid   51 and name hb2  )( resid   64 and name hz2  ) 0.0 0.0 5.0
assign ( resid   51 and name hb1  )( resid   64 and name hz2  ) 0.0 0.0 5.0
assign ( resid   51 and name hb1  )( resid   58 and name he#  ) 0.0 0.0 5.0
assign ( resid   51 and name hb1  )( resid   58 and name hd#  ) 0.0 0.0 5.0
assign ( resid   51 and name hb2  )( resid   64 and name hd1  ) 0.0 0.0 5.0
assign ( resid   51 and name ha   )( resid   86 and name hh2  ) 0.0 0.0 5.0
assign ( resid   51 and name ha   )( resid   58 and name hd#  ) 0.0 0.0 6.0
assign ( resid   51 and name ha   )( resid   86 and name hz3  ) 0.0 0.0 5.0
assign ( resid   51 and name hb2  )( resid   86 and name hz2  ) 0.0 0.0 6.0
assign ( resid   51 and name hb1  )( resid   86 and name hh2  ) 0.0 0.0 6.0
assign ( resid   51 and name hb2  )( resid   86 and name hz3  ) 0.0 0.0 5.0
assign ( resid   51 and name hn   )( resid   57 and name ha   ) 0.0 0.0 5.0
assign ( resid   51 and name hn   )( resid   56 and name hn   ) 0.0 0.0 5.0
assign ( resid   51 and name hb2  )( resid   58 and name hd#  ) 0.0 0.0 5.0
assign ( resid   53 and name hb2  )( resid   66 and name hg#  ) 0.0 0.0 5.0
assign ( resid   53 and name hb1  )( resid   66 and name hg#  ) 0.0 0.0 5.0
assign ( resid   53 and name hb1  )( resid   66 and name hd#  ) 0.0 0.0 3.5
assign ( resid   54 and name hg2# )( resid   64 and name hb1  ) 0.0 0.0 5.0
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assign ( resid   54 and name hg2# )( resid   66 and name ha   ) 0.0 0.0 5.0
assign ( resid   54 and name hg2# )( resid   64 and name hd1  ) 0.0 0.0 5.0
assign ( resid   54 and name hg2# )( resid   66 and name hd#  ) 0.0 0.0 5.0
assign ( resid   54 and name hb   )( resid   64 and name hd1  ) 0.0 0.0 6.0
assign ( resid   58 and name hd#  )( resid   64 and name hz2  ) 0.0 0.0 5.0
assign ( resid   58 and name he#  )( resid   64 and name hb2  ) 0.0 0.0 5.0
assign ( resid   58 and name hb1  )( resid   64 and name hz3  ) 0.0 0.0 5.0
assign ( resid   58 and name hd#  )( resid   64 and name ha   ) 0.0 0.0 5.0
assign ( resid   58 and name hb1  )( resid   64 and name he3  ) 0.0 0.0 5.0
assign ( resid   58 and name hb1  )( resid   64 and name hh2  ) 0.0 0.0 5.0
assign ( resid   58 and name hd#  )( resid   63 and name ha   ) 0.0 0.0 5.0
assign ( resid   58 and name he#  )( resid   64 and name hb1  ) 0.0 0.0 5.0
assign ( resid   58 and name he#  )( resid   64 and name hd1  ) 0.0 0.0 5.0
assign ( resid   58 and name hd#  )( resid   64 and name hd1  ) 0.0 0.0 6.0
assign ( resid   58 and name hd#  )( resid   64 and name he3  ) 0.0 0.0 5.0
assign ( resid   58 and name he#  )( resid   64 and name ha   ) 0.0 0.0 5.0
assign ( resid   58 and name he#  )( resid   64 and name he1  ) 0.0 0.0 5.0
assign ( resid   60 and name hg1# )( resid   80 and name ha2  ) 0.0 0.0 3.5
assign ( resid   60 and name ha   )( resid   78 and name hb2  ) 0.0 0.0 3.5
assign ( resid   60 and name hg1# )( resid   80 and name ha1  ) 0.0 0.0 5.0
assign ( resid   60 and name hg1# )( resid   81 and name hn   ) 0.0 0.0 5.0
assign ( resid   61 and name hn   )( resid   77 and name hg12 ) 0.0 0.0 5.0
assign ( resid   62 and name hn   )( resid   78 and name hb1  ) 0.0 0.0 5.0
assign ( resid   63 and name ha   )( resid   77 and name ha   ) 0.0 0.0 2.8
assign ( resid   63 and name ha   )( resid   77 and name hb   ) 0.0 0.0 5.0
assign ( resid   63 and name ha   )( resid   77 and name hg12 ) 0.0 0.0 5.0
assign ( resid   63 and name ha   )( resid   77 and name hd1# ) 0.0 0.0 5.0
assign ( resid   63 and name hg2# )( resid   77 and name ha   ) 0.0 0.0 3.5
assign ( resid   63 and name hb   )( resid   77 and name ha   ) 0.0 0.0 5.0
assign ( resid   63 and name hb   )( resid   75 and name hg#  ) 0.0 0.0 5.0
assign ( resid   63 and name hg2# )( resid   75 and name hg#  ) 0.0 0.0 5.0
assign ( resid   63 and name ha   )( resid   78 and name hn   ) 0.0 0.0 5.0
assign ( resid   63 and name hg2# )( resid   78 and name hn   ) 0.0 0.0 5.0
assign ( resid   64 and name hz3  )( resid   78 and name hb2  ) 0.0 0.0 3.5
assign ( resid   64 and name hh2  )( resid   86 and name hb1  ) 0.0 0.0 3.5
assign ( resid   64 and name hh2  )( resid   86 and name hb2  ) 0.0 0.0 3.5
assign ( resid   64 and name hh2  )( resid   86 and name he3  ) 0.0 0.0 5.0
assign ( resid   64 and name hz2  )( resid   86 and name he3  ) 0.0 0.0 5.0
assign ( resid   64 and name hz2  )( resid   86 and name hz3  ) 0.0 0.0 5.0
assign ( resid   64 and name hh2  )( resid   86 and name ha   ) 0.0 0.0 5.0
assign ( resid   64 and name hh2  )( resid   78 and name ha   ) 0.0 0.0 5.0
assign ( resid   64 and name hz3  )( resid   78 and name ha   ) 0.0 0.0 5.0
assign ( resid   64 and name hh2  )( resid   78 and name hb2  ) 0.0 0.0 5.0
assign ( resid   64 and name hz2  )( resid   86 and name hb1  ) 0.0 0.0 5.0
assign ( resid   64 and name hz2  )( resid   76 and name hb2  ) 0.0 0.0 5.0
assign ( resid   64 and name hz2  )( resid   76 and name hb1  ) 0.0 0.0 5.0
assign ( resid   64 and name he3  )( resid   76 and name hb1  ) 0.0 0.0 5.0
assign ( resid   64 and name hz2  )( resid   86 and name hb2  ) 0.0 0.0 5.0
assign ( resid   64 and name hz2  )( resid   78 and name ha   ) 0.0 0.0 6.0
assign ( resid   64 and name hn   )( resid   75 and name hb1  ) 0.0 0.0 5.0
assign ( resid   64 and name he1  )( resid   76 and name hb2  ) 0.0 0.0 5.0
assign ( resid   64 and name hn   )( resid   76 and name hb1  ) 0.0 0.0 5.0
assign ( resid   64 and name hn   )( resid   76 and name hn   ) 0.0 0.0 3.5
assign ( resid   64 and name he3  )( resid   78 and name hn   ) 0.0 0.0 5.0
assign ( resid   64 and name hh2  )( resid   86 and name hz3  ) 0.0 0.0 6.0
assign ( resid   65 and name ha   )( resid   75 and name ha   ) 0.0 0.0 2.8
assign ( resid   65 and name hb#  )( resid   73 and name hg   ) 0.0 0.0 5.0
assign ( resid   65 and name hb#  )( resid   73 and name hd2# ) 0.0 0.0 2.8
assign ( resid   65 and name hb#  )( resid   73 and name hd1# ) 0.0 0.0 3.5
assign ( resid   65 and name ha   )( resid   73 and name hd2# ) 0.0 0.0 5.0
assign ( resid   65 and name ha   )( resid   75 and name hb1  ) 0.0 0.0 5.0
assign ( resid   65 and name ha   )( resid   75 and name hb2  ) 0.0 0.0 5.0
assign ( resid   65 and name ha   )( resid   76 and name hn   ) 0.0 0.0 5.0
assign ( resid   65 and name hb#  )( resid   76 and name hn   ) 0.0 0.0 5.0
assign ( resid   66 and name hn   )( resid   74 and name hn   ) 0.0 0.0 3.5
assign ( resid   66 and name hd#  )( resid   74 and name hd2# ) 0.0 0.0 6.0
assign ( resid   67 and name ha   )( resid   73 and name ha   ) 0.0 0.0 2.8
assign ( resid   67 and name ha   )( resid   73 and name hg   ) 0.0 0.0 5.0
assign ( resid   67 and name ha   )( resid   73 and name hd2# ) 0.0 0.0 5.0
assign ( resid   67 and name ha   )( resid   73 and name hd1# ) 0.0 0.0 5.0
assign ( resid   67 and name hd#  )( resid   73 and name ha   ) 0.0 0.0 5.0
```

```
assign ( resid   67 and name ha   )( resid   74 and name hn   ) 0.0 0.0 5.0
assign ( resid   67 and name hg1  )( resid   73 and name hd2# ) 0.0 0.0 2.8
assign ( resid   68 and name hb2  )( resid   74 and name hd2# ) 0.0 0.0 5.0
assign ( resid   68 and name hb2  )( resid   74 and name hd1# ) 0.0 0.0 5.0
assign ( resid   68 and name hn   )( resid   73 and name ha   ) 0.0 0.0 5.0
assign ( resid   68 and name hb1  )( resid   74 and name hd1# ) 0.0 0.0 3.5
assign ( resid   68 and name hb1  )( resid   74 and name hd2# ) 0.0 0.0 3.5
assign ( resid   68 and name ha   )( resid   74 and name hd2# ) 0.0 0.0 5.0
assign ( resid   72 and name hb2  )( resid   90 and name hd#  ) 0.0 0.0 5.0
assign ( resid   72 and name hb1  )( resid   90 and name hd#  ) 0.0 0.0 5.0
assign ( resid   72 and name ha   )( resid   91 and name hn   ) 0.0 0.0 3.5
assign ( resid   72 and name ha   )( resid   92 and name hn   ) 0.0 0.0 2.8
assign ( resid   72 and name ha   )( resid   93 and name hn   ) 0.0 0.0 5.0
assign ( resid   74 and name ha   )( resid   90 and name ha   ) 0.0 0.0 2.8
assign ( resid   74 and name hd2# )( resid   88 and name ha   ) 0.0 0.0 5.0
assign ( resid   74 and name ha   )( resid   88 and name hb1  ) 0.0 0.0 5.0
assign ( resid   74 and name hd1# )( resid   90 and name ha   ) 0.0 0.0 3.5
assign ( resid   74 and name hd1# )( resid   88 and name hb2  ) 0.0 0.0 3.5
assign ( resid   74 and name hd2# )( resid   88 and name hb2  ) 0.0 0.0 3.5
assign ( resid   74 and name hb1  )( resid   88 and name hb1  ) 0.0 0.0 3.5
assign ( resid   74 and name ha   )( resid   91 and name hn   ) 0.0 0.0 5.0
assign ( resid   74 and name hd2# )( resid   90 and name hn   ) 0.0 0.0 5.0
assign ( resid   74 and name hd1# )( resid   91 and name hn   ) 0.0 0.0 5.0
assign ( resid   74 and name hd1# )( resid   90 and name hn   ) 0.0 0.0 5.0
assign ( resid   74 and name hb1  )( resid   89 and name hn   ) 0.0 0.0 5.0
assign ( resid   74 and name hb2  )( resid   88 and name hb1  ) 0.0 0.0 5.0
assign ( resid   74 and name hg   )( resid   91 and name hn   ) 0.0 0.0 5.0
assign ( resid   75 and name hn   )( resid   90 and name ha   ) 0.0 0.0 5.0
assign ( resid   75 and name hn   )( resid   89 and name hn   ) 0.0 0.0 3.5
assign ( resid   76 and name ha   )( resid   88 and name ha   ) 0.0 0.0 2.8
assign ( resid   76 and name hb2  )( resid   86 and name hd1  ) 0.0 0.0 3.5
assign ( resid   76 and name hb1  )( resid   86 and name hd1  ) 0.0 0.0 5.0
assign ( resid   76 and name ha   )( resid   88 and name hb1  ) 0.0 0.0 5.0
assign ( resid   76 and name ha   )( resid   88 and name hb2  ) 0.0 0.0 5.0
assign ( resid   76 and name hb2  )( resid   88 and name ha   ) 0.0 0.0 5.0
assign ( resid   76 and name hb1  )( resid   88 and name ha   ) 0.0 0.0 5.0
assign ( resid   76 and name ha   )( resid   86 and name hd1  ) 0.0 0.0 5.0
assign ( resid   76 and name ha   )( resid   89 and name hn   ) 0.0 0.0 3.5
assign ( resid   77 and name hn   )( resid   88 and name ha   ) 0.0 0.0 5.0
assign ( resid   77 and name hg12 )( resid   87 and name hn   ) 0.0 0.0 3.5
assign ( resid   78 and name ha   )( resid   86 and name ha   ) 0.0 0.0 3.5
assign ( resid   78 and name ha   )( resid   86 and name hd1  ) 0.0 0.0 5.0
assign ( resid   78 and name ha   )( resid   86 and name he3  ) 0.0 0.0 5.0
assign ( resid   78 and name ha   )( resid   86 and name hb2  ) 0.0 0.0 3.5
assign ( resid   78 and name ha   )( resid   86 and name hb1  ) 0.0 0.0 3.5
assign ( resid   78 and name hb1  )( resid   86 and name hb2  ) 0.0 0.0 5.0
assign ( resid   79 and name hg2# )( resid   86 and name ha   ) 0.0 0.0 5.0
assign ( resid   79 and name hn   )( resid   86 and name ha   ) 0.0 0.0 3.5
assign ( resid   79 and name hn   )( resid   86 and name hb1  ) 0.0 0.0 5.0
assign ( resid   79 and name hg2# )( resid   87 and name hn   ) 0.0 0.0 5.0
assign ( resid   80 and name hn   )( resid   86 and name ha   ) 0.0 0.0 5.0
assign ( resid   80 and name ha1  )( resid   85 and name hn   ) 0.0 0.0 5.0
assign ( resid   80 and name ha2  )( resid   85 and name hn   ) 0.0 0.0 5.0
assign ( resid   80 and name hn   )( resid   85 and name hn   ) 0.0 0.0 5.0
```

H-bonds

```
assign (resid   32 and   name O    )(resid   42 and   name N    ) 0.0 0.0 3.2
assign (resid   32 and   name O    )(resid   42 and   name HN   ) 0.0 0.0 2.3
assign (resid   58 and   name O    )(resid   49 and   name N    ) 0.0 0.0 3.2
assign (resid   58 and   name O    )(resid   49 and   name HN   ) 0.0 0.0 2.3
assign (resid   76 and   name O    )(resid   64 and   name N    ) 0.0 0.0 3.2
assign (resid   76 and   name O    )(resid   64 and   name HN   ) 0.0 0.0 2.3
assign (resid   66 and   name O    )(resid   74 and   name N    ) 0.0 0.0 3.2
assign (resid   66 and   name O    )(resid   74 and   name HN   ) 0.0 0.0 2.3
assign (resid   87 and   name O    )(resid   77 and   name N    ) 0.0 0.0 3.2
assign (resid   87 and   name O    )(resid   77 and   name HN   ) 0.0 0.0 2.4
```

CDIH restraints

```
f5,  8.924/9.087
assign  (resid  4 and name c ) (resid  5 and name n)
       (resid  5 and name ca) (resid  5 and name c) 1 -130.0 40 2
d6,  9.109
assign  (resid  5 and name c ) (resid  6 and name n)
       (resid  6 and name ca) (resid  6 and name c) 1 -120.0 40 2
s12, 8.715
assign  (resid 11 and name c ) (resid 12 and name n)
       (resid 12 and name ca) (resid 12 and name c) 1 -120.0 40 2

y13, 9.917
assign  (resid 12 and name c ) (resid 13 and name n)
       (resid 13 and name ca) (resid 13 and name c) 1 -120.0 40 2
t18, 9.79
assign  (resid 17 and name c ) (resid 18 and name n)
       (resid 18 and name ca) (resid 18 and name c) 1 -110.0 40 2
k21, 8.174/8.373
assign  (resid 20 and name c ) (resid 21 and name n)
       (resid 21 and name ca) (resid 21 and name c) 1 -110.0 40 2
m28, 9.164/9.438
assign  (resid 27 and name c ) (resid 28 and name n)
       (resid 28 and name ca) (resid 28 and name c) 1 -120.0 40 2
v29, 9.426/9.516
assign  (resid 28 and name c ) (resid 29 and name n)
       (resid 29 and name ca) (resid 29 and name c) 1 -120.0 40 2
d30, 9.51/9.029
assign  (resid 29 and name c ) (resid 30 and name n)
       (resid 30 and name ca) (resid 30 and name c) 1 -110.0 40 2
c31, 8.995/9.199
assign  (resid 30 and name c ) (resid 31 and name n)
       (resid 31 and name ca) (resid 31 and name c) 1 -120.0 40 2
i41, 8.709/9.005
assign  (resid 40 and name c ) (resid 41 and name n)
       (resid 41 and name ca) (resid 41 and name c) 1 -120.0 40 2
c49, 9.626
assign  (resid 48 and name c ) (resid 49 and name n)
       (resid 49 and name ca) (resid 49 and name c) 1 -120 40 2
t56, 9.223
assign  (resid 55 and name c ) (resid 56 and name n)
       (resid 56 and name ca) (resid 56 and name c) 1 -130 40 2
t63, 9.587
assign  (resid 62 and name c ) (resid 63 and name n)
       (resid 63 and name ca) (resid 63 and name c) 1 -120 40 2
s65, 9.507/9.64
assign  (resid 64 and name c ) (resid 65 and name n)
       (resid 65 and name ca) (resid 65 and name c) 1 -120 40 2
k66, 8.88
assign  (resid 65 and name c ) (resid 66 and name n)
       (resid 66 and name ca) (resid 66 and name c) 1 -130 40 2
k67, 8.801/8.952
assign  (resid 66 and name c ) (resid 67 and name n)
       (resid 67 and name ca) (resid 67 and name c) 1 -110 40 2
174, 9.222/9.272
assign  (resid 73 and name c ) (resid 74 and name n)
       (resid 74 and name ca) (resid 74 and name c) 1 -120 40 2
q75, 9.342
assign  (resid 74 and name c ) (resid 75 and name n)
       (resid 75 and name ca) (resid 75 and name c) 1 -110 40 2
c88, 8.857/9.251
assign  (resid 87 and name c ) (resid 88 and name n)
       (resid 88 and name ca) (resid 88 and name c) 1 -130 40 2
e89, 8.962
assign  (resid 88 and name c ) (resid 89 and name n)
       (resid 89 and name ca) (resid 89 and name c) 1 -120 40 2
```

ISAC restraints

```
assign ( resid 5    and name N   )
       ( resid 5    and name HN  )   10.4    0.5
assign ( resid 10   and name N   )
```

```
       ( resid 10   and name HN  )   14.6    0.5
assign ( resid 13   and name N   )
       ( resid 13   and name HN  )   5.3    0.5
assign ( resid 18   and name N   )
       ( resid 18   and name HN  )   17.9    0.5
assign ( resid 19   and name N   )
       ( resid 19   and name HN  )  15.35    0.5
assign ( resid 21   and name N   )
       ( resid 21   and name HN  )  10.6    0.5
assign ( resid 27   and name N   )
       ( resid 27   and name HN  )   -1.6    0.5
assign ( resid 28   and name N   )
       ( resid 28   and name HN  )   -7.4    0.5
assign ( resid 29   and name N   )
       ( resid 29   and name HN  )   6.5    0.5
assign ( resid 30   and name N   )
       ( resid 30   and name HN  )   12.4    0.5
assign ( resid 31   and name N   )
       ( resid 31   and name HN  )   15.8    0.5
assign ( resid 34   and name N   )
       ( resid 34   and name HN  )   10.95    0.5
assign ( resid 50   and name N   )
       ( resid 50   and name HN  )   13.8    0.5
assign ( resid 55   and name N   )
       ( resid 55   and name HN  )   15.1    0.5
assign ( resid 58   and name N   )
       ( resid 58   and name HN  )   15.9    0.5
assign ( resid 59   and name N   )
       ( resid 59   and name HN  )  -2.7    0.5
assign ( resid 63   and name N   )
       ( resid 63   and name HN  )   18.25    0.5
assign ( resid 64   and name N   )
       ( resid 64   and name HN  )   16.2    0.5
assign ( resid 65   and name N   )
       ( resid 65   and name HN  )   8.7    0.5
assign ( resid 66   and name N   )
       ( resid 66   and name HN  )  -2.3    0.5
assign ( resid 76   and name N   )
       ( resid 76   and name HN  )  15.3    0.5
assign ( resid 88   and name N   )
       ( resid 88   and name HN  )  19.1    0.5
```

DANI restraints

```
assign ( resid 6    and name N   )
       ( resid 6    and name HN  )  3.43    0.328
assign ( resid 7    and name N   )
       ( resid 7    and name HN  )  3.25    0.237
assign ( resid 10   and name N   )
       ( resid 10   and name HN  )  3.41    0.262
assign ( resid 13   and name N   )
       ( resid 13   and name HN  )  3.52    0.343
assign ( resid 18   and name N   )
       ( resid 18   and name HN  )  3.84    0.35
assign ( resid 21   and name N   )
       ( resid 21   and name HN  )  3.46    0.312
assign ( resid 27   and name N   )
       ( resid 27   and name HN  )  3.64    0.31
assign ( resid 29   and name N   )
       ( resid 29   and name HN  )  3.87    0.492
assign ( resid 30   and name N   )
       ( resid 30   and name HN  )  3.89    0.46
assign ( resid 31   and name N   )
       ( resid 31   and name HN  )  3.68    0.476
assign ( resid 32   and name N   )
       ( resid 32   and name HN  )  3.5    0.232
assign ( resid 34   and name N   )
       ( resid 34   and name HN  )  3.35    0.529
assign ( resid 41   and name N   )
       ( resid 41   and name HN  )  4.15    0.483
assign ( resid 44   and name N   )
```

```
       ( resid 44   and name HN  )   3.39    0.32
assign ( resid 48   and name N   )
       ( resid 48   and name HN  )   3.63    0.29
assign ( resid 51   and name N   )
       ( resid 51   and name HN  )   3.63    0.487
assign ( resid 52   and name N   )
       ( resid 52   and name HN  )   3.32    0.265
assign ( resid 55   and name N   )
       ( resid 55   and name HN  )   4.07    0.438
assign ( resid 56   and name N   )
       ( resid 56   and name HN  )   4.15    0.355
assign ( resid 58   and name N   )
       ( resid 58   and name HN  )   3.71    0.482
assign ( resid 59   and name N   )
       ( resid 59   and name HN  )   4.06    0.559
assign ( resid 63   and name N   )
       ( resid 63   and name HN  )   3.47    0.361
assign ( resid 64   and name N   )
       ( resid 64   and name HN  )   3.45    0.313
assign ( resid 66   and name N   )
       ( resid 66   and name HN  )   3.7     0.285
assign ( resid 67   and name N   )
       ( resid 67   and name HN  )   3.98    0.317
assign ( resid 68   and name N   )
       ( resid 68   and name HN  )   4.19    0.35
assign ( resid 74   and name N   )
       ( resid 74   and name HN  )   3.54    0.452
assign ( resid 76   and name N   )
       ( resid 76   and name HN  )   3.69    0.475
assign ( resid 77   and name N   )
       ( resid 77   and name HN  )   3.52    0.383
assign ( resid 78   and name N   )
       ( resid 78   and name HN  )   3.43    0.519
assign ( resid 79   and name N   )
       ( resid 79   and name HN  )   3.88    0.539
assign ( resid 86   and name N   )
       ( resid 86   and name HN  )   3.93    0.327
assign ( resid 87   and name N   )
       ( resid 87   and name HN  )   3.74    0.41
assign ( resid 88   and name N   )
       ( resid 88   and name HN  )   3.37    0.296
assign ( resid 89   and name N   )
       ( resid 89   and name HN  )   3.97    0.365
```

APPENDIX 3B

Restraints not found in the present spectra of free $^{4}F1^{5}F1$ (in comparison to Williams, 1992 - * indicates NOEs present in NOESY recorded with the longer mixing times).

```
assign ( resid  1 and name   ha ) ( resid 14 and name hg1# ) 0.0 1.5 6.5*
assign ( resid  1 and name   ha ) ( resid 14 and name hg2# ) 0.0 1.5 6.5*
assign ( resid  2 and name   hn ) ( resid  2 and name  hg2 ) 0.0 0.0 5.0
assign ( resid  2 and name  hb# ) ( resid 15 and name   hn ) 0.0 1.0 6.0
assign ( resid  2 and name  hg# ) ( resid 35 and name  ha2 ) 0.0 1.0 6.0
assign ( resid  3 and name   hn ) ( resid  3 and name  he# ) 0.0 1.0 6.0
assign ( resid  3 and name   ha ) ( resid 13 and name   hn ) 0.0 0.0 5.0
assign ( resid  3 and name  hb# ) ( resid 15 and name   hn ) 0.0 1.0 6.0
assign ( resid  3 and name  hg# ) ( resid 15 and name   hn ) 0.0 1.0 6.0
assign ( resid  3 and name  he# ) ( resid 14 and name   ha ) 0.0 1.0 6.0
assign ( resid  5 and name   hn ) ( resid  6 and name   hn ) 0.0 0.0 5.0
assign ( resid  5 and name  he# ) ( resid  7 and name  hd2 ) 0.0 2.0 7.0
assign ( resid  5 and name  hd# ) ( resid 11 and name hg2# ) 0.0 3.5 8.5
assign ( resid  6 and name  hb# ) ( resid  9 and name   hn ) 0.0 1.0 6.0
assign ( resid  7 and name   hn ) ( resid  8 and name   hn ) 0.0 0.0 5.0
assign ( resid  7 and name   ha ) ( resid 11 and name   hn ) 0.0 0.0 5.0
assign ( resid  8 and name   hn ) ( resid 10 and name   hn ) 0.0 0.0 5.0
assign ( resid  8 and name  hb# ) ( resid 10 and name   hn ) 0.0 1.5 6.5
assign ( resid  9 and name   hn ) ( resid 10 and name  ha1 ) 0.0 0.0 5.0
assign ( resid  9 and name   hn ) ( resid 10 and name  ha2 ) 0.0 0.0 5.0
assign ( resid 11 and name   ha ) ( resid 13 and name  hd# ) 0.0 2.0 7.0
assign ( resid 12 and name   hn ) ( resid 13 and name  hd# ) 0.0 2.0 7.0
assign ( resid 12 and name   hn ) ( resid 13 and name  he# ) 0.0 2.0 7.0
assign ( resid 13 and name   hn ) ( resid 13 and name  he# ) 0.0 2.0 7.0
assign ( resid 13 and name   hn ) ( resid 14 and name   hn ) 0.0 0.0 5.0
assign ( resid 13 and name   ha ) ( resid 17 and name  hb1 ) 0.0 0.0 5.0
assign ( resid 13 and name   ha ) ( resid 17 and name  hb2 ) 0.0 0.0 5.0*
assign ( resid 13 and name  hd# ) ( resid 17 and name   ha ) 0.0 2.0 7.0*
assign ( resid 13 and name   ha ) ( resid 19 and name  hz3 ) 0.0 0.0 5.0
assign ( resid 13 and name  hd# ) ( resid 19 and name   ha ) 0.0 2.0 7.0*
assign ( resid 13 and name  hd# ) ( resid 19 and name  hd1 ) 0.0 2.0 7.0*
assign ( resid 14 and name   hb ) ( resid 17 and name  hb# ) 0.0 1.0 6.0
assign ( resid 17 and name  hb2 ) ( resid 18 and name   ha ) 0.0 0.0 5.0*
assign ( resid 18 and name   hn ) ( resid 19 and name  he3 ) 0.0 0.0 5.0
assign ( resid 18 and name hg2# ) ( resid 33 and name   hn ) 0.0 1.5 6.5
assign ( resid 19 and name   hn ) ( resid 19 and name  hd1 ) 0.0 0.0 5.0
assign ( resid 19 and name  hz2 ) ( resid 33 and name   ha ) 0.0 0.0 5.0*
assign ( resid 19 and name  hz3 ) ( resid 32 and name   hn ) 0.0 0.0 5.0
assign ( resid 20 and name   hn ) ( resid 21 and name   hn ) 0.0 0.0 5.0
assign ( resid 20 and name   hn ) ( resid 31 and name   hn ) 0.0 0.0 5.0
assign ( resid 21 and name   hn ) ( resid 31 and name   hn ) 0.0 0.0 5.0
assign ( resid 23 and name  hd# ) ( resid 24 and name   ha ) 0.0 2.0 7.0*
assign ( resid 23 and name  he# ) ( resid 24 and name   hn ) 0.0 2.0 7.0*
assign ( resid 23 and name  he# ) ( resid 24 and name   ha ) 0.0 2.0 7.0
assign ( resid 23 and name  hd# ) ( resid 27 and name   ha ) 0.0 2.0 7.0*
assign ( resid 23 and name   hn ) ( resid 28 and name   hn ) 0.0 0.0 5.0
assign ( resid 23 and name  hb# ) ( resid 29 and name   hn ) 0.0 1.0 6.0
assign ( resid 25 and name   hn ) ( resid 26 and name  hd1 ) 0.0 0.0 5.0
assign ( resid 26 and name   hn ) ( resid 27 and name  hg# ) 0.0 1.0 6.0
assign ( resid 26 and name  he1 ) ( resid 27 and name  hg# ) 0.0 1.0 6.0
assign ( resid 26 and name  he3 ) ( resid 27 and name  he# ) 0.0 1.5 6.5*
assign ( resid 26 and name  he1 ) ( resid 52 and name   ha ) 0.0 0.0 5.0*
assign ( resid 26 and name  he1 ) ( resid 55 and name  hg2 ) 0.0 0.0 5.0*
assign ( resid 26 and name  hz3 ) ( resid 56 and name hg2# ) 0.0 1.5 6.5*
assign ( resid 26 and name  hh2 ) ( resid 57 and name   ha ) 0.0 0.0 5.0*
assign ( resid 28 and name   hn ) ( resid 29 and name   hn ) 0.0 0.0 5.0
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assign ( resid 28 and name  hg1 ) ( resid 29 and name   hn ) 0.0 0.0 5.0
assign ( resid 28 and name  hg2 ) ( resid 29 and name   hn ) 0.0 0.0 5.0
assign ( resid 29 and name  hg# ) ( resid 44 and name   ha ) 0.0 2.4 7.4*
assign ( resid 29 and name  hg# ) ( resid 44 and name   hb ) 0.0 2.4 7.4*
assign ( resid 34 and name  hb# ) ( resid 36 and name  hb# ) 0.0 2.0 7.0*
assign ( resid 34 and name   hn ) ( resid 42 and name   hn ) 0.0 0.0 5.0
assign ( resid 34 and name   ha ) ( resid 42 and name hg2# ) 0.0 1.5 6.5*
assign ( resid 46 and name   hn ) ( resid 48 and name   hn ) 0.0 0.0 5.0
assign ( resid 46 and name   ha ) ( resid 83 and name  hb2 ) 0.0 0.0 5.0*
assign ( resid 47 and name  hb1 ) ( resid 48 and name   hn ) 0.0 0.0 5.0
assign ( resid 47 and name   ha ) ( resid 49 and name   hn ) 0.0 0.0 5.0
assign ( resid 47 and name  hb2 ) ( resid 48 and name   hn ) 0.0 0.0 5.0
assign ( resid 48 and name   ha ) ( resid 59 and name   ha ) 0.0 0.0 5.0*
assign ( resid 48 and name   ha ) ( resid 59 and name  hd# ) 0.0 1.0 6.0*
assign ( resid 48 and name  hd2 ) ( resid 59 and name   ha ) 0.0 0.0 3.5*
assign ( resid 51 and name  hb1 ) ( resid 86 and name  hh2 ) 0.0 0.0 5.0*
assign ( resid 52 and name   hn ) ( resid 54 and name   ha ) 0.0 0.0 5.0
assign ( resid 52 and name   ha ) ( resid 55 and name  hg2 ) 0.0 0.0 5.0*
assign ( resid 53 and name   hn ) ( resid 54 and name   ha ) 0.0 0.0 5.0
assign ( resid 54 and name   hn ) ( resid 56 and name   hn ) 0.0 0.0 5.0
assign ( resid 55 and name   hn ) ( resid 55 and name  hb1 ) 0.0 0.0 5.0
assign ( resid 55 and name  hg# ) ( resid 56 and name   hb ) 0.0 1.0 6.0*
assign ( resid 55 and name  hb# ) ( resid 56 and name   hn ) 0.0 1.0 6.0
assign ( resid 57 and name  hb# ) ( resid 58 and name   hn ) 0.0 1.0 6.0
assign ( resid 58 and name   hn ) ( resid 58 and name  he# ) 0.0 2.0 7.0
assign ( resid 58 and name   ha ) ( resid 62 and name  hb1 ) 0.0 0.0 5.0*
assign ( resid 58 and name  he# ) ( resid 63 and name   ha ) 0.0 2.0 7.0
assign ( resid 58 and name  he# ) ( resid 64 and name  hz3 ) 0.0 2.0 7.0*
assign ( resid 58 and name  he# ) ( resid 65 and name   hn ) 0.0 2.0 7.0
assign ( resid 61 and name  ha# ) ( resid 62 and name   ha ) 0.0 1.0 6.0*
assign ( resid 62 and name   hn ) ( resid 63 and name   hn ) 0.0 0.0 5.0
assign ( resid 63 and name   hn ) ( resid 64 and name  he3 ) 0.0 0.0 5.0
assign ( resid 63 and name   hn ) ( resid 77 and name   ha ) 0.0 0.0 5.0
assign ( resid 64 and name   hn ) ( resid 64 and name  hz3 ) 0.0 0.0 5.0
assign ( resid 64 and name  hd1 ) ( resid 66 and name   ha ) 0.0 0.0 5.0
assign ( resid 64 and name  he3 ) ( resid 76 and name  hb2 ) 0.0 0.0 5.0*
assign ( resid 64 and name  he3 ) ( resid 77 and name  hd# ) 0.0 1.5 6.5*
assign ( resid 64 and name  hh2 ) ( resid 78 and name   hn ) 0.0 0.0 5.0
assign ( resid 66 and name   hn ) ( resid 76 and name   hn ) 0.0 0.0 5.0
assign ( resid 66 and name   ha ) ( resid 73 and name  hd# ) 0.0 2.4 7.4
assign ( resid 67 and name  hd# ) ( resid 68 and name   hn ) 0.0 1.0 6.0
assign ( resid 68 and name   hn ) ( resid 71 and name   hn ) 0.0 0.0 5.0
assign ( resid 69 and name   hn ) ( resid 71 and name   hn ) 0.0 0.0 3.5
assign ( resid 69 and name   hn ) ( resid 72 and name   hn ) 0.0 0.0 5.0
assign ( resid 72 and name   hn ) ( resid 73 and name   hn ) 0.0 0.0 5.0
assign ( resid 72 and name  hb1 ) ( resid 74 and name hd2# ) 0.0 1.5 6.5
assign ( resid 73 and name   hn ) ( resid 73 and name hd1# ) 0.0 1.5 6.5
assign ( resid 73 and name  hd# ) ( resid 74 and name   ha ) 0.0 2.4 7.4*
assign ( resid 73 and name   hn ) ( resid 91 and name  hd1 ) 0.0 1.5 6.5
assign ( resid 73 and name   hn ) ( resid 91 and name  hd2 ) 0.0 2.4 7.4
assign ( resid 73 and name hd1# ) ( resid 91 and name  hd2 ) 0.0 1.5 6.5
assign ( resid 73 and name hd2# ) ( resid 91 and name  hd2 ) 0.0 2.4 7.4
assign ( resid 74 and name   hn ) ( resid 75 and name   hn ) 0.0 0.0 5.0
assign ( resid 74 and name hd1# ) ( resid 88 and name   ha ) 0.0 1.5 6.5*
assign ( resid 75 and name  hb1 ) ( resid 91 and name  hd2 ) 0.0 0.0 5.0
assign ( resid 75 and name  hb2 ) ( resid 91 and name  hd2 ) 0.0 0.0 5.0
assign ( resid 77 and name   hn ) ( resid 77 and name hg2# ) 0.0 1.5 5.0
assign ( resid 77 and name hg2# ) ( resid 79 and name   ha ) 0.0 1.5 5.0
assign ( resid 77 and name hg2# ) ( resid 79 and name   hb ) 0.0 1.5 6.5*
assign ( resid 79 and name   hb ) ( resid 85 and name  hb# ) 0.0 1.0 6.0*
assign ( resid 79 and name hg2# ) ( resid 86 and name  hd1 ) 0.0 1.5 6.5*
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assign ( resid 81 and name   hn ) ( resid 81 and name  hd# ) 0.0 1.0 6.0
assign ( resid 81 and name  hb# ) ( resid 82 and name   hn ) 0.0 1.0 6.0
assign ( resid 85 and name  hg# ) ( resid 86 and name  hd1 ) 0.0 1.0 6.0*
assign ( resid 87 and name   hn ) ( resid 88 and name   hn ) 0.0 0.0 5.0
assign ( resid 87 and name  hd# ) ( resid 88 and name   hn ) 0.0 1.0 6.0
assign ( resid 91 and name   hn ) ( resid 93 and name   hn ) 0.0 0.0 5.0
assign ( resid 92 and name   ha ) ( resid 93 and name   hn ) 0.0 0.0 3.5
```

* indicates NOEs present in either 150ms NOESY in D_2O or 200ms NOESY in water.

APPENDIX 3C

Additional restraints for free $^{4}F1^{5}F1$ (in comparison to Williams, 1992).

```
assign ( resid  5 and name  he# ) ( resid  4 and name   ha ) 0.0 0.0 6.0
assign ( resid  5 and name   hz ) ( resid 10 and name  ha1 ) 0.0 0.0 6.0
assign ( resid 12 and name   hn ) ( resid  5 and name   ha ) 0.0 0.0 6.0
assign ( resid 13 and name   hn ) ( resid  3 and name  hb# ) 0.0 0.0 5.0
assign ( resid 13 and name   hn ) ( resid  3 and name  hd# ) 0.0 0.0 5.0
assign ( resid 13 and name  hd# ) ( resid 12 and name   ha ) 0.0 0.0 3.5
assign ( resid 13 and name  hd# ) ( resid  4 and name  hb2 ) 0.0 0.0 5.0
assign ( resid 13 and name  hd# ) ( resid  6 and name  hb2 ) 0.0 0.0 5.0
assign ( resid 13 and name  hd# ) ( resid  4 and name  hb1 ) 0.0 0.0 5.0
assign ( resid 13 and name  hd# ) ( resid 19 and name  hb2 ) 0.0 0.0 3.5
assign ( resid 13 and name  he# ) ( resid 19 and name  hb1 ) 0.0 0.0 3.5
assign ( resid 13 and name  he# ) ( resid 19 and name  hb2 ) 0.0 0.0 3.5
assign ( resid 15 and name hg1# ) ( resid 16 and name  ha2 ) 0.0 0.0 5.0
assign ( resid 15 and name hg2# ) ( resid  2 and name  hb# ) 0.0 0.0 2.8
assign ( resid 15 and name hg2# ) ( resid 35 and name  ha1 ) 0.0 0.0 3.5
assign ( resid 15 and name hg1# ) ( resid 34 and name   ha ) 0.0 0.0 5.0
assign ( resid 15 and name hg2# ) ( resid 34 and name   ha ) 0.0 0.0 5.0
assign ( resid 15 and name hg2# ) ( resid  2 and name   ha ) 0.0 0.0 5.0
assign ( resid 15 and name hg2# ) ( resid 35 and name  ha2 ) 0.0 0.0 3.5
assign ( resid 15 and name hg2# ) ( resid  2 and name  hg2 ) 0.0 0.0 5.0
assign ( resid 15 and name hg2# ) ( resid 16 and name   hn ) 0.0 0.0 5.0
assign ( resid 19 and name  he3 ) ( resid 17 and name  hb1 ) 0.0 0.0 5.0
assign ( resid 19 and name  he3 ) ( resid 31 and name  hb1 ) 0.0 0.0 6.0
assign ( resid 19 and name  he3 ) ( resid 17 and name  hb2 ) 0.0 0.0 5.0
assign ( resid 23 and name  hb2 ) ( resid 24 and name  hg2 ) 0.0 0.0 6.0
assign ( resid 23 and name  he# ) ( resid 27 and name  hg# ) 0.0 0.0 5.0
assign ( resid 23 and name  he# ) ( resid 24 and name  hg1 ) 0.0 0.0 5.0
assign ( resid 23 and name  he# ) ( resid 27 and name  hb2 ) 0.0 0.0 5.0
assign ( resid 28 and name  hg2 ) ( resid 22 and name  hd2 ) 0.0 0.0 5.0
assign ( resid 29 and name hg1# ) ( resid 23 and name  hb2 ) 0.0 0.0 3.5
assign ( resid 29 and name hg2# ) ( resid 23 and name  hb1 ) 0.0 0.0 5.0
assign ( resid 29 and name   hn ) ( resid 28 and name  hb# ) 0.0 0.0 5.0
assign ( resid 29 and name   hn ) ( resid 29 and name   hb ) 0.0 0.0 5.0
assign ( resid 34 and name hd1# ) ( resid 42 and name   hb ) 0.0 0.0 3.5
assign ( resid 34 and name hd2# ) ( resid 42 and name   hb ) 0.0 0.0 3.5
assign ( resid 34 and name hd1# ) ( resid 32 and name   hb ) 0.0 0.0 3.5
assign ( resid 34 and name hd1# ) ( resid 34 and name   ha ) 0.0 0.0 2.8
assign ( resid 34 and name hd2# ) ( resid 36 and name  hg2 ) 0.0 0.0 5.0
assign ( resid 34 and name hd2# ) ( resid 42 and name hg2# ) 0.0 0.0 2.8
assign ( resid 34 and name   hg ) ( resid 34 and name   ha ) 0.0 0.0 3.5
assign ( resid 34 and name  hb# ) ( resid 42 and name   hb ) 0.0 0.0 5.0
assign ( resid 34 and name hd1# ) ( resid 34 and name   hn ) 0.0 0.0 5.0
assign ( resid 35 and name  ha1 ) ( resid 39 and name  ha1 ) 0.0 0.0 5.0
assign ( resid 35 and name   hn ) ( resid 15 and name hg2# ) 0.0 0.0 5.0
assign ( resid 35 and name   hn ) ( resid 34 and name hd1# ) 0.0 0.0 6.0
assign ( resid 35 and name   hn ) ( resid 34 and name hd2# ) 0.0 0.0 6.0
assign ( resid 35 and name   hn ) ( resid 34 and name  hb# ) 0.0 0.0 3.5
assign ( resid 36 and name   hn ) ( resid 15 and name hg2# ) 0.0 0.0 6.0
assign ( resid 36 and name   hn ) ( resid 36 and name  hb# ) 0.0 0.0 5.0
assign ( resid 36 and name   hn ) ( resid 36 and name  hg1 ) 0.0 0.0 5.0
assign ( resid 37 and name   hn ) ( resid 36 and name  hb# ) 0.0 0.0 5.0
assign ( resid 41 and name hg1# ) ( resid  4 and name  hb2 ) 0.0 0.0 2.8
assign ( resid 41 and name hg2# ) ( resid 43 and name   ha ) 0.0 0.0 5.0
assign ( resid 42 and name   hn ) ( resid 34 and name hd1# ) 0.0 0.0 5.0
assign ( resid 42 and name   hn ) ( resid 41 and name hg1# ) 0.0 0.0 5.0
assign ( resid 42 and name   hn ) ( resid 42 and name hg2# ) 0.0 0.0 5.0
assign ( resid 44 and name hg2# ) ( resid 43 and name   ha ) 0.0 0.0 5.0
assign ( resid 46 and name   hn ) ( resid 45 and name   hn ) 0.0 0.0 5.0
```

```
assign ( resid  5 and name   hn ) ( resid  4 and name   ha ) 0.0 0.0 3.5
assign ( resid 13 and name  hd# ) ( resid 19 and name  he3 ) 0.0 0.0 5.0
assign ( resid 13 and name  hb1 ) ( resid 33 and name  hb2 ) 0.0 0.0 5.0
assign ( resid 15 and name   ha ) ( resid 33 and name   ha ) 0.0 0.0 5.0
assign ( resid 17 and name   hn ) ( resid 16 and name  ha2 ) 0.0 0.0 5.0
assign ( resid 17 and name   hn ) ( resid 17 and name   ha ) 0.0 0.0 5.0
assign ( resid 19 and name  hz2 ) ( resid 41 and name   ha ) 0.0 0.0 5.0
assign ( resid 19 and name  hz3 ) ( resid 13 and name  hb2 ) 0.0 0.0 5.0
assign ( resid 19 and name  hz3 ) ( resid 33 and name  hb2 ) 0.0 0.0 3.5
assign ( resid 19 and name  he3 ) ( resid 13 and name  hb2 ) 0.0 0.0 5.0
assign ( resid 19 and name  he3 ) ( resid 17 and name   ha ) 0.0 0.0 5.0
assign ( resid 19 and name  he3 ) ( resid 33 and name  hb2 ) 0.0 0.0 5.0
assign ( resid 19 and name  hh2 ) ( resid 32 and name   ha ) 0.0 0.0 5.0
assign ( resid 19 and name  he3 ) ( resid 17 and name  hg# ) 0.0 0.0 3.5
assign ( resid 28 and name  hb# ) ( resid 22 and name   ha ) 0.0 0.0 5.0
assign ( resid 29 and name hg2# ) ( resid 21 and name  hg# ) 0.0 0.0 5.0
assign ( resid 29 and name hg2# ) ( resid 21 and name  hb# ) 0.0 0.0 3.5
assign ( resid 29 and name hg2# ) ( resid 21 and name  he# ) 0.0 0.0 6.0
assign ( resid 36 and name   hn ) ( resid 35 and name  ha2 ) 0.0 0.0 3.5
assign ( resid 36 and name   hn ) ( resid 36 and name   ha ) 0.0 0.0 3.5
assign ( resid 41 and name hg2# ) ( resid 42 and name   ha ) 0.0 0.0 5.0
assign ( resid 41 and name hg2# ) ( resid  4 and name  hb2 ) 0.0 0.0 5.0
assign ( resid 54 and name hg2# ) ( resid 54 and name   ha ) 0.0 0.0 5.0
assign ( resid 56 and name   hn ) ( resid 51 and name   hn ) 0.0 0.0 5.0
assign ( resid 58 and name  he# ) ( resid 56 and name   hb ) 0.0 0.0 3.5
assign ( resid 58 and name  hd# ) ( resid 58 and name  hb2 ) 0.0 0.0 3.5
assign ( resid 58 and name  hd# ) ( resid 51 and name  hb2 ) 0.0 0.0 5.0
assign ( resid 58 and name  hd# ) ( resid 56 and name   hb ) 0.0 0.0 5.0
assign ( resid 64 and name  hd1 ) ( resid 66 and name  hb1 ) 0.0 0.0 5.0
assign ( resid 64 and name  hd1 ) ( resid 54 and name hg2# ) 0.0 0.0 5.0
assign ( resid 64 and name  hh2 ) ( resid 86 and name  hz3 ) 0.0 0.0 6.0
assign ( resid 64 and name  hz3 ) ( resid 62 and name  hb2 ) 0.0 0.0 5.0
assign ( resid 64 and name  he3 ) ( resid 62 and name  hb1 ) 0.0 0.0 5.0
assign ( resid 64 and name  hz3 ) ( resid 60 and name   ha ) 0.0 0.0 6.0
assign ( resid 72 and name hd21 ) ( resid 72 and name  hb2 ) 0.0 0.0 5.0
assign ( resid 74 and name hd1# ) ( resid 68 and name  hb1 ) 0.0 0.0 3.5
assign ( resid 74 and name hd2# ) ( resid 68 and name  hb1 ) 0.0 0.0 3.5
assign ( resid 74 and name hd1# ) ( resid 74 and name  hb2 ) 0.0 0.0 3.5
assign ( resid 77 and name   hn ) ( resid 77 and name hg12 ) 0.0 0.0 5.0
assign ( resid 78 and name  hb1 ) ( resid 86 and name  hb2 ) 0.0 0.0 5.0
assign ( resid 83 and name  hb1 ) ( resid 82 and name  ha1 ) 0.0 0.0 5.0
assign ( resid 86 and name  he3 ) ( resid 85 and name   ha ) 0.0 0.0 5.0
assign ( resid 89 and name   hn ) ( resid 74 and name  hb1 ) 0.0 0.0 5.0
assign ( resid 51 and name   hn ) ( resid 51 and name  hb2 ) 0.0 0.0 5.0
assign ( resid 54 and name   hn ) ( resid 54 and name   ha ) 0.0 0.0 5.0
assign ( resid 54 and name   hn ) ( resid 54 and name   hb ) 0.0 0.0 5.0
assign ( resid 54 and name hg2# ) ( resid 66 and name  hd# ) 0.0 0.0 5.0
assign ( resid 55 and name   hn ) ( resid 54 and name   ha ) 0.0 0.0 5.0
assign ( resid 55 and name   hn ) ( resid 54 and name   hb ) 0.0 0.0 5.0
assign ( resid 56 and name   hn ) ( resid 54 and name   ha ) 0.0 0.0 5.0
assign ( resid 56 and name   hn ) ( resid 54 and name   hb ) 0.0 0.0 5.0
assign ( resid 56 and name   hb ) ( resid 54 and name   hb ) 0.0 0.0 5.0
assign ( resid 64 and name  hd1 ) ( resid 54 and name   hb ) 0.0 0.0 6.0
assign ( resid 64 and name  hz2 ) ( resid 49 and name  hb2 ) 0.0 0.0 5.0
assign ( resid 64 and name  hd1 ) ( resid 66 and name  hd# ) 0.0 0.0 3.5
assign ( resid 66 and name  hd# ) ( resid 53 and name  hb1 ) 0.0 0.0 3.5
assign ( resid 67 and name   hn ) ( resid 67 and name  hg1 ) 0.0 0.0 5.0
assign ( resid 70 and name   hn ) ( resid 69 and name   ha ) 0.0 0.0 3.5
assign ( resid 70 and name   hn ) ( resid 70 and name   ha ) 0.0 0.0 3.5
assign ( resid 73 and name hd2# ) ( resid 67 and name  hg1 ) 0.0 0.0 2.8
assign ( resid 74 and name hd1# ) ( resid 74 and name   ha ) 0.0 0.0 5.0
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```
assign ( resid 74 and name hd2# ) ( resid 68 and name   ha ) 0.0 0.0 5.0
assign ( resid 74 and name hd2# ) ( resid 66 and name  hd# ) 0.0 0.0 6.0
assign ( resid 74 and name  hb2 ) ( resid 74 and name   hg ) 0.0 0.0 3.5
assign ( resid 74 and name  hb2 ) ( resid 88 and name  hb1 ) 0.0 0.0 5.0
assign ( resid 74 and name   hn ) ( resid 74 and name  hb2 ) 0.0 0.0 3.5
assign ( resid 74 and name   hn ) ( resid 74 and name   hg ) 0.0 0.0 3.5
assign ( resid 74 and name   hn ) ( resid 73 and name   ha ) 0.0 0.0 2.8
assign ( resid 75 and name   hn ) ( resid 74 and name  hb2 ) 0.0 0.0 5.0
assign ( resid 78 and name   hn ) ( resid 63 and name hg2# ) 0.0 0.0 5.0
assign ( resid 80 and name   hn ) ( resid 79 and name   ha ) 0.0 0.0 3.5
assign ( resid 80 and name   hn ) ( resid 80 and name  ha2 ) 0.0 0.0 3.5
assign ( resid 81 and name   hn ) ( resid 80 and name  ha2 ) 0.0 0.0 2.8
assign ( resid 81 and name   hn ) ( resid 81 and name   ha ) 0.0 0.0 2.8
assign ( resid 91 and name   hn ) ( resid 74 and name   hg ) 0.0 0.0 5.0
```

APPENDIX 4

Restraints for the calculation of the structure of $^{4}F1^{5}F1$ module pair in complex with D3

Distance restraints

number of intraresidue NOEs: 411
number of sequential NOEs: 295
number of medium range NOEs: 77
number of long range NOEs: 320

INTRARESIDUE NOES

```
assign ( resid    2 and name ha   )( resid    2 and name hg#  ) 0.0 0.0 5.0
assign ( resid    2 and name ha   )( resid    2 and name hn   ) 0.0 0.0 3.5
assign ( resid    2 and name hb#  )( resid    2 and name hn   ) 0.0 0.0 3.5
assign ( resid    2 and name hg#  )( resid    2 and name hn   ) 0.0 0.0 5.0
assign ( resid    3 and name he#  )( resid    3 and name hg#  ) 0.0 0.0 3.5
assign ( resid    3 and name ha   )( resid    3 and name hg#  ) 0.0 0.0 3.5
assign ( resid    3 and name ha   )( resid    3 and name hd#  ) 0.0 0.0 5.0
assign ( resid    3 and name ha   )( resid    3 and name hn   ) 0.0 0.0 3.5
assign ( resid    3 and name hb#  )( resid    3 and name hn   ) 0.0 0.0 3.5
assign ( resid    3 and name hd#  )( resid    3 and name hn   ) 0.0 0.0 3.5
assign ( resid    3 and name hg#  )( resid    3 and name hn   ) 0.0 0.0 3.5
assign ( resid    3 and name hb#  )( resid    3 and name he#  ) 0.0 0.0 5.0
assign ( resid    4 and name hb2  )( resid    4 and name hn   ) 0.0 0.0 5.0
assign ( resid    4 and name hb1  )( resid    4 and name hn   ) 0.0 0.0 3.5
assign ( resid    4 and name ha   )( resid    4 and name hn   ) 0.0 0.0 3.5
assign ( resid    5 and name hb2  )( resid    5 and name hd#  ) 0.0 0.0 3.5
assign ( resid    5 and name hb1  )( resid    5 and name hd#  ) 0.0 0.0 3.5
assign ( resid    5 and name hb2  )( resid    5 and name he#  ) 0.0 0.0 5.0
assign ( resid    5 and name ha   )( resid    5 and name hn   ) 0.0 0.0 3.5
assign ( resid    5 and name hb1  )( resid    5 and name hn   ) 0.0 0.0 3.5
assign ( resid    5 and name hb2  )( resid    5 and name hn   ) 0.0 0.0 3.5
assign ( resid    5 and name hd#  )( resid    5 and name hn   ) 0.0 0.0 5.0
assign ( resid    5 and name ha   )( resid    5 and name hd#  ) 0.0 0.0 5.0
assign ( resid    6 and name hb2  )( resid    6 and name hn   ) 0.0 0.0 3.5
assign ( resid    6 and name hb1  )( resid    6 and name hn   ) 0.0 0.0 3.5
assign ( resid    6 and name ha   )( resid    6 and name hn   ) 0.0 0.0 3.5
assign ( resid    7 and name ha   )( resid    7 and name hn   ) 0.0 0.0 3.5
assign ( resid    7 and name hb#  )( resid    7 and name hn   ) 0.0 0.0 3.5
assign ( resid    8 and name ha   )( resid    8 and name hn   ) 0.0 0.0 2.8
assign ( resid    8 and name hb#  )( resid    8 and name hn   ) 0.0 0.0 2.8
assign ( resid    9 and name ha   )( resid    9 and name hn   ) 0.0 0.0 3.5
assign ( resid    9 and name hb#  )( resid    9 and name hn   ) 0.0 0.0 2.8
assign ( resid   10 and name ha2  )( resid   10 and name hn   ) 0.0 0.0 2.8
assign ( resid   10 and name ha1  )( resid   10 and name hn   ) 0.0 0.0 2.8
assign ( resid   11 and name ha   )( resid   11 and name hg2# ) 0.0 0.0 2.8
assign ( resid   11 and name ha   )( resid   11 and name hn   ) 0.0 0.0 3.5
assign ( resid   11 and name hb   )( resid   11 and name hn   ) 0.0 0.0 3.5
assign ( resid   11 and name hg2# )( resid   11 and name hn   ) 0.0 0.0 3.5
assign ( resid   12 and name hb2  )( resid   12 and name hn   ) 0.0 0.0 3.5
assign ( resid   12 and name hb1  )( resid   12 and name hn   ) 0.0 0.0 3.5
assign ( resid   12 and name ha   )( resid   12 and name hn   ) 0.0 0.0 5.0
assign ( resid   13 and name hb1  )( resid   13 and name hd#  ) 0.0 0.0 3.5
assign ( resid   13 and name ha   )( resid   13 and name hd#  ) 0.0 0.0 5.0
assign ( resid   13 and name hb2  )( resid   13 and name hd#  ) 0.0 0.0 3.5
assign ( resid   13 and name hb1  )( resid   13 and name he#  ) 0.0 0.0 5.0
assign ( resid   13 and name hd#  )( resid   13 and name hn   ) 0.0 0.0 3.5
assign ( resid   13 and name ha   )( resid   13 and name hn   ) 0.0 0.0 5.0
assign ( resid   14 and name ha   )( resid   14 and name hg2# ) 0.0 0.0 2.8
assign ( resid   14 and name ha   )( resid   14 and name hg1# ) 0.0 0.0 3.5
assign ( resid   14 and name hg1# )( resid   14 and name hn   ) 0.0 0.0 2.8
assign ( resid   14 and name hb   )( resid   14 and name hn   ) 0.0 0.0 3.5
assign ( resid   14 and name ha   )( resid   14 and name hn   ) 0.0 0.0 3.5
assign ( resid   14 and name hg2# )( resid   14 and name hn   ) 0.0 0.0 5.0
assign ( resid   15 and name ha   )( resid   15 and name hn   ) 0.0 0.0 3.5
assign ( resid   15 and name hb   )( resid   15 and name hn   ) 0.0 0.0 2.8
assign ( resid   15 and name hg1# )( resid   15 and name hn   ) 0.0 0.0 5.0
assign ( resid   15 and name hg2# )( resid   15 and name hn   ) 0.0 0.0 3.5
assign ( resid   16 and name ha1  )( resid   16 and name hn   ) 0.0 0.0 3.5
assign ( resid   16 and name ha2  )( resid   16 and name hn   ) 0.0 0.0 2.8
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assign ( resid   17 and name ha   )( resid   17 and name hg#  ) 0.0 0.0 5.0
assign ( resid   17 and name hb2  )( resid   17 and name hn   ) 0.0 0.0 3.5
assign ( resid   17 and name ha   )( resid   17 and name hn   ) 0.0 0.0 3.5
assign ( resid   17 and name hg#  )( resid   17 and name hn   ) 0.0 0.0 3.5
assign ( resid   17 and name hb1  )( resid   17 and name hn   ) 0.0 0.0 3.5
assign ( resid   18 and name ha   )( resid   18 and name hg2# ) 0.0 0.0 3.5
assign ( resid   18 and name ha   )( resid   18 and name hn   ) 0.0 0.0 3.5
assign ( resid   18 and name hb   )( resid   18 and name hn   ) 0.0 0.0 3.5
assign ( resid   19 and name hb1  )( resid   19 and name hd1  ) 0.0 0.0 5.0
assign ( resid   19 and name hb2  )( resid   19 and name hd1  ) 0.0 0.0 3.5
assign ( resid   19 and name ha   )( resid   19 and name hd1  ) 0.0 0.0 5.0
assign ( resid   19 and name hb1  )( resid   19 and name he3  ) 0.0 0.0 5.0
assign ( resid   19 and name ha   )( resid   19 and name hn   ) 0.0 0.0 5.0
assign ( resid   19 and name hb2  )( resid   19 and name hn   ) 0.0 0.0 5.0
assign ( resid   19 and name he3  )( resid   19 and name hn   ) 0.0 0.0 3.5
assign ( resid   19 and name hd1  )( resid   19 and name he1  ) 0.0 0.0 2.8
assign ( resid   19 and name hz2  )( resid   19 and name he1  ) 0.0 0.0 3.5
assign ( resid   20 and name ha   )( resid   20 and name hn   ) 0.0 0.0 3.5
assign ( resid   20 and name hg2  )( resid   20 and name hn   ) 0.0 0.0 3.5
assign ( resid   20 and name hb#  )( resid   20 and name hn   ) 0.0 0.0 3.5
assign ( resid   20 and name ha   )( resid   20 and name hg2  ) 0.0 0.0 5.0
assign ( resid   20 and name ha   )( resid   20 and name hg1  ) 0.0 0.0 5.0
assign ( resid   21 and name ha   )( resid   21 and name hg#  ) 0.0 0.0 3.5
assign ( resid   21 and name he#  )( resid   21 and name hg#  ) 0.0 0.0 3.5
assign ( resid   21 and name ha   )( resid   21 and name hn   ) 0.0 0.0 3.5
assign ( resid   21 and name hb#  )( resid   21 and name hn   ) 0.0 0.0 3.5
assign ( resid   21 and name hd#  )( resid   21 and name hn   ) 0.0 0.0 3.5
assign ( resid   21 and name ha   )( resid   21 and name hd#  ) 0.0 0.0 3.5
assign ( resid   22 and name ha   )( resid   22 and name hg1  ) 0.0 0.0 5.0
assign ( resid   22 and name hd1  )( resid   22 and name hb1  ) 0.0 0.0 5.0
assign ( resid   22 and name ha   )( resid   22 and name hg2  ) 0.0 0.0 3.5
assign ( resid   23 and name ha   )( resid   23 and name hd#  ) 0.0 0.0 3.5
assign ( resid   23 and name ha   )( resid   23 and name he#  ) 0.0 0.0 5.0
assign ( resid   23 and name ha   )( resid   23 and name hd#  ) 0.0 0.0 3.5
assign ( resid   23 and name ha   )( resid   23 and name he#  ) 0.0 0.0 5.0
assign ( resid   23 and name hb2  )( resid   23 and name hd#  ) 0.0 0.0 3.5
assign ( resid   23 and name hb2  )( resid   23 and name he#  ) 0.0 0.0 5.0
assign ( resid   23 and name hb1  )( resid   23 and name hd#  ) 0.0 0.0 3.5
assign ( resid   23 and name hb1  )( resid   23 and name he#  ) 0.0 0.0 5.0
assign ( resid   23 and name hb1  )( resid   23 and name hn   ) 0.0 0.0 3.5
assign ( resid   23 and name ha   )( resid   23 and name hn   ) 0.0 0.0 3.5
assign ( resid   24 and name ha   )( resid   24 and name hg2  ) 0.0 0.0 5.0
assign ( resid   24 and name ha   )( resid   24 and name hn   ) 0.0 0.0 2.8
assign ( resid   24 and name ha   )( resid   24 and name hg1  ) 0.0 0.0 3.5
assign ( resid   24 and name hb1  )( resid   24 and name hn   ) 0.0 0.0 3.5
assign ( resid   24 and name hb2  )( resid   24 and name hn   ) 0.0 0.0 5.0
assign ( resid   25 and name ha1  )( resid   25 and name hn   ) 0.0 0.0 3.5
assign ( resid   25 and name ha2  )( resid   25 and name hn   ) 0.0 0.0 2.8
assign ( resid   26 and name hz2  )( resid   26 and name hz3  ) 0.0 0.0 5.0
assign ( resid   26 and name he3  )( resid   26 and name hh2  ) 0.0 0.0 5.0
assign ( resid   26 and name hb2  )( resid   26 and name he3  ) 0.0 0.0 5.0
assign ( resid   26 and name hb1  )( resid   26 and name he3  ) 0.0 0.0 5.0
assign ( resid   26 and name hb1  )( resid   26 and name hd1  ) 0.0 0.0 5.0
assign ( resid   26 and name hb2  )( resid   26 and name hd1  ) 0.0 0.0 5.0
assign ( resid   26 and name he3  )( resid   26 and name hz2  ) 0.0 0.0 5.0
assign ( resid   26 and name hd1  )( resid   26 and name he1  ) 0.0 0.0 2.8
assign ( resid   26 and name hb1  )( resid   26 and name hn   ) 0.0 0.0 3.5
assign ( resid   26 and name hb2  )( resid   26 and name hn   ) 0.0 0.0 5.0
assign ( resid   27 and name ha   )( resid   27 and name hg#  ) 0.0 0.0 5.0
assign ( resid   27 and name ha   )( resid   27 and name he#  ) 0.0 0.0 5.0
assign ( resid   27 and name he#  )( resid   27 and name hg#  ) 0.0 0.0 5.0
assign ( resid   27 and name hg1  )( resid   27 and name hn   ) 0.0 0.0 3.5
assign ( resid   27 and name hg2  )( resid   27 and name hn   ) 0.0 0.0 3.5
assign ( resid   27 and name hb2  )( resid   27 and name hn   ) 0.0 0.0 3.5
assign ( resid   27 and name hb1  )( resid   27 and name hn   ) 0.0 0.0 3.5
assign ( resid   27 and name ha   )( resid   27 and name hn   ) 0.0 0.0 3.5
assign ( resid   28 and name ha   )( resid   28 and name he#  ) 0.0 0.0 3.5
assign ( resid   28 and name hg2  )( resid   28 and name he#  ) 0.0 0.0 3.5
assign ( resid   28 and name ha   )( resid   28 and name hg2  ) 0.0 0.0 5.0
assign ( resid   28 and name ha   )( resid   28 and name hn   ) 0.0 0.0 3.5
assign ( resid   28 and name hg2  )( resid   28 and name hn   ) 0.0 0.0 5.0
assign ( resid   28 and name hb#  )( resid   28 and name hn   ) 0.0 0.0 5.0
assign ( resid   28 and name he#  )( resid   28 and name hn   ) 0.0 0.0 3.5
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assign ( resid   28 and name ha    )( resid   28 and name hg1  ) 0.0 0.0 3.5
assign ( resid   28 and name hg1   )( resid   28 and name hn   ) 0.0 0.0 2.8
assign ( resid   28 and name hb#   )( resid   28 and name he#  ) 0.0 0.0 3.5
assign ( resid   29 and name ha    )( resid   29 and name hg1# ) 0.0 0.0 3.5
assign ( resid   29 and name ha    )( resid   29 and name hg2# ) 0.0 0.0 5.0
assign ( resid   29 and name ha    )( resid   29 and name hn   ) 0.0 0.0 5.0
assign ( resid   29 and name hg1#  )( resid   29 and name hn   ) 0.0 0.0 3.5
assign ( resid   29 and name hg2#  )( resid   29 and name hn   ) 0.0 0.0 2.8
assign ( resid   29 and name hb    )( resid   29 and name hn   ) 0.0 0.0 3.5
assign ( resid   30 and name ha    )( resid   30 and name hn   ) 0.0 0.0 3.5
assign ( resid   30 and name hb#   )( resid   30 and name hn   ) 0.0 0.0 3.5
assign ( resid   31 and name hb1   )( resid   31 and name hn   ) 0.0 0.0 3.5
assign ( resid   31 and name hb2   )( resid   31 and name hn   ) 0.0 0.0 3.5
assign ( resid   31 and name ha    )( resid   31 and name hn   ) 0.0 0.0 5.0
assign ( resid   32 and name ha    )( resid   32 and name hg2# ) 0.0 0.0 3.5
assign ( resid   32 and name hb    )( resid   32 and name hn   ) 0.0 0.0 3.5
assign ( resid   32 and name hg2#  )( resid   32 and name hn   ) 0.0 0.0 5.0
assign ( resid   32 and name ha    )( resid   32 and name hn   ) 0.0 0.0 5.0
assign ( resid   33 and name ha    )( resid   33 and name hn   ) 0.0 0.0 3.5
assign ( resid   33 and name hb1   )( resid   33 and name hn   ) 0.0 0.0 3.5
assign ( resid   33 and name hb2   )( resid   33 and name hn   ) 0.0 0.0 3.5
assign ( resid   34 and name ha    )( resid   34 and name hn   ) 0.0 0.0 3.5
assign ( resid   34 and name ha    )( resid   34 and name hg   ) 0.0 0.0 5.0
assign ( resid   34 and name ha    )( resid   34 and name hd1# ) 0.0 0.0 5.0
assign ( resid   34 and name ha    )( resid   34 and name hd2# ) 0.0 0.0 5.0
assign ( resid   34 and name hb#   )( resid   34 and name hn   ) 0.0 0.0 3.5
assign ( resid   34 and name hd2#  )( resid   34 and name hn   ) 0.0 0.0 5.0
assign ( resid   35 and name ha2   )( resid   35 and name hn   ) 0.0 0.0 3.5
assign ( resid   35 and name ha1   )( resid   35 and name hn   ) 0.0 0.0 3.5
assign ( resid   36 and name ha    )( resid   36 and name hg2  ) 0.0 0.0 3.5
assign ( resid   36 and name ha    )( resid   36 and name hn   ) 0.0 0.0 2.8
assign ( resid   36 and name hg2   )( resid   36 and name hn   ) 0.0 0.0 3.5
assign ( resid   36 and name hb#   )( resid   36 and name hn   ) 0.0 0.0 3.5
assign ( resid   36 and name hg1   )( resid   36 and name hn   ) 0.0 0.0 5.0
assign ( resid   36 and name hb#   )( resid   36 and name hn   ) 0.0 0.0 3.5
assign ( resid   36 and name hg1   )( resid   36 and name hn   ) 0.0 0.0 5.0
assign ( resid   37 and name ha1   )( resid   37 and name hn   ) 0.0 0.0 2.8
assign ( resid   37 and name ha2   )( resid   37 and name hn   ) 0.0 0.0 3.5
assign ( resid   38 and name ha    )( resid   38 and name hn   ) 0.0 0.0 3.5
assign ( resid   38 and name hb1   )( resid   38 and name hn   ) 0.0 0.0 5.0
assign ( resid   39 and name ha1   )( resid   39 and name hn   ) 0.0 0.0 5.0
assign ( resid   39 and name ha2   )( resid   39 and name hn   ) 0.0 0.0 5.0
assign ( resid   40 and name ha    )( resid   40 and name hn   ) 0.0 0.0 5.0
assign ( resid   40 and name hb2   )( resid   40 and name hn   ) 0.0 0.0 3.5
assign ( resid   40 and name hg#   )( resid   40 and name hn   ) 0.0 0.0 3.5
assign ( resid   41 and name ha    )( resid   41 and name hg2# ) 0.0 0.0 5.0
assign ( resid   41 and name ha    )( resid   41 and name hg1# ) 0.0 0.0 3.5
assign ( resid   41 and name ha    )( resid   41 and name hd1# ) 0.0 0.0 3.5
assign ( resid   41 and name hd1#  )( resid   41 and name hn   ) 0.0 0.0 5.0
assign ( resid   41 and name hb    )( resid   41 and name hn   ) 0.0 0.0 3.5
assign ( resid   41 and name ha    )( resid   41 and name hn   ) 0.0 0.0 5.0
assign ( resid   41 and name hg1#  )( resid   41 and name hn   ) 0.0 0.0 5.0
assign ( resid   41 and name hg2#  )( resid   41 and name hn   ) 0.0 0.0 5.0
assign ( resid   42 and name ha    )( resid   42 and name hg2# ) 0.0 0.0 3.5
assign ( resid   42 and name hb    )( resid   42 and name hg2# ) 0.0 0.0 2.8
assign ( resid   42 and name hg2#  )( resid   42 and name hn   ) 0.0 0.0 2.8
assign ( resid   42 and name ha    )( resid   42 and name hn   ) 0.0 0.0 3.5
assign ( resid   42 and name hb    )( resid   42 and name hn   ) 0.0 0.0 3.5
assign ( resid   43 and name ha    )( resid   43 and name hn   ) 0.0 0.0 5.0
assign ( resid   44 and name ha    )( resid   44 and name hg2# ) 0.0 0.0 5.0
assign ( resid   44 and name hg2#  )( resid   44 and name hn   ) 0.0 0.0 3.5
assign ( resid   44 and name hb    )( resid   44 and name hn   ) 0.0 0.0 5.0
assign ( resid   44 and name ha    )( resid   44 and name hn   ) 0.0 0.0 5.0
assign ( resid   46 and name ha    )( resid   46 and name hg2  ) 0.0 0.0 3.5
assign ( resid   46 and name ha    )( resid   46 and name hg1  ) 0.0 0.0 5.0
assign ( resid   46 and name hd#   )( resid   46 and name hb#  ) 0.0 0.0 5.0
assign ( resid   46 and name ha    )( resid   46 and name hn   ) 0.0 0.0 3.5
assign ( resid   46 and name hb#   )( resid   46 and name hn   ) 0.0 0.0 3.5
assign ( resid   46 and name hg1   )( resid   46 and name hn   ) 0.0 0.0 5.0
assign ( resid   47 and name ha    )( resid   47 and name hn   ) 0.0 0.0 3.5
assign ( resid   47 and name hb1   )( resid   47 and name hn   ) 0.0 0.0 3.5
assign ( resid   47 and name hb2   )( resid   47 and name hn   ) 0.0 0.0 3.5
assign ( resid   47 and name hb1   )( resid   47 and name hd21 ) 0.0 0.0 3.5
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assign ( resid   47 and name hb2  )( resid   47 and name hd21 ) 0.0 0.0 3.5
assign ( resid   47 and name hb2  )( resid   47 and name hd22 ) 0.0 0.0 3.5
assign ( resid   47 and name hb1  )( resid   47 and name hd22 ) 0.0 0.0 3.5
assign ( resid   47 and name hd22 )( resid   47 and name hd21 ) 0.0 0.0 2.8
assign ( resid   48 and name hg2  )( resid   48 and name hn   ) 0.0 0.0 3.5
assign ( resid   48 and name ha   )( resid   48 and name hn   ) 0.0 0.0 3.5
assign ( resid   48 and name hg1  )( resid   48 and name hn   ) 0.0 0.0 5.0
assign ( resid   48 and name hb#  )( resid   48 and name hn   ) 0.0 0.0 5.0
assign ( resid   49 and name hb2  )( resid   49 and name hn   ) 0.0 0.0 5.0
assign ( resid   49 and name hb1  )( resid   49 and name hn   ) 0.0 0.0 5.0
assign ( resid   50 and name hb2  )( resid   50 and name hn   ) 0.0 0.0 3.5
assign ( resid   50 and name ha   )( resid   50 and name hn   ) 0.0 0.0 3.5
assign ( resid   50 and name hb1  )( resid   50 and name hn   ) 0.0 0.0 5.0
assign ( resid   52 and name ha   )( resid   52 and name hn   ) 0.0 0.0 3.5
assign ( resid   52 and name hg#  )( resid   52 and name hn   ) 0.0 0.0 3.5
assign ( resid   52 and name hb#  )( resid   52 and name hn   ) 0.0 0.0 3.5
assign ( resid   52 and name ha   )( resid   52 and name hg1  ) 0.0 0.0 5.0
assign ( resid   52 and name ha   )( resid   52 and name hg2  ) 0.0 0.0 3.5
assign ( resid   53 and name ha   )( resid   53 and name hn   ) 0.0 0.0 3.5
assign ( resid   54 and name hg2# )( resid   54 and name hn   ) 0.0 0.0 3.5
assign ( resid   54 and name ha   )( resid   54 and name hn   ) 0.0 0.0 3.5
assign ( resid   55 and name hd#  )( resid   55 and name hb1  ) 0.0 0.0 5.0
assign ( resid   55 and name hd#  )( resid   55 and name hb2  ) 0.0 0.0 5.0
assign ( resid   55 and name hd#  )( resid   55 and name ha   ) 0.0 0.0 5.0
assign ( resid   55 and name ha   )( resid   55 and name hg1  ) 0.0 0.0 5.0
assign ( resid   55 and name ha   )( resid   55 and name hn   ) 0.0 0.0 2.8
assign ( resid   55 and name hg2  )( resid   55 and name hn   ) 0.0 0.0 3.5
assign ( resid   55 and name hb2  )( resid   55 and name hn   ) 0.0 0.0 3.5
assign ( resid   55 and name hd#  )( resid   55 and name hn   ) 0.0 0.0 5.0
assign ( resid   56 and name hg2# )( resid   56 and name hn   ) 0.0 0.0 3.5
assign ( resid   56 and name hb   )( resid   56 and name hn   ) 0.0 0.0 3.5
assign ( resid   56 and name ha   )( resid   56 and name hn   ) 0.0 0.0 3.5
assign ( resid   56 and name ha   )( resid   56 and name hg2# ) 0.0 0.0 5.0
assign ( resid   57 and name hb1  )( resid   57 and name hn   ) 0.0 0.0 3.5
assign ( resid   57 and name hb2  )( resid   57 and name hn   ) 0.0 0.0 3.5
assign ( resid   57 and name ha   )( resid   57 and name hn   ) 0.0 0.0 5.0
assign ( resid   58 and name ha   )( resid   58 and name he#  ) 0.0 0.0 5.0
assign ( resid   58 and name hb1  )( resid   58 and name hd#  ) 0.0 0.0 5.0
assign ( resid   58 and name ha   )( resid   58 and name hn   ) 0.0 0.0 5.0
assign ( resid   58 and name hb1  )( resid   58 and name hn   ) 0.0 0.0 5.0
assign ( resid   59 and name hd#  )( resid   59 and name hb#  ) 0.0 0.0 5.0
assign ( resid   59 and name hd#  )( resid   59 and name hn   ) 0.0 0.0 3.5
assign ( resid   59 and name hg2  )( resid   59 and name hn   ) 0.0 0.0 3.5
assign ( resid   59 and name hb#  )( resid   59 and name hn   ) 0.0 0.0 5.0
assign ( resid   59 and name hg1  )( resid   59 and name hn   ) 0.0 0.0 5.0
assign ( resid   60 and name ha   )( resid   60 and name hg1# ) 0.0 0.0 3.5
assign ( resid   60 and name ha   )( resid   60 and name hn   ) 0.0 0.0 2.8
assign ( resid   60 and name hg1# )( resid   60 and name hn   ) 0.0 0.0 3.5
assign ( resid   60 and name hb   )( resid   60 and name hn   ) 0.0 0.0 3.5
assign ( resid   60 and name ha   )( resid   60 and name hg2# ) 0.0 0.0 3.5
assign ( resid   60 and name ha   )( resid   60 and name hd1# ) 0.0 0.0 5.0
assign ( resid   61 and name ha1  )( resid   61 and name hn   ) 0.0 0.0 5.0
assign ( resid   61 and name ha2  )( resid   61 and name hn   ) 0.0 0.0 2.8
assign ( resid   62 and name ha   )( resid   62 and name hn   ) 0.0 0.0 3.5
assign ( resid   62 and name hb1  )( resid   62 and name hn   ) 0.0 0.0 3.5
assign ( resid   62 and name hb2  )( resid   62 and name hn   ) 0.0 0.0 3.5
assign ( resid   63 and name ha   )( resid   63 and name hg2# ) 0.0 0.0 3.5
assign ( resid   63 and name ha   )( resid   63 and name hn   ) 0.0 0.0 3.5
assign ( resid   63 and name hb   )( resid   63 and name hn   ) 0.0 0.0 3.5
assign ( resid   63 and name hg2# )( resid   63 and name hn   ) 0.0 0.0 3.5
assign ( resid   64 and name hb2  )( resid   64 and name he3  ) 0.0 0.0 5.0
assign ( resid   64 and name hb1  )( resid   64 and name he3  ) 0.0 0.0 5.0
assign ( resid   64 and name ha   )( resid   64 and name hd1  ) 0.0 0.0 5.0
assign ( resid   64 and name hb2  )( resid   64 and name hd1  ) 0.0 0.0 5.0
assign ( resid   64 and name hb1  )( resid   64 and name hd1  ) 0.0 0.0 3.5
assign ( resid   64 and name ha   )( resid   64 and name he3  ) 0.0 0.0 5.0
assign ( resid   64 and name he3  )( resid   64 and name hn   ) 0.0 0.0 3.5
assign ( resid   64 and name hd1  )( resid   64 and name he1  ) 0.0 0.0 3.5
assign ( resid   64 and name ha   )( resid   64 and name hn   ) 0.0 0.0 3.5
assign ( resid   65 and name ha   )( resid   65 and name hn   ) 0.0 0.0 3.5
assign ( resid   65 and name hb#  )( resid   65 and name hn   ) 0.0 0.0 3.5
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assign ( resid   66 and name hd#  )( resid   66 and name hb1  ) 0.0 0.0 3.5
assign ( resid   66 and name hb2  )( resid   66 and name hn   ) 0.0 0.0 5.0
assign ( resid   66 and name hb1  )( resid   66 and name hn   ) 0.0 0.0 3.5
assign ( resid   66 and name hg#  )( resid   66 and name hn   ) 0.0 0.0 3.5
assign ( resid   66 and name hd#  )( resid   66 and name hn   ) 0.0 0.0 3.5
assign ( resid   67 and name ha   )( resid   67 and name hd#  ) 0.0 0.0 3.5
assign ( resid   67 and name hd#  )( resid   67 and name hn   ) 0.0 0.0 3.5
assign ( resid   67 and name hg1  )( resid   67 and name hn   ) 0.0 0.0 3.5
assign ( resid   67 and name ha   )( resid   67 and name hn   ) 0.0 0.0 3.5
assign ( resid   67 and name he#  )( resid   67 and name hg1  ) 0.0 0.0 5.0
assign ( resid   68 and name hb1  )( resid   68 and name hn   ) 0.0 0.0 3.5
assign ( resid   68 and name hb2  )( resid   68 and name hn   ) 0.0 0.0 3.5
assign ( resid   68 and name ha   )( resid   68 and name hn   ) 0.0 0.0 3.5
assign ( resid   69 and name hb2  )( resid   69 and name hn   ) 0.0 0.0 3.5
assign ( resid   69 and name ha   )( resid   69 and name hn   ) 0.0 0.0 3.5
assign ( resid   69 and name hb1  )( resid   69 and name hn   ) 0.0 0.0 5.0
assign ( resid   70 and name ha   )( resid   70 and name hg2  ) 0.0 0.0 5.0
assign ( resid   70 and name ha   )( resid   70 and name hg1  ) 0.0 0.0 3.5
assign ( resid   70 and name hd#  )( resid   70 and name hb2  ) 0.0 0.0 5.0
assign ( resid   70 and name hd#  )( resid   70 and name hb1  ) 0.0 0.0 3.5
assign ( resid   70 and name ha   )( resid   70 and name hd#  ) 0.0 0.0 5.0
assign ( resid   70 and name hb2  )( resid   70 and name hn   ) 0.0 0.0 3.5
assign ( resid   70 and name hb1  )( resid   70 and name hn   ) 0.0 0.0 2.8
assign ( resid   70 and name hg1  )( resid   70 and name hn   ) 0.0 0.0 3.5
assign ( resid   70 and name hg2  )( resid   70 and name hn   ) 0.0 0.0 3.5
assign ( resid   71 and name ha1  )( resid   71 and name hn   ) 0.0 0.0 3.5
assign ( resid   71 and name ha2  )( resid   71 and name hn   ) 0.0 0.0 3.5
assign ( resid   72 and name hb2  )( resid   72 and name hn   ) 0.0 0.0 2.8
assign ( resid   72 and name hb1  )( resid   72 and name hn   ) 0.0 0.0 3.5
assign ( resid   72 and name hd22 )( resid   72 and name hd21 ) 0.0 0.0 2.8
assign ( resid   72 and name hb2  )( resid   72 and name hd21 ) 0.0 0.0 3.5
assign ( resid   73 and name ha   )( resid   73 and name hg   ) 0.0 0.0 3.5
assign ( resid   73 and name hb#  )( resid   73 and name hd2# ) 0.0 0.0 2.8
assign ( resid   73 and name ha   )( resid   73 and name hn   ) 0.0 0.0 3.5
assign ( resid   73 and name hb#  )( resid   73 and name hn   ) 0.0 0.0 2.8
assign ( resid   73 and name hg   )( resid   73 and name hn   ) 0.0 0.0 2.8
assign ( resid   73 and name ha   )( resid   73 and name hd2# ) 0.0 0.0 3.5
assign ( resid   73 and name hb#  )( resid   73 and name hd1# ) 0.0 0.0 2.8
assign ( resid   73 and name hg   )( resid   73 and name hd1# ) 0.0 0.0 2.8
assign ( resid   73 and name hg   )( resid   73 and name hd2# ) 0.0 0.0 2.8
assign ( resid   73 and name hd1# )( resid   73 and name hn   ) 0.0 0.0 3.5
assign ( resid   73 and name hd1# )( resid   73 and name ha   ) 0.0 0.0 5.0
assign ( resid   74 and name ha   )( resid   74 and name hd1# ) 0.0 0.0 5.0
assign ( resid   74 and name hb1  )( resid   74 and name hd1# ) 0.0 0.0 3.5
assign ( resid   74 and name ha   )( resid   74 and name hd2# ) 0.0 0.0 5.0
assign ( resid   74 and name ha   )( resid   74 and name hn   ) 0.0 0.0 5.0
assign ( resid   74 and name hb1  )( resid   74 and name hn   ) 0.0 0.0 3.5
assign ( resid   74 and name hd2# )( resid   74 and name hn   ) 0.0 0.0 5.0
assign ( resid   74 and name hd1# )( resid   74 and name hn   ) 0.0 0.0 3.5
assign ( resid   74 and name hg   )( resid   74 and name hn   ) 0.0 0.0 3.5
assign ( resid   74 and name hd1# )( resid   74 and name hb2  ) 0.0 0.0 3.5
assign ( resid   74 and name ha   )( resid   74 and name hg   ) 0.0 0.0 5.0
assign ( resid   75 and name ha   )( resid   75 and name hg1  ) 0.0 0.0 5.0
assign ( resid   75 and name ha   )( resid   75 and name hg2  ) 0.0 0.0 5.0
assign ( resid   75 and name ha   )( resid   75 and name hn   ) 0.0 0.0 3.5
assign ( resid   75 and name hb2  )( resid   75 and name hn   ) 0.0 0.0 3.5
assign ( resid   75 and name hb1  )( resid   75 and name hn   ) 0.0 0.0 3.5
assign ( resid   75 and name hg1  )( resid   75 and name hn   ) 0.0 0.0 5.0
assign ( resid   75 and name hg2  )( resid   75 and name hn   ) 0.0 0.0 5.0
assign ( resid   76 and name hb1  )( resid   76 and name hn   ) 0.0 0.0 3.5
assign ( resid   76 and name hb2  )( resid   76 and name hn   ) 0.0 0.0 5.0
assign ( resid   76 and name ha   )( resid   76 and name hn   ) 0.0 0.0 5.0
assign ( resid   77 and name ha   )( resid   77 and name hg11 ) 0.0 0.0 5.0
assign ( resid   77 and name ha   )( resid   77 and name hg12 ) 0.0 0.0 3.5
assign ( resid   77 and name ha   )( resid   77 and name hd1# ) 0.0 0.0 5.0
assign ( resid   77 and name hg12 )( resid   77 and name hn   ) 0.0 0.0 3.5
assign ( resid   77 and name ha   )( resid   77 and name hn   ) 0.0 0.0 3.5
assign ( resid   77 and name hg2# )( resid   77 and name hn   ) 0.0 0.0 3.5
assign ( resid   78 and name ha   )( resid   78 and name hn   ) 0.0 0.0 3.5
assign ( resid   79 and name ha   )( resid   79 and name hg2# ) 0.0 0.0 3.5
assign ( resid   79 and name hg2# )( resid   79 and name hn   ) 0.0 0.0 5.0
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assign ( resid   79 and name ha   )( resid   79 and name hn   ) 0.0 0.0 5.0
assign ( resid   80 and name ha1  )( resid   80 and name hn   ) 0.0 0.0 5.0
assign ( resid   81 and name hb2  )( resid   81 and name hn   ) 0.0 0.0 3.5
assign ( resid   81 and name hb1  )( resid   81 and name hn   ) 0.0 0.0 3.5
assign ( resid   81 and name hb1  )( resid   81 and name hd22 ) 0.0 0.0 3.5
assign ( resid   81 and name hb2  )( resid   81 and name hd22 ) 0.0 0.0 3.5
assign ( resid   81 and name hb1  )( resid   81 and name hd21 ) 0.0 0.0 3.5
assign ( resid   81 and name hb2  )( resid   81 and name hd21 ) 0.0 0.0 3.5
assign ( resid   81 and name hd22 )( resid   81 and name hd21 ) 0.0 0.0 2.8
assign ( resid   82 and name ha2  )( resid   82 and name hn   ) 0.0 0.0 3.5
assign ( resid   82 and name ha1  )( resid   82 and name hn   ) 0.0 0.0 3.5
assign ( resid   83 and name hb1  )( resid   83 and name hn   ) 0.0 0.0 3.5
assign ( resid   83 and name hb2  )( resid   83 and name hn   ) 0.0 0.0 3.5
assign ( resid   83 and name hd#  )( resid   83 and name hn   ) 0.0 0.0 5.0
assign ( resid   84 and name ha1  )( resid   84 and name hn   ) 0.0 0.0 3.5
assign ( resid   84 and name ha2  )( resid   84 and name hn   ) 0.0 0.0 5.0
assign ( resid   86 and name hz2  )( resid   86 and name hz3  ) 0.0 0.0 5.0
assign ( resid   86 and name ha   )( resid   86 and name he3  ) 0.0 0.0 5.0
assign ( resid   86 and name hb2  )( resid   86 and name he3  ) 0.0 0.0 5.0
assign ( resid   86 and name hb1  )( resid   86 and name he3  ) 0.0 0.0 5.0
assign ( resid   86 and name hb1  )( resid   86 and name hd1  ) 0.0 0.0 5.0
assign ( resid   86 and name hb2  )( resid   86 and name hd1  ) 0.0 0.0 5.0
assign ( resid   86 and name hd1  )( resid   86 and name he1  ) 0.0 0.0 3.5
assign ( resid   86 and name hz2  )( resid   86 and name he1  ) 0.0 0.0 3.5
assign ( resid   86 and name ha   )( resid   86 and name hn   ) 0.0 0.0 5.0
assign ( resid   87 and name hb#  )( resid   87 and name hn   ) 0.0 0.0 5.0
assign ( resid   87 and name hg#  )( resid   87 and name ha   ) 0.0 0.0 3.5
assign ( resid   87 and name he#  )( resid   87 and name ha   ) 0.0 0.0 5.0
assign ( resid   87 and name hg#  )( resid   87 and name he#  ) 0.0 0.0 3.5
assign ( resid   87 and name ha   )( resid   87 and name hd#  ) 0.0 0.0 5.0
assign ( resid   87 and name ha   )( resid   87 and name hn   ) 0.0 0.0 3.5
assign ( resid   88 and name hb2  )( resid   88 and name hn   ) 0.0 0.0 3.5
assign ( resid   89 and name ha   )( resid   89 and name hg2  ) 0.0 0.0 5.0
assign ( resid   89 and name ha   )( resid   89 and name hg1  ) 0.0 0.0 5.0
assign ( resid   89 and name hg2  )( resid   89 and name hn   ) 0.0 0.0 3.5
assign ( resid   89 and name hg1  )( resid   89 and name hn   ) 0.0 0.0 3.5
assign ( resid   90 and name ha   )( resid   90 and name hn   ) 0.0 0.0 3.5
assign ( resid   90 and name hb#  )( resid   90 and name hn   ) 0.0 0.0 3.5
assign ( resid   90 and name hg#  )( resid   90 and name hn   ) 0.0 0.0 3.5
assign ( resid   91 and name ha   )( resid   91 and name hn   ) 0.0 0.0 3.5
assign ( resid   91 and name hb2  )( resid   91 and name hn   ) 0.0 0.0 5.0
assign ( resid   91 and name hb1  )( resid   91 and name hn   ) 0.0 0.0 5.0
assign ( resid   92 and name ha   )( resid   92 and name hg2# ) 0.0 0.0 3.5
assign ( resid   92 and name hg2# )( resid   92 and name hn   ) 0.0 0.0 3.5
assign ( resid   92 and name ha   )( resid   92 and name hn   ) 0.0 0.0 3.5
assign ( resid   92 and name hb   )( resid   92 and name hn   ) 0.0 0.0 3.5
assign ( resid   93 and name hb#  )( resid   93 and name hn   ) 0.0 0.0 3.5
assign ( resid   93 and name ha   )( resid   93 and name hn   ) 0.0 0.0 5.0

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assign ( resid    1 and name hb#  )( resid    2 and name hn   ) 0.0 0.0 3.5
assign ( resid    1 and name ha   )( resid    2 and name hb#  ) 0.0 0.0 5.0
assign ( resid    1 and name ha   )( resid    2 and name hn   ) 0.0 0.0 3.5
assign ( resid    2 and name ha   )( resid    3 and name ha   ) 0.0 0.0 5.0
assign ( resid    2 and name hb#  )( resid    3 and name hn   ) 0.0 0.0 3.5
assign ( resid    2 and name hg#  )( resid    3 and name hn   ) 0.0 0.0 3.5
assign ( resid    2 and name ha   )( resid    3 and name hn   ) 0.0 0.0 2.8
assign ( resid    3 and name ha   )( resid    4 and name hn   ) 0.0 0.0 2.8
assign ( resid    3 and name hb#  )( resid    4 and name hn   ) 0.0 0.0 3.5
assign ( resid    3 and name hd#  )( resid    4 and name hn   ) 0.0 0.0 5.0
assign ( resid    3 and name hg#  )( resid    4 and name hn   ) 0.0 0.0 3.5
assign ( resid    3 and name hn   )( resid    4 and name hn   ) 0.0 0.0 5.0
assign ( resid    4 and name ha   )( resid    5 and name hn   ) 0.0 0.0 3.5
assign ( resid    4 and name hb2  )( resid    5 and name hn   ) 0.0 0.0 3.5
assign ( resid    4 and name hb1  )( resid    5 and name hn   ) 0.0 0.0 5.0
assign ( resid    5 and name ha   )( resid    6 and name hn   ) 0.0 0.0 3.5
assign ( resid    5 and name hd#  )( resid    6 and name hn   ) 0.0 0.0 5.0
assign ( resid    5 and name hb1  )( resid    6 and name hn   ) 0.0 0.0 3.5
assign ( resid    6 and name ha   )( resid    7 and name hn   ) 0.0 0.0 2.8
assign ( resid    7 and name ha   )( resid    8 and name hn   ) 0.0 0.0 2.8
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assign ( resid    7 and name hb#  )( resid    8 and name hn   ) 0.0 0.0 5.0
assign ( resid    8 and name ha   )( resid    9 and name hn   ) 0.0 0.0 3.5
assign ( resid    8 and name hn   )( resid    9 and name hn   ) 0.0 0.0 2.8
assign ( resid    8 and name hb#  )( resid    9 and name hn   ) 0.0 0.0 2.8
assign ( resid    9 and name ha   )( resid   10 and name hn   ) 0.0 0.0 3.5
assign ( resid    9 and name hb#  )( resid   10 and name hn   ) 0.0 0.0 3.5
assign ( resid    9 and name hb#  )( resid   10 and name ha2  ) 0.0 0.0 5.0
assign ( resid    9 and name hn   )( resid   10 and name hn   ) 0.0 0.0 2.8
assign ( resid   10 and name ha1  )( resid   11 and name hn   ) 0.0 0.0 3.5
assign ( resid   10 and name ha2  )( resid   11 and name hn   ) 0.0 0.0 3.5
assign ( resid   10 and name hn   )( resid   11 and name hn   ) 0.0 0.0 2.8
assign ( resid   11 and name ha   )( resid   12 and name hn   ) 0.0 0.0 2.8
assign ( resid   11 and name hg2# )( resid   12 and name hn   ) 0.0 0.0 3.5
assign ( resid   11 and name hb   )( resid   12 and name hn   ) 0.0 0.0 2.8
assign ( resid   12 and name ha   )( resid   13 and name he#  ) 0.0 0.0 5.0
assign ( resid   12 and name ha   )( resid   13 and name hd#  ) 0.0 0.0 5.0
assign ( resid   12 and name hb1  )( resid   13 and name hd#  ) 0.0 0.0 5.0
assign ( resid   12 and name ha   )( resid   13 and name hn   ) 0.0 0.0 3.5
assign ( resid   12 and name hb2  )( resid   13 and name hn   ) 0.0 0.0 3.5
assign ( resid   12 and name hb1  )( resid   13 and name hn   ) 0.0 0.0 3.5
assign ( resid   12 and name hn   )( resid   13 and name hn   ) 0.0 0.0 5.0
assign ( resid   13 and name ha   )( resid   14 and name hg1# ) 0.0 0.0 5.0
assign ( resid   13 and name ha   )( resid   14 and name hn   ) 0.0 0.0 3.5
assign ( resid   13 and name hb2  )( resid   14 and name hn   ) 0.0 0.0 2.8
assign ( resid   13 and name hd#  )( resid   14 and name hn   ) 0.0 0.0 5.0
assign ( resid   14 and name ha   )( resid   15 and name hn   ) 0.0 0.0 2.8
assign ( resid   14 and name hg1# )( resid   15 and name hn   ) 0.0 0.0 3.5
assign ( resid   14 and name hb   )( resid   15 and name hn   ) 0.0 0.0 2.8
assign ( resid   14 and name hg2# )( resid   15 and name hn   ) 0.0 0.0 3.5
assign ( resid   15 and name hb   )( resid   16 and name hn   ) 0.0 0.0 3.5
assign ( resid   15 and name hn   )( resid   16 and name hn   ) 0.0 0.0 5.0
assign ( resid   15 and name hg1# )( resid   16 and name ha2  ) 0.0 0.0 5.0
assign ( resid   15 and name ha   )( resid   16 and name hn   ) 0.0 0.0 3.5
assign ( resid   16 and name ha2  )( resid   17 and name hn   ) 0.0 0.0 3.5
assign ( resid   16 and name ha1  )( resid   17 and name hn   ) 0.0 0.0 3.5
assign ( resid   16 and name hn   )( resid   17 and name hn   ) 0.0 0.0 2.8
assign ( resid   17 and name ha   )( resid   18 and name hg2# ) 0.0 0.0 5.0
assign ( resid   17 and name ha   )( resid   18 and name ha   ) 0.0 0.0 5.0
assign ( resid   17 and name hb2  )( resid   18 and name ha   ) 0.0 0.0 5.0
assign ( resid   17 and name hb1  )( resid   18 and name ha   ) 0.0 0.0 5.0
assign ( resid   17 and name hg#  )( resid   18 and name hn   ) 0.0 0.0 3.5
assign ( resid   17 and name hb1  )( resid   18 and name hn   ) 0.0 0.0 3.5
assign ( resid   17 and name hb2  )( resid   18 and name hn   ) 0.0 0.0 3.5
assign ( resid   17 and name ha   )( resid   18 and name hn   ) 0.0 0.0 2.8
assign ( resid   18 and name ha   )( resid   19 and name he3  ) 0.0 0.0 3.5
assign ( resid   18 and name hb   )( resid   19 and name he3  ) 0.0 0.0 5.0
assign ( resid   18 and name hg2# )( resid   19 and name he3  ) 0.0 0.0 5.0
assign ( resid   18 and name ha   )( resid   19 and name hn   ) 0.0 0.0 2.8
assign ( resid   18 and name hb   )( resid   19 and name hn   ) 0.0 0.0 3.5
assign ( resid   18 and name hg2# )( resid   19 and name hn   ) 0.0 0.0 3.5
assign ( resid   19 and name hd1  )( resid   20 and name ha   ) 0.0 0.0 5.0
assign ( resid   19 and name ha   )( resid   20 and name hn   ) 0.0 0.0 2.8
assign ( resid   19 and name hd1  )( resid   20 and name hn   ) 0.0 0.0 3.5
assign ( resid   19 and name hb2  )( resid   20 and name hn   ) 0.0 0.0 3.5
assign ( resid   19 and name hb1  )( resid   20 and name hn   ) 0.0 0.0 3.5
assign ( resid   20 and name ha   )( resid   21 and name hn   ) 0.0 0.0 2.8
assign ( resid   21 and name ha   )( resid   22 and name hg1  ) 0.0 0.0 5.0
assign ( resid   21 and name ha   )( resid   22 and name hg2  ) 0.0 0.0 5.0
assign ( resid   21 and name ha   )( resid   22 and name ha   ) 0.0 0.0 5.0
assign ( resid   21 and name ha   )( resid   22 and name hd1  ) 0.0 0.0 3.5
assign ( resid   21 and name ha   )( resid   22 and name hd2  ) 0.0 0.0 2.8
assign ( resid   21 and name hg#  )( resid   22 and name hd1  ) 0.0 0.0 5.0
assign ( resid   21 and name hg#  )( resid   22 and name hd2  ) 0.0 0.0 5.0
assign ( resid   21 and name hb#  )( resid   22 and name hd2  ) 0.0 0.0 5.0
assign ( resid   21 and name hd2  )( resid   22 and name hd1  ) 0.0 0.0 5.0
assign ( resid   22 and name ha   )( resid   23 and name hn   ) 0.0 0.0 2.8
assign ( resid   23 and name he#  )( resid   24 and name hb1  ) 0.0 0.0 5.0
assign ( resid   23 and name hd#  )( resid   24 and name hg1  ) 0.0 0.0 5.0
assign ( resid   23 and name he#  )( resid   24 and name hg1  ) 0.0 0.0 5.0
assign ( resid   23 and name hd#  )( resid   24 and name hn   ) 0.0 0.0 3.5
assign ( resid   23 and name ha   )( resid   24 and name hn   ) 0.0 0.0 3.5
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assign ( resid   24 and name ha   )( resid   25 and name hn   ) 0.0 0.0 3.5
assign ( resid   25 and name ha2  )( resid   26 and name hd1  ) 0.0 0.0 5.0
assign ( resid   25 and name ha2  )( resid   26 and name hn   ) 0.0 0.0 3.5
assign ( resid   25 and name ha1  )( resid   26 and name hn   ) 0.0 0.0 3.5
assign ( resid   25 and name hn   )( resid   26 and name hn   ) 0.0 0.0 5.0
assign ( resid   25 and name ha1  )( resid   26 and name hd1  ) 0.0 0.0 5.0
assign ( resid   26 and name hh2  )( resid   27 and name hg#  ) 0.0 0.0 5.0
assign ( resid   26 and name hz3  )( resid   27 and name hg#  ) 0.0 0.0 5.0
assign ( resid   26 and name hz2  )( resid   27 and name hg#  ) 0.0 0.0 5.0
assign ( resid   26 and name hz2  )( resid   27 and name he#  ) 0.0 0.0 3.5
assign ( resid   26 and name he3  )( resid   27 and name hg#  ) 0.0 0.0 5.0
assign ( resid   26 and name hh2  )( resid   27 and name he#  ) 0.0 0.0 5.0
assign ( resid   26 and name he1  )( resid   27 and name hg#  ) 0.0 0.0 5.0
assign ( resid   26 and name ha   )( resid   27 and name hn   ) 0.0 0.0 3.5
assign ( resid   26 and name hn   )( resid   27 and name hn   ) 0.0 0.0 2.8
assign ( resid   27 and name ha   )( resid   28 and name hn   ) 0.0 0.0 3.5
assign ( resid   27 and name hb1  )( resid   28 and name hn   ) 0.0 0.0 3.5
assign ( resid   28 and name ha   )( resid   29 and name hg1# ) 0.0 0.0 5.0
assign ( resid   28 and name ha   )( resid   29 and name hg2# ) 0.0 0.0 5.0
assign ( resid   28 and name ha   )( resid   29 and name hn   ) 0.0 0.0 2.8
assign ( resid   28 and name hb#  )( resid   29 and name hn   ) 0.0 0.0 3.5
assign ( resid   28 and name he#  )( resid   29 and name hn   ) 0.0 0.0 5.0
assign ( resid   29 and name ha   )( resid   30 and name ha   ) 0.0 0.0 5.0
assign ( resid   29 and name ha   )( resid   30 and name hn   ) 0.0 0.0 2.8
assign ( resid   29 and name hg1# )( resid   30 and name hn   ) 0.0 0.0 3.5
assign ( resid   29 and name hg2# )( resid   30 and name hn   ) 0.0 0.0 3.5
assign ( resid   29 and name hb   )( resid   30 and name hn   ) 0.0 0.0 3.5
assign ( resid   30 and name ha   )( resid   31 and name hn   ) 0.0 0.0 2.8
assign ( resid   30 and name hb#  )( resid   31 and name hn   ) 0.0 0.0 5.0
assign ( resid   31 and name ha   )( resid   32 and name hn   ) 0.0 0.0 2.8
assign ( resid   31 and name hb1  )( resid   32 and name hn   ) 0.0 0.0 5.0
assign ( resid   31 and name hb2  )( resid   32 and name hn   ) 0.0 0.0 3.5
assign ( resid   32 and name ha   )( resid   33 and name ha   ) 0.0 0.0 5.0
assign ( resid   32 and name ha   )( resid   33 and name hn   ) 0.0 0.0 2.8
assign ( resid   32 and name hg2# )( resid   33 and name hn   ) 0.0 0.0 3.5
assign ( resid   33 and name ha   )( resid   34 and name hn   ) 0.0 0.0 3.5
assign ( resid   34 and name ha   )( resid   35 and name hn   ) 0.0 0.0 3.5
assign ( resid   34 and name hd1# )( resid   35 and name hn   ) 0.0 0.0 5.0
assign ( resid   35 and name ha2  )( resid   36 and name hn   ) 0.0 0.0 3.5
assign ( resid   35 and name ha1  )( resid   36 and name hn   ) 0.0 0.0 3.5
assign ( resid   36 and name ha   )( resid   37 and name hn   ) 0.0 0.0 3.5
assign ( resid   36 and name hn   )( resid   37 and name hn   ) 0.0 0.0 3.5
assign ( resid   36 and name hg1  )( resid   37 and name hn   ) 0.0 0.0 5.0
assign ( resid   36 and name hb#  )( resid   37 and name hn   ) 0.0 0.0 5.0
assign ( resid   37 and name ha2  )( resid   38 and name hn   ) 0.0 0.0 3.5
assign ( resid   37 and name ha1  )( resid   38 and name hn   ) 0.0 0.0 3.5
assign ( resid   37 and name hn   )( resid   38 and name hn   ) 0.0 0.0 3.5
assign ( resid   38 and name ha   )( resid   39 and name hn   ) 0.0 0.0 5.0
assign ( resid   38 and name hn   )( resid   39 and name hn   ) 0.0 0.0 5.0
assign ( resid   39 and name ha1  )( resid   40 and name hn   ) 0.0 0.0 5.0
assign ( resid   40 and name ha   )( resid   41 and name hn   ) 0.0 0.0 5.0
assign ( resid   41 and name ha   )( resid   42 and name hn   ) 0.0 0.0 2.8
assign ( resid   41 and name hg2# )( resid   42 and name hn   ) 0.0 0.0 3.5
assign ( resid   41 and name hg2# )( resid   42 and name ha   ) 0.0 0.0 5.0
assign ( resid   41 and name hb   )( resid   42 and name hn   ) 0.0 0.0 5.0
assign ( resid   41 and name hg1# )( resid   42 and name hn   ) 0.0 0.0 5.0
assign ( resid   42 and name ha   )( resid   43 and name hn   ) 0.0 0.0 3.5
assign ( resid   42 and name hb   )( resid   43 and name hn   ) 0.0 0.0 5.0
assign ( resid   42 and name hg2# )( resid   43 and name hn   ) 0.0 0.0 3.5
assign ( resid   43 and name ha   )( resid   44 and name hg2# ) 0.0 0.0 5.0
assign ( resid   43 and name ha   )( resid   44 and name hn   ) 0.0 0.0 2.8
assign ( resid   43 and name hb#  )( resid   44 and name hn   ) 0.0 0.0 3.5
assign ( resid   44 and name ha   )( resid   45 and name hn   ) 0.0 0.0 2.8
assign ( resid   44 and name hg2# )( resid   45 and name hn   ) 0.0 0.0 5.0
assign ( resid   45 and name hn   )( resid   46 and name hn   ) 0.0 0.0 3.5
assign ( resid   45 and name ha   )( resid   46 and name hn   ) 0.0 0.0 3.5
assign ( resid   45 and name hn   )( resid   46 and name hg1  ) 0.0 0.0 5.0
assign ( resid   46 and name ha   )( resid   47 and name hn   ) 0.0 0.0 5.0
assign ( resid   47 and name ha   )( resid   48 and name hn   ) 0.0 0.0 5.0
assign ( resid   47 and name hn   )( resid   48 and name hn   ) 0.0 0.0 5.0
assign ( resid   48 and name hg2  )( resid   49 and name hn   ) 0.0 0.0 3.5
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assign ( resid   48 and name ha   )( resid   49 and name hn   ) 0.0 0.0 5.0
assign ( resid   49 and name ha   )( resid   50 and name hn   ) 0.0 0.0 2.8
assign ( resid   50 and name ha   )( resid   51 and name hn   ) 0.0 0.0 3.5
assign ( resid   50 and name hb2  )( resid   51 and name hn   ) 0.0 0.0 3.5
assign ( resid   50 and name hb1  )( resid   51 and name hn   ) 0.0 0.0 5.0
assign ( resid   51 and name ha   )( resid   52 and name hn   ) 0.0 0.0 3.5
assign ( resid   52 and name ha   )( resid   53 and name hn   ) 0.0 0.0 5.0
assign ( resid   53 and name hb#  )( resid   54 and name hn   ) 0.0 0.0 3.5
assign ( resid   53 and name ha   )( resid   54 and name hn   ) 0.0 0.0 5.0
assign ( resid   53 and name hn   )( resid   54 and name hn   ) 0.0 0.0 3.5
assign ( resid   54 and name hn   )( resid   55 and name ha   ) 0.0 0.0 3.5
assign ( resid   54 and name hb   )( resid   55 and name hn   ) 0.0 0.0 5.0
assign ( resid   54 and name ha   )( resid   55 and name hn   ) 0.0 0.0 5.0
assign ( resid   55 and name ha   )( resid   56 and name hn   ) 0.0 0.0 3.5
assign ( resid   55 and name hg1  )( resid   56 and name hn   ) 0.0 0.0 5.0
assign ( resid   56 and name hg2# )( resid   57 and name hn   ) 0.0 0.0 2.8
assign ( resid   56 and name hg2# )( resid   57 and name ha   ) 0.0 0.0 5.0
assign ( resid   56 and name ha   )( resid   57 and name hn   ) 0.0 0.0 5.0
assign ( resid   57 and name ha   )( resid   58 and name hn   ) 0.0 0.0 3.5
assign ( resid   58 and name ha   )( resid   59 and name hn   ) 0.0 0.0 5.0
assign ( resid   58 and name hb1  )( resid   59 and name hn   ) 0.0 0.0 5.0
assign ( resid   58 and name hd#  )( resid   59 and name hn   ) 0.0 0.0 5.0
assign ( resid   59 and name ha   )( resid   60 and name hn   ) 0.0 0.0 5.0
assign ( resid   59 and name hb#  )( resid   60 and name hn   ) 0.0 0.0 5.0
assign ( resid   60 and name ha   )( resid   61 and name hn   ) 0.0 0.0 2.8
assign ( resid   60 and name hb   )( resid   61 and name hn   ) 0.0 0.0 3.5
assign ( resid   60 and name hn   )( resid   61 and name hn   ) 0.0 0.0 5.0
assign ( resid   60 and name hg1# )( resid   61 and name hn   ) 0.0 0.0 5.0
assign ( resid   60 and name hg2# )( resid   61 and name hn   ) 0.0 0.0 3.5
assign ( resid   61 and name ha1  )( resid   62 and name hn   ) 0.0 0.0 3.5
assign ( resid   61 and name hn   )( resid   62 and name hn   ) 0.0 0.0 3.5
assign ( resid   62 and name ha   )( resid   63 and name hg2# ) 0.0 0.0 5.0
assign ( resid   62 and name ha   )( resid   63 and name hn   ) 0.0 0.0 2.8
assign ( resid   62 and name hb1  )( resid   63 and name hn   ) 0.0 0.0 3.5
assign ( resid   62 and name hb2  )( resid   63 and name hn   ) 0.0 0.0 5.0
assign ( resid   62 and name hn   )( resid   63 and name hn   ) 0.0 0.0 5.0
assign ( resid   63 and name ha   )( resid   64 and name he3  ) 0.0 0.0 3.5
assign ( resid   63 and name ha   )( resid   64 and name hz2  ) 0.0 0.0 5.0
assign ( resid   63 and name hb   )( resid   64 and name he3  ) 0.0 0.0 5.0
assign ( resid   63 and name hg2# )( resid   64 and name he3  ) 0.0 0.0 5.0
assign ( resid   63 and name ha   )( resid   64 and name hn   ) 0.0 0.0 2.8
assign ( resid   63 and name hb   )( resid   64 and name hn   ) 0.0 0.0 3.5
assign ( resid   63 and name hg2# )( resid   64 and name hn   ) 0.0 0.0 3.5
assign ( resid   63 and name hn   )( resid   64 and name he3  ) 0.0 0.0 3.5
assign ( resid   64 and name hd1  )( resid   65 and name ha   ) 0.0 0.0 5.0
assign ( resid   64 and name ha   )( resid   65 and name hn   ) 0.0 0.0 2.8
assign ( resid   64 and name hd1  )( resid   65 and name hn   ) 0.0 0.0 3.5
assign ( resid   64 and name hb2  )( resid   65 and name hn   ) 0.0 0.0 3.5
assign ( resid   64 and name hb1  )( resid   65 and name hn   ) 0.0 0.0 3.5
assign ( resid   65 and name hb#  )( resid   66 and name ha   ) 0.0 0.0 5.0
assign ( resid   65 and name ha   )( resid   66 and name hn   ) 0.0 0.0 2.8
assign ( resid   65 and name hb#  )( resid   66 and name hn   ) 0.0 0.0 3.5
assign ( resid   66 and name hb2  )( resid   67 and name hn   ) 0.0 0.0 3.5
assign ( resid   66 and name hd#  )( resid   67 and name hn   ) 0.0 0.0 3.5
assign ( resid   66 and name ha   )( resid   67 and name hn   ) 0.0 0.0 5.0
assign ( resid   66 and name hg#  )( resid   67 and name hn   ) 0.0 0.0 5.0
assign ( resid   67 and name hg2  )( resid   68 and name hb2  ) 0.0 0.0 5.0
assign ( resid   67 and name ha   )( resid   68 and name hn   ) 0.0 0.0 2.8
assign ( resid   67 and name hb#  )( resid   68 and name hn   ) 0.0 0.0 3.5
assign ( resid   67 and name hd#  )( resid   68 and name hn   ) 0.0 0.0 3.5
assign ( resid   68 and name hb1  )( resid   69 and name hn   ) 0.0 0.0 3.5
assign ( resid   68 and name ha   )( resid   69 and name hn   ) 0.0 0.0 3.5
assign ( resid   68 and name hb2  )( resid   69 and name hn   ) 0.0 0.0 3.5
assign ( resid   68 and name ha   )( resid   69 and name hn   ) 0.0 0.0 5.0
assign ( resid   69 and name hn   )( resid   70 and name hn   ) 0.0 0.0 2.8
assign ( resid   70 and name ha   )( resid   71 and name hn   ) 0.0 0.0 3.5
assign ( resid   70 and name hb2  )( resid   71 and name hn   ) 0.0 0.0 3.5
assign ( resid   70 and name hb1  )( resid   71 and name hn   ) 0.0 0.0 3.5
assign ( resid   70 and name hn   )( resid   71 and name ha1  ) 0.0 0.0 5.0
assign ( resid   70 and name hg1  )( resid   71 and name hn   ) 0.0 0.0 5.0
assign ( resid   70 and name hn   )( resid   71 and name hn   ) 0.0 0.0 2.8
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assign ( resid   71 and name ha1  )( resid   72 and name hn   ) 0.0 0.0 3.5
assign ( resid   71 and name ha2  )( resid   72 and name hn   ) 0.0 0.0 5.0
assign ( resid   71 and name hn   )( resid   72 and name hn   ) 0.0 0.0 2.8
assign ( resid   72 and name hb1  )( resid   73 and name hn   ) 0.0 0.0 3.5
assign ( resid   72 and name hb2  )( resid   73 and name hn   ) 0.0 0.0 3.5
assign ( resid   73 and name ha   )( resid   74 and name hn   ) 0.0 0.0 2.8
assign ( resid   73 and name hb#  )( resid   74 and name hn   ) 0.0 0.0 3.5
assign ( resid   73 and name hg   )( resid   74 and name hn   ) 0.0 0.0 3.5
assign ( resid   73 and name hd2# )( resid   74 and name hn   ) 0.0 0.0 3.5
assign ( resid   73 and name hn   )( resid   74 and name hn   ) 0.0 0.0 5.0
assign ( resid   74 and name ha   )( resid   75 and name hn   ) 0.0 0.0 2.8
assign ( resid   74 and name hb1  )( resid   75 and name hn   ) 0.0 0.0 3.5
assign ( resid   74 and name hd1# )( resid   75 and name hn   ) 0.0 0.0 5.0
assign ( resid   74 and name hb2  )( resid   75 and name hn   ) 0.0 0.0 3.5
assign ( resid   75 and name ha   )( resid   76 and name hn   ) 0.0 0.0 2.8
assign ( resid   75 and name hb1  )( resid   76 and name hn   ) 0.0 0.0 3.5
assign ( resid   75 and name hb2  )( resid   76 and name hn   ) 0.0 0.0 5.0
assign ( resid   75 and name hg1  )( resid   76 and name hn   ) 0.0 0.0 5.0
assign ( resid   75 and name hg2  )( resid   76 and name hn   ) 0.0 0.0 5.0
assign ( resid   76 and name hb2  )( resid   77 and name hn   ) 0.0 0.0 3.5
assign ( resid   76 and name ha   )( resid   77 and name hn   ) 0.0 0.0 3.5
assign ( resid   77 and name ha   )( resid   78 and name hb1  ) 0.0 0.0 5.0
assign ( resid   77 and name ha   )( resid   78 and name hn   ) 0.0 0.0 3.5
assign ( resid   77 and name hg12 )( resid   78 and name hn   ) 0.0 0.0 3.5
assign ( resid   77 and name hg11 )( resid   78 and name hn   ) 0.0 0.0 5.0
assign ( resid   78 and name ha   )( resid   79 and name hn   ) 0.0 0.0 3.5
assign ( resid   79 and name hn   )( resid   80 and name hn   ) 0.0 0.0 2.8
assign ( resid   80 and name ha1  )( resid   81 and name hn   ) 0.0 0.0 3.5
assign ( resid   81 and name ha   )( resid   82 and name hn   ) 0.0 0.0 3.5
assign ( resid   81 and name hn   )( resid   82 and name hn   ) 0.0 0.0 2.8
assign ( resid   81 and name hb1  )( resid   82 and name hn   ) 0.0 0.0 5.0
assign ( resid   82 and name ha1  )( resid   83 and name hb1  ) 0.0 0.0 5.0
assign ( resid   82 and name ha2  )( resid   83 and name hn   ) 0.0 0.0 5.0
assign ( resid   82 and name ha1  )( resid   83 and name hn   ) 0.0 0.0 3.5
assign ( resid   82 and name hn   )( resid   83 and name hn   ) 0.0 0.0 5.0
assign ( resid   83 and name hn   )( resid   84 and name hn   ) 0.0 0.0 3.5
assign ( resid   83 and name ha   )( resid   84 and name hn   ) 0.0 0.0 3.5
assign ( resid   83 and name hb1  )( resid   84 and name hn   ) 0.0 0.0 3.5
assign ( resid   84 and name ha1  )( resid   85 and name hn   ) 0.0 0.0 5.0
assign ( resid   84 and name ha2  )( resid   85 and name hn   ) 0.0 0.0 5.0
assign ( resid   85 and name hb#  )( resid   86 and name hn   ) 0.0 0.0 5.0
assign ( resid   85 and name ha   )( resid   86 and name hn   ) 0.0 0.0 2.8
assign ( resid   86 and name ha   )( resid   87 and name hn   ) 0.0 0.0 3.5
assign ( resid   86 and name hb1  )( resid   87 and name hn   ) 0.0 0.0 3.5
assign ( resid   86 and name hb2  )( resid   87 and name hn   ) 0.0 0.0 3.5
assign ( resid   87 and name hb2  )( resid   88 and name hn   ) 0.0 0.0 5.0
assign ( resid   88 and name ha   )( resid   89 and name hn   ) 0.0 0.0 3.5
assign ( resid   88 and name hb1  )( resid   89 and name hn   ) 0.0 0.0 3.5
assign ( resid   89 and name hg2  )( resid   90 and name hn   ) 0.0 0.0 3.5
assign ( resid   89 and name hb#  )( resid   90 and name hn   ) 0.0 0.0 3.5
assign ( resid   89 and name hg1  )( resid   90 and name hn   ) 0.0 0.0 3.5
assign ( resid   89 and name ha   )( resid   90 and name hn   ) 0.0 0.0 3.5
assign ( resid   90 and name ha   )( resid   91 and name hn   ) 0.0 0.0 3.5
assign ( resid   90 and name hg1  )( resid   91 and name hn   ) 0.0 0.0 3.5
assign ( resid   90 and name hb#  )( resid   91 and name hn   ) 0.0 0.0 5.0
assign ( resid   91 and name ha   )( resid   92 and name hn   ) 0.0 0.0 2.8
assign ( resid   91 and name hb2  )( resid   92 and name hn   ) 0.0 0.0 3.5
assign ( resid   91 and name hb1  )( resid   92 and name hn   ) 0.0 0.0 5.0
assign ( resid   92 and name ha   )( resid   93 and name hn   ) 0.0 0.0 2.8
assign ( resid   92 and name hg2# )( resid   93 and name hn   ) 0.0 0.0 3.5
assign ( resid   92 and name hb   )( resid   93 and name hn   ) 0.0 0.0 5.0
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MEDIUM RANGE NOES

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assign ( resid    3 and name hb#  )( resid    5 and name hd#  ) 0.0 0.0 5.0
assign ( resid    5 and name hd#  )( resid    7 and name ha   ) 0.0 0.0 5.0
assign ( resid    6 and name hb2  )( resid    9 and name hb#  ) 0.0 0.0 5.0
assign ( resid    6 and name hb1  )( resid    9 and name hb#  ) 0.0 0.0 5.0
assign ( resid    7 and name ha   )( resid   10 and name ha2  ) 0.0 0.0 5.0
assign ( resid    8 and name hn   )( resid   10 and name hn   ) 0.0 0.0 3.5
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assign ( resid    9 and name hb#  )( resid   13 and name he#  ) 0.0 0.0 5.0
assign ( resid    9 and name hb#  )( resid   13 and name hd#  ) 0.0 0.0 5.0
assign ( resid    9 and name hb#  )( resid   11 and name hb   ) 0.0 0.0 5.0
assign ( resid    9 and name hb#  )( resid   11 and name hn   ) 0.0 0.0 2.8
assign ( resid   11 and name ha   )( resid   13 and name he#  ) 0.0 0.0 5.0
assign ( resid   11 and name hb   )( resid   13 and name he#  ) 0.0 0.0 3.5
assign ( resid   11 and name hb   )( resid   13 and name hd#  ) 0.0 0.0 5.0
assign ( resid   11 and name hg2# )( resid   13 and name hd#  ) 0.0 0.0 5.0
assign ( resid   11 and name hg2# )( resid   13 and name he#  ) 0.0 0.0 3.5
assign ( resid   13 and name hd#  )( resid   17 and name hb2  ) 0.0 0.0 5.0
assign ( resid   13 and name hd#  )( resid   17 and name hb1  ) 0.0 0.0 5.0
assign ( resid   13 and name hd#  )( resid   17 and name hg#  ) 0.0 0.0 5.0
assign ( resid   13 and name hb2  )( resid   17 and name hb2  ) 0.0 0.0 5.0
assign ( resid   13 and name he#  )( resid   17 and name hb1  ) 0.0 0.0 5.0
assign ( resid   13 and name hb1  )( resid   17 and name hb2  ) 0.0 0.0 5.0
assign ( resid   14 and name hn   )( resid   17 and name hg#  ) 0.0 0.0 3.5
assign ( resid   14 and name hn   )( resid   17 and name hb2  ) 0.0 0.0 3.5
assign ( resid   14 and name hn   )( resid   17 and name hb1  ) 0.0 0.0 3.5
assign ( resid   14 and name hg1# )( resid   17 and name hn   ) 0.0 0.0 3.5
assign ( resid   14 and name hg2# )( resid   17 and name hn   ) 0.0 0.0 3.5
assign ( resid   17 and name hg#  )( resid   19 and name he3  ) 0.0 0.0 5.0
assign ( resid   17 and name ha   )( resid   19 and name he3  ) 0.0 0.0 5.0
assign ( resid   17 and name hb1  )( resid   19 and name hz3  ) 0.0 0.0 5.0
assign ( resid   17 and name hb1  )( resid   19 and name he3  ) 0.0 0.0 5.0
assign ( resid   19 and name hd1  )( resid   21 and name hd#  ) 0.0 0.0 5.0
assign ( resid   19 and name hd1  )( resid   21 and name hb#  ) 0.0 0.0 5.0
assign ( resid   19 and name hd1  )( resid   21 and name hg#  ) 0.0 0.0 3.5
assign ( resid   19 and name hd1  )( resid   21 and name ha   ) 0.0 0.0 5.0
assign ( resid   19 and name he1  )( resid   21 and name hd#  ) 0.0 0.0 5.0
assign ( resid   19 and name he1  )( resid   21 and name hg#  ) 0.0 0.0 3.5
assign ( resid   23 and name he#  )( resid   27 and name he#  ) 0.0 0.0 3.5
assign ( resid   23 and name hd#  )( resid   27 and name hb2  ) 0.0 0.0 5.0
assign ( resid   23 and name he#  )( resid   27 and name hb2  ) 0.0 0.0 5.0
assign ( resid   23 and name hd#  )( resid   27 and name hb1  ) 0.0 0.0 5.0
assign ( resid   23 and name hb2  )( resid   27 and name hb2  ) 0.0 0.0 5.0
assign ( resid   23 and name hb1  )( resid   27 and name hb1  ) 0.0 0.0 5.0
assign ( resid   23 and name hn   )( resid   27 and name hn   ) 0.0 0.0 3.5
assign ( resid   23 and name hd#  )( resid   27 and name hg#  ) 0.0 0.0 5.0
assign ( resid   23 and name he#  )( resid   27 and name hg#  ) 0.0 0.0 5.0
assign ( resid   24 and name hb1  )( resid   26 and name hd1  ) 0.0 0.0 5.0
assign ( resid   34 and name hd1# )( resid   36 and name hg2  ) 0.0 0.0 5.0
assign ( resid   35 and name ha1  )( resid   39 and name ha1  ) 0.0 0.0 5.0
assign ( resid   44 and name hg2# )( resid   47 and name hn   ) 0.0 0.0 5.0
assign ( resid   46 and name ha   )( resid   48 and name hn   ) 0.0 0.0 5.0
assign ( resid   52 and name ha   )( resid   55 and name hg1  ) 0.0 0.0 5.0
assign ( resid   52 and name ha   )( resid   55 and name hn   ) 0.0 0.0 3.5
assign ( resid   52 and name ha   )( resid   54 and name hn   ) 0.0 0.0 5.0
assign ( resid   52 and name ha   )( resid   55 and name ha   ) 0.0 0.0 5.0
assign ( resid   53 and name hn   )( resid   55 and name hn   ) 0.0 0.0 5.0
assign ( resid   54 and name hg2# )( resid   58 and name he#  ) 0.0 0.0 5.0
assign ( resid   54 and name hg2# )( resid   56 and name hg2# ) 0.0 0.0 3.5
assign ( resid   56 and name hg2# )( resid   58 and name he#  ) 0.0 0.0 3.5
assign ( resid   56 and name hg2# )( resid   58 and name hd#  ) 0.0 0.0 5.0
assign ( resid   58 and name hd#  )( resid   62 and name hb2  ) 0.0 0.0 5.0
assign ( resid   59 and name hn   )( resid   62 and name hb1  ) 0.0 0.0 3.5
assign ( resid   59 and name hn   )( resid   62 and name hb2  ) 0.0 0.0 3.5
assign ( resid   60 and name hg2# )( resid   62 and name hn   ) 0.0 0.0 5.0
assign ( resid   62 and name hb1  )( resid   64 and name he3  ) 0.0 0.0 5.0
assign ( resid   62 and name ha   )( resid   64 and name he3  ) 0.0 0.0 5.0
assign ( resid   64 and name hd1  )( resid   66 and name hb2  ) 0.0 0.0 5.0
assign ( resid   64 and name hd1  )( resid   66 and name hd#  ) 0.0 0.0 5.0
assign ( resid   64 and name hd1  )( resid   66 and name hb1  ) 0.0 0.0 5.0
assign ( resid   64 and name hd1  )( resid   66 and name hg#  ) 0.0 0.0 5.0
assign ( resid   64 and name he1  )( resid   66 and name hd#  ) 0.0 0.0 3.5
assign ( resid   67 and name hb#  )( resid   71 and name ha1  ) 0.0 0.0 5.0
assign ( resid   68 and name hn   )( resid   72 and name hn   ) 0.0 0.0 3.5
assign ( resid   70 and name hb1  )( resid   72 and name hn   ) 0.0 0.0 3.5
assign ( resid   73 and name hd2# )( resid   75 and name ha   ) 0.0 0.0 5.0
assign ( resid   77 and name hg11 )( resid   79 and name hb   ) 0.0 0.0 5.0
assign ( resid   78 and name ha   )( resid   80 and name hn   ) 0.0 0.0 5.0
assign ( resid   79 and name hb   )( resid   81 and name hb1  ) 0.0 0.0 5.0
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LONG RANGE NOES

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assign ( resid    1 and name hb#  )( resid   15 and name hg2# ) 0.0 0.0 3.5
assign ( resid    2 and name hn   )( resid   15 and name hg2# ) 0.0 0.0 3.5
assign ( resid    2 and name ha   )( resid   15 and name hg2# ) 0.0 0.0 5.0
assign ( resid    2 and name hb#  )( resid   15 and name hg2# ) 0.0 0.0 2.8
assign ( resid    3 and name ha   )( resid   14 and name ha   ) 0.0 0.0 2.8
assign ( resid    3 and name ha   )( resid   14 and name hg2# ) 0.0 0.0 3.5
assign ( resid    3 and name ha   )( resid   14 and name hg1# ) 0.0 0.0 5.0
assign ( resid    3 and name he#  )( resid   14 and name hg2# ) 0.0 0.0 3.5
assign ( resid    3 and name he#  )( resid   14 and name hg1# ) 0.0 0.0 5.0
assign ( resid    3 and name hd#  )( resid   14 and name ha   ) 0.0 0.0 5.0
assign ( resid    3 and name hg#  )( resid   14 and name ha   ) 0.0 0.0 5.0
assign ( resid    3 and name hg#  )( resid   12 and name hb1  ) 0.0 0.0 5.0
assign ( resid    3 and name hb#  )( resid   12 and name hb1  ) 0.0 0.0 5.0
assign ( resid    3 and name hb#  )( resid   12 and name hb2  ) 0.0 0.0 5.0
assign ( resid    3 and name he#  )( resid   12 and name hb2  ) 0.0 0.0 5.0
assign ( resid    3 and name hd#  )( resid   12 and name hb1  ) 0.0 0.0 5.0
assign ( resid    3 and name hb#  )( resid   13 and name hn   ) 0.0 0.0 3.5
assign ( resid    3 and name ha   )( resid   15 and name hn   ) 0.0 0.0 5.0
assign ( resid    3 and name hd#  )( resid   13 and name hn   ) 0.0 0.0 5.0
assign ( resid    4 and name ha   )( resid   39 and name ha2  ) 0.0 0.0 3.5
assign ( resid    4 and name ha   )( resid   39 and name ha1  ) 0.0 0.0 5.0
assign ( resid    4 and name hb2  )( resid   19 and name hh2  ) 0.0 0.0 5.0
assign ( resid    4 and name hb1  )( resid   19 and name hh2  ) 0.0 0.0 5.0
assign ( resid    4 and name hb2  )( resid   41 and name hg1# ) 0.0 0.0 3.5
assign ( resid    4 and name hb1  )( resid   41 and name hg1# ) 0.0 0.0 5.0
assign ( resid    4 and name hb2  )( resid   33 and name ha   ) 0.0 0.0 5.0
assign ( resid    4 and name hb1  )( resid   33 and name ha   ) 0.0 0.0 5.0
assign ( resid    4 and name hn   )( resid   14 and name ha   ) 0.0 0.0 3.5
assign ( resid    4 and name hn   )( resid   13 and name hn   ) 0.0 0.0 3.5
assign ( resid    4 and name hn   )( resid   13 and name hd#  ) 0.0 0.0 5.0
assign ( resid    5 and name he#  )( resid   12 and name hb2  ) 0.0 0.0 5.0
assign ( resid    5 and name he#  )( resid   12 and name hb1  ) 0.0 0.0 5.0
assign ( resid    5 and name hd#  )( resid   12 and name hb1  ) 0.0 0.0 5.0
assign ( resid    5 and name hd#  )( resid   12 and name hb2  ) 0.0 0.0 5.0
assign ( resid    5 and name he#  )( resid   12 and name ha   ) 0.0 0.0 5.0
assign ( resid    5 and name hb2  )( resid   13 and name he#  ) 0.0 0.0 5.0
assign ( resid    5 and name ha   )( resid   12 and name hn   ) 0.0 0.0 5.0
assign ( resid    5 and name hn   )( resid   41 and name hg1# ) 0.0 0.0 3.5
assign ( resid    5 and name he#  )( resid   10 and name ha1  ) 0.0 0.0 5.0
assign ( resid    5 and name he#  )( resid   11 and name ha   ) 0.0 0.0 5.0
assign ( resid    5 and name hd#  )( resid   12 and name ha   ) 0.0 0.0 5.0
assign ( resid    6 and name hb1  )( resid   13 and name he#  ) 0.0 0.0 3.5
assign ( resid    6 and name hb1  )( resid   13 and name hd#  ) 0.0 0.0 5.0
assign ( resid    6 and name hb2  )( resid   13 and name he#  ) 0.0 0.0 3.5
assign ( resid    6 and name hb2  )( resid   13 and name hd#  ) 0.0 0.0 5.0
assign ( resid    6 and name hb2  )( resid   19 and name hz2  ) 0.0 0.0 5.0
assign ( resid    6 and name hn   )( resid   13 and name he#  ) 0.0 0.0 3.5
assign ( resid    6 and name hn   )( resid   11 and name hn   ) 0.0 0.0 3.5
assign ( resid    6 and name hn   )( resid   13 and name hd#  ) 0.0 0.0 3.5
assign ( resid    6 and name hb2  )( resid   11 and name hn   ) 0.0 0.0 3.5
assign ( resid    6 and name hb1  )( resid   11 and name hn   ) 0.0 0.0 5.0
assign ( resid    6 and name ha   )( resid   19 and name hz2  ) 0.0 0.0 5.0
assign ( resid    6 and name hb2  )( resid   19 and name he1  ) 0.0 0.0 5.0
assign ( resid   13 and name hd#  )( resid   19 and name hh2  ) 0.0 0.0 5.0
assign ( resid   13 and name hd#  )( resid   19 and name he3  ) 0.0 0.0 5.0
assign ( resid   13 and name hb1  )( resid   19 and name hh2  ) 0.0 0.0 5.0
assign ( resid   13 and name he#  )( resid   19 and name ha   ) 0.0 0.0 5.0
assign ( resid   13 and name he#  )( resid   19 and name hb2  ) 0.0 0.0 5.0
assign ( resid   13 and name hd#  )( resid   19 and name hb1  ) 0.0 0.0 5.0
assign ( resid   13 and name he#  )( resid   41 and name hg1# ) 0.0 0.0 5.0
assign ( resid   13 and name hd#  )( resid   19 and name hz2  ) 0.0 0.0 5.0
assign ( resid   13 and name he#  )( resid   19 and name hz2  ) 0.0 0.0 5.0
assign ( resid   13 and name he#  )( resid   19 and name hd1  ) 0.0 0.0 5.0
assign ( resid   13 and name he#  )( resid   19 and name hb1  ) 0.0 0.0 5.0
assign ( resid   13 and name hd#  )( resid   19 and name hb2  ) 0.0 0.0 5.0
assign ( resid   13 and name hd#  )( resid   41 and name hg1# ) 0.0 0.0 5.0
assign ( resid   13 and name hb1  )( resid   33 and name hb2  ) 0.0 0.0 5.0
assign ( resid   13 and name he#  )( resid   19 and name he1  ) 0.0 0.0 5.0
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assign ( resid   13 and name hb2  )( resid   19 and name he3  ) 0.0 0.0 5.0
assign ( resid   13 and name hb2  )( resid   19 and name hz3  ) 0.0 0.0 5.0
assign ( resid   13 and name hb1  )( resid   19 and name he3  ) 0.0 0.0 5.0
assign ( resid   13 and name hb1  )( resid   19 and name hz3  ) 0.0 0.0 5.0
assign ( resid   13 and name hd#  )( resid   19 and name hz3  ) 0.0 0.0 5.0
assign ( resid   13 and name he#  )( resid   19 and name he3  ) 0.0 0.0 5.0
assign ( resid   15 and name ha   )( resid   33 and name ha   ) 0.0 0.0 5.0
assign ( resid   15 and name hg1# )( resid   35 and name ha1  ) 0.0 0.0 5.0
assign ( resid   15 and name hg1# )( resid   34 and name ha   ) 0.0 0.0 3.5
assign ( resid   15 and name ha   )( resid   33 and name hb1  ) 0.0 0.0 5.0
assign ( resid   15 and name ha   )( resid   33 and name hb2  ) 0.0 0.0 3.5
assign ( resid   15 and name hg1# )( resid   35 and name ha2  ) 0.0 0.0 3.5
assign ( resid   15 and name hg1# )( resid   35 and name hn   ) 0.0 0.0 resid
assign ( resid   16 and name hn   )( resid   33 and name hb2  ) 0.0 0.0 3.5
assign ( resid   17 and name hn   )( resid   33 and name hb1  ) 0.0 0.0 3.5
assign ( resid   17 and name hn   )( resid   33 and name hb2  ) 0.0 0.0 2.8
assign ( resid   18 and name ha   )( resid   32 and name hb   ) 0.0 0.0 5.0
assign ( resid   18 and name hb   )( resid   32 and name ha   ) 0.0 0.0 5.0
assign ( resid   18 and name ha   )( resid   32 and name hg2# ) 0.0 0.0 5.0
assign ( resid   18 and name hg2# )( resid   32 and name ha   ) 0.0 0.0 3.5
assign ( resid   18 and name ha   )( resid   32 and name ha   ) 0.0 0.0 2.8
assign ( resid   18 and name hg2# )( resid   32 and name hb   ) 0.0 0.0 5.0
assign ( resid   18 and name hg2# )( resid   30 and name ha   ) 0.0 0.0 5.0
assign ( resid   18 and name hg2# )( resid   31 and name hn   ) 0.0 0.0 5.0
assign ( resid   18 and name ha   )( resid   33 and name hn   ) 0.0 0.0 5.0
assign ( resid   19 and name hd1  )( resid   31 and name hb1  ) 0.0 0.0 5.0
assign ( resid   19 and name hz3  )( resid   33 and name ha   ) 0.0 0.0 5.0
assign ( resid   19 and name hh2  )( resid   33 and name ha   ) 0.0 0.0 5.0
assign ( resid   19 and name he3  )( resid   33 and name hb2  ) 0.0 0.0 5.0
assign ( resid   19 and name hh2  )( resid   33 and name hb1  ) 0.0 0.0 5.0
assign ( resid   19 and name he3  )( resid   32 and name hg2# ) 0.0 0.0 5.0
assign ( resid   19 and name he3  )( resid   31 and name hb1  ) 0.0 0.0 5.0
assign ( resid   19 and name hh2  )( resid   41 and name hg1# ) 0.0 0.0 3.5
assign ( resid   19 and name he3  )( resid   31 and name hb1  ) 0.0 0.0 5.0
assign ( resid   19 and name hh2  )( resid   41 and name hg1# ) 0.0 0.0 3.5
assign ( resid   19 and name hz3  )( resid   41 and name hg1# ) 0.0 0.0 5.0
assign ( resid   19 and name hh2  )( resid   41 and name hg2# ) 0.0 0.0 5.0
assign ( resid   19 and name hh2  )( resid   32 and name ha   ) 0.0 0.0 5.0
assign ( resid   19 and name hh2  )( resid   41 and name hd1# ) 0.0 0.0 5.0
assign ( resid   19 and name hh2  )( resid   31 and name hb1  ) 0.0 0.0 5.0
assign ( resid   19 and name hz2  )( resid   41 and name hg1# ) 0.0 0.0 3.5
assign ( resid   19 and name hz2  )( resid   41 and name hg2# ) 0.0 0.0 3.5
assign ( resid   19 and name hz2  )( resid   41 and name hd1# ) 0.0 0.0 5.0
assign ( resid   19 and name hz2  )( resid   41 and name ha   ) 0.0 0.0 5.0
assign ( resid   19 and name hn   )( resid   31 and name hn   ) 0.0 0.0 3.5
assign ( resid   19 and name he1  )( resid   41 and name hd1# ) 0.0 0.0 5.0
assign ( resid   19 and name he3  )( resid   33 and name hn   ) 0.0 0.0 3.5
assign ( resid   19 and name hz3  )( resid   32 and name ha   ) 0.0 0.0 3.5
assign ( resid   19 and name hz3  )( resid   33 and name hb2  ) 0.0 0.0 3.5
assign ( resid   19 and name hh2  )( resid   31 and name hb2  ) 0.0 0.0 5.0
assign ( resid   19 and name hh2  )( resid   41 and name hb   ) 0.0 0.0 5.0
assign ( resid   19 and name hz2  )( resid   41 and name hb   ) 0.0 0.0 5.0
assign ( resid   19 and name hz2  )( resid   31 and name hb2  ) 0.0 0.0 5.0
assign ( resid   19 and name hn   )( resid   32 and name ha   ) 0.0 0.0 3.5
assign ( resid   19 and name hz3  )( resid   33 and name hn   ) 0.0 0.0 3.5
assign ( resid   19 and name hz3  )( resid   33 and name hb1  ) 0.0 0.0 3.5
assign ( resid   20 and name ha   )( resid   31 and name hn   ) 0.0 0.0 3.5
assign ( resid   20 and name ha   )( resid   30 and name ha   ) 0.0 0.0 2.8
assign ( resid   20 and name hb2  )( resid   30 and name ha   ) 0.0 0.0 5.0
assign ( resid   21 and name hg#  )( resid   29 and name hg2# ) 0.0 0.0 3.5
assign ( resid   21 and name hb#  )( resid   29 and name hg2# ) 0.0 0.0 3.5
assign ( resid   21 and name he#  )( resid   29 and name hg2# ) 0.0 0.0 5.0
assign ( resid   21 and name hn   )( resid   29 and name hg2# ) 0.0 0.0 3.5
assign ( resid   21 and name hn   )( resid   29 and name hg1# ) 0.0 0.0 5.0
assign ( resid   21 and name hn   )( resid   30 and name ha   ) 0.0 0.0 3.5
assign ( resid   21 and name hn   )( resid   29 and name hn   ) 0.0 0.0 3.5
assign ( resid   22 and name ha   )( resid   29 and name hn   ) 0.0 0.0 3.5
assign ( resid   22 and name ha   )( resid   28 and name ha   ) 0.0 0.0 2.8
assign ( resid   23 and name hd#  )( resid   29 and name hg2# ) 0.0 0.0 5.0
assign ( resid   23 and name he#  )( resid   29 and name hg2# ) 0.0 0.0 5.0
assign ( resid   23 and name hb2  )( resid   29 and name hg1# ) 0.0 0.0 5.0
assign ( resid   23 and name hb2  )( resid   29 and name hg2# ) 0.0 0.0 5.0
assign ( resid   23 and name hb1  )( resid   29 and name hg1# ) 0.0 0.0 5.0
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assign ( resid   23 and name hb1  )( resid   29 and name hg2# ) 0.0 0.0 5.0
assign ( resid   23 and name ha   )( resid   29 and name hg2# ) 0.0 0.0 5.0
assign ( resid   23 and name ha   )( resid   29 and name hg1# ) 0.0 0.0 5.0
assign ( resid   23 and name hn   )( resid   28 and name ha   ) 0.0 0.0 3.5
assign ( resid   23 and name hd#  )( resid   29 and name hg1# ) 0.0 0.0 5.0
assign ( resid   23 and name he#  )( resid   29 and name hg1# ) 0.0 0.0 5.0
assign ( resid   26 and name hz2  )( resid   55 and name hg1  ) 0.0 0.0 5.0
assign ( resid   26 and name hz2  )( resid   55 and name hg2  ) 0.0 0.0 5.0
assign ( resid   26 and name hd1  )( resid   55 and name hb2  ) 0.0 0.0 5.0
assign ( resid   26 and name hz2  )( resid   55 and name ha   ) 0.0 0.0 5.0
assign ( resid   26 and name hz2  )( resid   50 and name hb1  ) 0.0 0.0 5.0
assign ( resid   26 and name hd1  )( resid   55 and name hd#  ) 0.0 0.0 3.5
assign ( resid   26 and name hz2  )( resid   52 and name hg1  ) 0.0 0.0 5.0
assign ( resid   26 and name he3  )( resid   56 and name ha   ) 0.0 0.0 5.0
assign ( resid   26 and name hh2  )( resid   50 and name ha   ) 0.0 0.0 5.0
assign ( resid   26 and name hh2  )( resid   52 and name ha   ) 0.0 0.0 3.5
assign ( resid   26 and name hh2  )( resid   50 and name hb2  ) 0.0 0.0 5.0
assign ( resid   26 and name hh2  )( resid   52 and name hg#  ) 0.0 0.0 5.0
assign ( resid   26 and name hh2  )( resid   50 and name hb1  ) 0.0 0.0 5.0
assign ( resid   26 and name hh2  )( resid   52 and name hb#  ) 0.0 0.0 5.0
assign ( resid   26 and name hh2  )( resid   55 and name ha   ) 0.0 0.0 5.0
assign ( resid   26 and name he3  )( resid   50 and name ha   ) 0.0 0.0 5.0
assign ( resid   26 and name hz3  )( resid   50 and name ha   ) 0.0 0.0 5.0
assign ( resid   26 and name hz3  )( resid   50 and name hb2  ) 0.0 0.0 5.0
assign ( resid   26 and name hz3  )( resid   50 and name hb1  ) 0.0 0.0 5.0
assign ( resid   26 and name hz3  )( resid   55 and name ha   ) 0.0 0.0 5.0
assign ( resid   26 and name hz3  )( resid   52 and name ha   ) 0.0 0.0 5.0
assign ( resid   26 and name he3  )( resid   57 and name hb2  ) 0.0 0.0 5.0
assign ( resid   26 and name he3  )( resid   55 and name ha   ) 0.0 0.0 3.5
assign ( resid   26 and name he3  )( resid   55 and name hb2  ) 0.0 0.0 5.0
assign ( resid   26 and name he3  )( resid   55 and name hb1  ) 0.0 0.0 5.0
assign ( resid   26 and name hz3  )( resid   51 and name hn   ) 0.0 0.0 5.0
assign ( resid   26 and name he3  )( resid   57 and name hn   ) 0.0 0.0 3.5
assign ( resid   26 and name hh2  )( resid   52 and name hn   ) 0.0 0.0 5.0
assign ( resid   26 and name he3  )( resid   50 and name hb1  ) 0.0 0.0 5.0
assign ( resid   26 and name he1  )( resid   55 and name hd#  ) 0.0 0.0 5.0
assign ( resid   26 and name he1  )( resid   55 and name hg1  ) 0.0 0.0 5.0
assign ( resid   26 and name hz2  )( resid   52 and name hb#  ) 0.0 0.0 5.0
assign ( resid   26 and name hz2  )( resid   52 and name ha   ) 0.0 0.0 5.0
assign ( resid   26 and name he3  )( resid   57 and name ha   ) 0.0 0.0 5.0
assign ( resid   26 and name he3  )( resid   57 and name hb1  ) 0.0 0.0 5.0
assign ( resid   26 and name hz3  )( resid   56 and name hn   ) 0.0 0.0 5.0
assign ( resid   26 and name hd1  )( resid   55 and name hb1  ) 0.0 0.0 5.0
assign ( resid   27 and name he#  )( resid   50 and name hb2  ) 0.0 0.0 3.5
assign ( resid   27 and name hg#  )( resid   50 and name hb2  ) 0.0 0.0 5.0
assign ( resid   27 and name hg#  )( resid   50 and name hb1  ) 0.0 0.0 5.0
assign ( resid   27 and name he#  )( resid   50 and name hb1  ) 0.0 0.0 5.0
assign ( resid   28 and name hg1  )( resid   57 and name hb1  ) 0.0 0.0 5.0
assign ( resid   28 and name hn   )( resid   57 and name hb2  ) 0.0 0.0 3.5
assign ( resid   29 and name hg1# )( resid   45 and name ha   ) 0.0 0.0 5.0
assign ( resid   29 and name ha   )( resid   45 and name ha   ) 0.0 0.0 3.5
assign ( resid   29 and name hg1# )( resid   45 and name hb1  ) 0.0 0.0 5.0
assign ( resid   29 and name hg1# )( resid   45 and name hb2  ) 0.0 0.0 5.0
assign ( resid   29 and name ha   )( resid   45 and name hb1  ) 0.0 0.0 5.0
assign ( resid   29 and name hb   )( resid   44 and name hn   ) 0.0 0.0 3.5
assign ( resid   29 and name hg1# )( resid   44 and name hn   ) 0.0 0.0 5.0
assign ( resid   29 and name hg2# )( resid   44 and name hn   ) 0.0 0.0 5.0
assign ( resid   30 and name hn   )( resid   44 and name hn   ) 0.0 0.0 3.5
assign ( resid   31 and name ha   )( resid   43 and name ha   ) 0.0 0.0 2.8
assign ( resid   31 and name hb1  )( resid   43 and name ha   ) 0.0 0.0 5.0
assign ( resid   31 and name ha   )( resid   44 and name hn   ) 0.0 0.0 3.5
assign ( resid   32 and name hn   )( resid   42 and name hn   ) 0.0 0.0 3.5
assign ( resid   32 and name hn   )( resid   42 and name hg2# ) 0.0 0.0 2.8
assign ( resid   32 and name hn   )( resid   42 and name hg2# ) 0.0 0.0 3.5
assign ( resid   33 and name ha   )( resid   41 and name hg1# ) 0.0 0.0 3.5
assign ( resid   33 and name hb2  )( resid   41 and name hg1# ) 0.0 0.0 5.0
assign ( resid   33 and name ha   )( resid   41 and name ha   ) 0.0 0.0 3.5
assign ( resid   33 and name ha   )( resid   42 and name hn   ) 0.0 0.0 5.0
assign ( resid   34 and name hn   )( resid   40 and name hn   ) 0.0 0.0 5.0
assign ( resid   34 and name hb#  )( resid   42 and name hb   ) 0.0 0.0 5.0
assign ( resid   34 and name hd2# )( resid   42 and name hn   ) 0.0 0.0 5.0
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assign ( resid   34 and name hd2# )( resid   42 and name hg2# ) 0.0 0.0 2.8
assign ( resid   35 and name ha2  )( resid   40 and name hn   ) 0.0 0.0 5.0
assign ( resid   35 and name ha1  )( resid   40 and name hn   ) 0.0 0.0 5.0
assign ( resid   46 and name hb2  )( resid   83 and name ha   ) 0.0 0.0 5.0
assign ( resid   46 and name ha   )( resid   83 and name ha   ) 0.0 0.0 5.0
assign ( resid   47 and name ha   )( resid   60 and name hb   ) 0.0 0.0 5.0
assign ( resid   47 and name ha   )( resid   60 and name hg1# ) 0.0 0.0 5.0
assign ( resid   47 and name hb1  )( resid   60 and name hg1# ) 0.0 0.0 3.5
assign ( resid   49 and name hb2  )( resid   86 and name he3  ) 0.0 0.0 5.0
assign ( resid   49 and name hb1  )( resid   86 and name he3  ) 0.0 0.0 5.0
assign ( resid   49 and name hb2  )( resid   86 and name hz3  ) 0.0 0.0 5.0
assign ( resid   49 and name hb1  )( resid   64 and name hh2  ) 0.0 0.0 5.0
assign ( resid   49 and name hb2  )( resid   64 and name hh2  ) 0.0 0.0 5.0
assign ( resid   49 and name hb1  )( resid   64 and name hz2  ) 0.0 0.0 5.0
assign ( resid   49 and name hb1  )( resid   58 and name hd#  ) 0.0 0.0 5.0
assign ( resid   50 and name ha   )( resid   57 and name ha   ) 0.0 0.0 3.5
assign ( resid   50 and name ha   )( resid   57 and name hb2  ) 0.0 0.0 5.0
assign ( resid   50 and name ha   )( resid   57 and name hb1  ) 0.0 0.0 5.0
assign ( resid   51 and name hb1  )( resid   58 and name he#  ) 0.0 0.0 5.0
assign ( resid   51 and name hb1  )( resid   64 and name hz2  ) 0.0 0.0 5.0
assign ( resid   51 and name hb2  )( resid   64 and name hd1  ) 0.0 0.0 5.0
assign ( resid   51 and name hn   )( resid   56 and name hn   ) 0.0 0.0 5.0
assign ( resid   51 and name ha   )( resid   86 and name hz2  ) 0.0 0.0 5.0
assign ( resid   53 and name hb#  )( resid   66 and name hg#  ) 0.0 0.0 5.0
assign ( resid   54 and name hg2# )( resid   64 and name hd1  ) 0.0 0.0 3.5
assign ( resid   54 and name hg2# )( resid   64 and name hb1  ) 0.0 0.0 5.0
assign ( resid   54 and name hg2# )( resid   66 and name hd#  ) 0.0 0.0 2.8
assign ( resid   54 and name hb   )( resid   64 and name hd1  ) 0.0 0.0 5.0
assign ( resid   58 and name he#  )( resid   64 and name hd1  ) 0.0 0.0 5.0
assign ( resid   58 and name hd#  )( resid   64 and name ha   ) 0.0 0.0 5.0
assign ( resid   58 and name he#  )( resid   64 and name hb2  ) 0.0 0.0 3.5
assign ( resid   58 and name he#  )( resid   64 and name hb1  ) 0.0 0.0 5.0
assign ( resid   58 and name he#  )( resid   64 and name he3  ) 0.0 0.0 5.0
assign ( resid   58 and name he#  )( resid   64 and name hb1  ) 0.0 0.0 5.0
assign ( resid   58 and name hb1  )( resid   64 and name he3  ) 0.0 0.0 5.0
assign ( resid   58 and name hb1  )( resid   64 and name hz3  ) 0.0 0.0 5.0
assign ( resid   58 and name hb1  )( resid   64 and name he3  ) 0.0 0.0 5.0
assign ( resid   60 and name ha   )( resid   78 and name hb2  ) 0.0 0.0 5.0
assign ( resid   60 and name hg1# )( resid   80 and name ha1  ) 0.0 0.0 5.0
assign ( resid   60 and name hg1# )( resid   80 and name ha2  ) 0.0 0.0 5.0
assign ( resid   60 and name hg1# )( resid   81 and name hn   ) 0.0 0.0 5.0
assign ( resid   62 and name hn   )( resid   78 and name hb1  ) 0.0 0.0 3.5
assign ( resid   63 and name hb   )( resid   77 and name ha   ) 0.0 0.0 5.0
assign ( resid   63 and name ha   )( resid   77 and name ha   ) 0.0 0.0 2.8
assign ( resid   63 and name ha   )( resid   77 and name hb   ) 0.0 0.0 5.0
assign ( resid   63 and name ha   )( resid   77 and name hg11 ) 0.0 0.0 5.0
assign ( resid   63 and name ha   )( resid   77 and name hd1# ) 0.0 0.0 5.0
assign ( resid   63 and name hg2# )( resid   77 and name hb   ) 0.0 0.0 2.8
assign ( resid   63 and name hg2# )( resid   75 and name hg1  ) 0.0 0.0 5.0
assign ( resid   63 and name hb   )( resid   75 and name hg2  ) 0.0 0.0 5.0
assign ( resid   63 and name hg2# )( resid   78 and name hn   ) 0.0 0.0 3.5
assign ( resid   63 and name hg2# )( resid   77 and name ha   ) 0.0 0.0 3.5
assign ( resid   64 and name hh2  )( resid   86 and name he3  ) 0.0 0.0 5.0
assign ( resid   64 and name hz2  )( resid   78 and name ha   ) 0.0 0.0 5.0
assign ( resid   64 and name hh2  )( resid   78 and name ha   ) 0.0 0.0 5.0
assign ( resid   64 and name hh2  )( resid   78 and name hb2  ) 0.0 0.0 5.0
assign ( resid   64 and name hz2  )( resid   86 and name hb2  ) 0.0 0.0 5.0
assign ( resid   64 and name hz2  )( resid   78 and name hb1  ) 0.0 0.0 5.0
assign ( resid   64 and name hh2  )( resid   86 and name hb2  ) 0.0 0.0 5.0
assign ( resid   64 and name hh2  )( resid   86 and name hb1  ) 0.0 0.0 5.0
assign ( resid   64 and name hh2  )( resid   86 and name ha   ) 0.0 0.0 5.0
assign ( resid   64 and name hn   )( resid   76 and name hn   ) 0.0 0.0 3.5
assign ( resid   64 and name hn   )( resid   76 and name hb1  ) 0.0 0.0 5.0
assign ( resid   65 and name hb#  )( resid   75 and name ha   ) 0.0 0.0 5.0
assign ( resid   65 and name ha   )( resid   75 and name ha   ) 0.0 0.0 2.8
assign ( resid   65 and name hb#  )( resid   73 and name hd1# ) 0.0 0.0 5.0
assign ( resid   65 and name hb#  )( resid   73 and name hd2# ) 0.0 0.0 3.5
assign ( resid   65 and name ha   )( resid   75 and name hb2  ) 0.0 0.0 5.0
assign ( resid   65 and name ha   )( resid   75 and name hb1  ) 0.0 0.0 5.0
assign ( resid   65 and name ha   )( resid   73 and name hd2# ) 0.0 0.0 5.0
assign ( resid   65 and name ha   )( resid   76 and name hn   ) 0.0 0.0 3.5
assign ( resid   66 and name hd#  )( resid   74 and name hd1# ) 0.0 0.0 5.0
assign ( resid   66 and name hn   )( resid   74 and name hn   ) 0.0 0.0 3.5
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assign ( resid   67 and name ha   )( resid   73 and name ha   ) 0.0 0.0 2.8
assign ( resid   67 and name ha   )( resid   73 and name hd2# ) 0.0 0.0 5.0
assign ( resid   67 and name ha   )( resid   73 and name hd1# ) 0.0 0.0 5.0
assign ( resid   67 and name hd#  )( resid   73 and name ha   ) 0.0 0.0 5.0
assign ( resid   67 and name ha   )( resid   74 and name hn   ) 0.0 0.0 3.5
assign ( resid   68 and name hb2  )( resid   74 and name hd1# ) 0.0 0.0 5.0
assign ( resid   68 and name hb1  )( resid   74 and name hd1# ) 0.0 0.0 5.0
assign ( resid   68 and name hn   )( resid   73 and name ha   ) 0.0 0.0 3.5
assign ( resid   72 and name hb1  )( resid   90 and name hd#  ) 0.0 0.0 5.0
assign ( resid   74 and name ha   )( resid   90 and name ha   ) 0.0 0.0 3.5
assign ( resid   74 and name ha   )( resid   88 and name hb1  ) 0.0 0.0 5.0
assign ( resid   74 and name hd1# )( resid   88 and name hb2  ) 0.0 0.0 3.5
assign ( resid   74 and name hd1# )( resid   88 and name hb1  ) 0.0 0.0 5.0
assign ( resid   74 and name hb1  )( resid   88 and name hb1  ) 0.0 0.0 5.0
assign ( resid   74 and name hb2  )( resid   88 and name hb1  ) 0.0 0.0 5.0
assign ( resid   74 and name ha   )( resid   91 and name hn   ) 0.0 0.0 3.5
assign ( resid   74 and name hb1  )( resid   89 and name hn   ) 0.0 0.0 5.0
assign ( resid   74 and name hd1# )( resid   90 and name hn   ) 0.0 0.0 5.0
assign ( resid   75 and name hn   )( resid   89 and name hn   ) 0.0 0.0 3.5
assign ( resid   76 and name ha   )( resid   88 and name hb2  ) 0.0 0.0 5.0
assign ( resid   76 and name ha   )( resid   88 and name hb1  ) 0.0 0.0 5.0
assign ( resid   76 and name ha   )( resid   88 and name ha   ) 0.0 0.0 2.8
assign ( resid   76 and name hb1  )( resid   86 and name hd1  ) 0.0 0.0 5.0
assign ( resid   76 and name hb2  )( resid   86 and name hd1  ) 0.0 0.0 5.0
assign ( resid   76 and name hb1  )( resid   88 and name ha   ) 0.0 0.0 5.0
assign ( resid   76 and name hb2  )( resid   88 and name ha   ) 0.0 0.0 5.0
assign ( resid   76 and name ha   )( resid   89 and name hn   ) 0.0 0.0 5.0
assign ( resid   77 and name hg12 )( resid   87 and name hn   ) 0.0 0.0 3.5
assign ( resid   77 and name hn   )( resid   88 and name ha   ) 0.0 0.0 3.5
assign ( resid   78 and name ha   )( resid   86 and name hb1  ) 0.0 0.0 5.0
assign ( resid   78 and name ha   )( resid   86 and name hb2  ) 0.0 0.0 5.0
assign ( resid   78 and name ha   )( resid   86 and name ha   ) 0.0 0.0 3.5
assign ( resid   79 and name hg2# )( resid   86 and name ha   ) 0.0 0.0 5.0
assign ( resid   79 and name hn   )( resid   86 and name ha   ) 0.0 0.0 3.5
assign ( resid   79 and name hg2# )( resid   87 and name hn   ) 0.0 0.0 3.5
assign ( resid   79 and name hn   )( resid   86 and name hb1  ) 0.0 0.0 5.0
assign ( resid   80 and name ha1  )( resid   85 and name hn   ) 0.0 0.0 5.0
assign ( resid   80 and name ha2  )( resid   85 and name hn   ) 0.0 0.0 5.0
```

H-bonds

```
assign (resid  31 and   name O    )(resid   19 and   name N    ) 0.0 0.0 3.2
assign (resid  31 and   name O    )(resid   19 and   name HN   ) 0.0 0.0 2.3
assign (resid  21 and   name O    )(resid   29 and   name N    ) 0.0 0.0 3.2
assign (resid  21 and   name O    )(resid   29 and   name HN   ) 0.0 0.0 2.3
assign (resid  29 and   name O    )(resid   21 and   name N    ) 0.0 0.0 3.2
assign (resid  29 and   name O    )(resid   21 and   name HN   ) 0.0 0.0 2.3
assign (resid  76 and   name O    )(resid   64 and   name N    ) 0.0 0.0 3.2
assign (resid  76 and   name O    )(resid   64 and   name HN   ) 0.0 0.0 2.3
```

CDIH restraints

```
c4,  8.745
assign  (resid  3 and name c ) (resid  4 and name n)
        (resid  4 and name ca) (resid  4 and name c) 1 -120.0 40 2
s12, 7.837
assign  (resid 11 and name c ) (resid 12 and name n)
        (resid 12 and name ca) (resid 12 and name c) 1 -100.0 40 2
y13, 9.492
assign  (resid 12 and name c ) (resid 13 and name n)
        (resid 13 and name ca) (resid 13 and name c) 1 -100.0 40 2
v14, 8.706
assign  (resid 13 and name c ) (resid 14 and name n)
        (resid 14 and name ca) (resid 14 and name c) 1 -120.0 40 2
t18, 8.889
assign  (resid 17 and name c ) (resid 18 and name n)
        (resid 18 and name ca) (resid 18 and name c) 1 -120.0 40 2
k21, 7.778
assign  (resid 20 and name c ) (resid 21 and name n)
        (resid 21 and name ca) (resid 21 and name c) 1 -100.0 40 2
t32, 8.393
assign  (resid 31 and name c ) (resid 32 and name n)
```

```
       (resid 32 and name ca) (resid 32 and name c) 1 -120.0 40 2
s67, 8.436
assign  (resid 66 and name c ) (resid 67 and name n)
       (resid 67 and name ca) (resid 67 and name c) 1 -110.0 40 2
s68, 8.265
assign  (resid 67 and name c ) (resid 68 and name n)
       (resid 68 and name ca) (resid 68 and name c) 1 -110.0 40 2
```

ISAC restraints

```
assign ( resid 4    and name N   )
       ( resid 4    and name HN  )   3.8    0.5
assign ( resid 5    and name N   )
       ( resid 5    and name HN  )   11.3    0.5
assign ( resid 10   and name N   )
       ( resid 10   and name HN  )   13.6    0.5
assign ( resid 13   and name N   )
       ( resid 13   and name HN  )   3.7    0.5
assign ( resid 16   and name N   )
       ( resid 16   and name HN  )   14.8    0.5
assign ( resid 18   and name N   )
       ( resid 18   and name HN  )   9.5    0.5
assign ( resid 19   and name N   )
       ( resid 19   and name HN  )   11.5    0.5
assign ( resid 21   and name N   )
       ( resid 21   and name HN  )   8.8    0.5
assign ( resid 23   and name N   )
       ( resid 23   and name HN  )   -25.3    0.5
assign ( resid 24   and name N   )
       ( resid 24   and name HN  )   -23.0    0.5
assign ( resid 27   and name N   )
       ( resid 27   and name HN  )   -5.6    0.5
assign ( resid 28   and name N   )
       ( resid 28   and name HN  )   -12    0.5
assign ( resid 29   and name N   )
       ( resid 29   and name HN  )   0.6    0.5
assign ( resid 31   and name N   )
       ( resid 31   and name HN  )   12.4    0.5
assign ( resid 32   and name N   )
       ( resid 32   and name HN  )   13.1    0.5
assign ( resid 33   and name N   )
       ( resid 33   and name HN  )   6.4    0.5
assign ( resid 37   and name N   )
       ( resid 37   and name HN  )   8.9    0.5
assign ( resid 38   and name N   )
       ( resid 38   and name HN  )   -12.6    0.5
assign ( resid 39   and name N   )
       ( resid 39   and name HN  )   3.6    0.5
assign ( resid 42   and name N   )
       ( resid 42   and name HN  )   13.8    0.5
assign ( resid 46   and name N   )
       ( resid 46   and name HN  )   -18.4    0.5
assign ( resid 47   and name N   )
       ( resid 47   and name HN  )   -19.4    0.5
assign ( resid 50   and name N   )
       ( resid 50   and name HN  )   8.5    0.5
assign ( resid 55   and name N   )
       ( resid 55   and name HN  )   14.2    0.5
assign ( resid 58   and name N   )
       ( resid 58   and name HN  )   9.6    0.5
assign ( resid 59   and name N   )
       ( resid 59   and name HN  )   -2    0.5
assign ( resid 61   and name N   )
       ( resid 61   and name HN  )   10.1    0.5
assign ( resid 62   and name N   )
       ( resid 62   and name HN  )   -15.7    0.5
assign ( resid 65   and name N   )
       ( resid 65   and name HN  )   4.4    0.5
assign ( resid 76   and name N   )
       ( resid 76   and name HN  )   11    0.5
assign ( resid 77   and name N   )
       ( resid 77   and name HN  )   10.2    0.5
```

```
assign ( resid 78   and name N    )
       ( resid 78   and name HN   )   11    0.5
assign ( resid 80   and name N    )
       ( resid 80   and name HN   )   6.5    0.5
assign ( resid 82   and name N    )
       ( resid 82   and name HN   )   13.3    0.5
assign ( resid 85   and name N    )
       ( resid 85   and name HN   )   12.9    0.5
assign ( resid 87   and name N    )
       ( resid 87   and name HN   )   12.3    0.5
assign ( resid 88   and name N    )
       ( resid 88   and name HN   )   12.4    0.5
```

DANI restraints

```
assign ( resid 4    and name N    )
       ( resid 4    and name HN   )   4.86    1.487
assign ( resid 5    and name N    )
       ( resid 5    and name HN   )   3.63    1.254
assign ( resid 7    and name N    )
       ( resid 7    and name HN   )   5.63    1.436
assign ( resid 10   and name N    )
       ( resid 10   and name HN   )   3.88    0.773
assign ( resid 13   and name N    )
       ( resid 13   and name HN   )   4.5     0.981
assign ( resid 16   and name N    )
       ( resid 16   and name HN   )   4.76    1.754
assign ( resid 18   and name N    )
       ( resid 18   and name HN   )   4.92    1.267
assign ( resid 19   and name N    )
       ( resid 19   and name HN   )   3.5     0.892
assign ( resid 23   and name N    )
       ( resid 23   and name HN   )   5.66    2.271
assign ( resid 27   and name N    )
       ( resid 27   and name HN   )   5.02    1.327
assign ( resid 30   and name N    )
       ( resid 30   and name HN   )   5.26    2.166
assign ( resid 31   and name N    )
       ( resid 31   and name HN   )   4.37    1.353
assign ( resid 32   and name N    )
       ( resid 32   and name HN   )   3.8     1.201
assign ( resid 35   and name N    )
       ( resid 35   and name HN   )   4.86    1.554
assign ( resid 37   and name N    )
       ( resid 37   and name HN   )   4.32    1.313
assign ( resid 38   and name N    )
       ( resid 38   and name HN   )   5.16    1.201
assign ( resid 39   and name N    )
       ( resid 39   and name HN   )   5.11    2.511
assign ( resid 42   and name N    )
       ( resid 42   and name HN   )   5.63    2.379
assign ( resid 44   and name N    )
       ( resid 44   and name HN   )   5.01    1.73
assign ( resid 46   and name N    )
       ( resid 46   and name HN   )   5.61    2.394
assign ( resid 50   and name N    )
       ( resid 50   and name HN   )   3.63    1.687
assign ( resid 55   and name N    )
       ( resid 55   and name HN   )   3.98    1.498
assign ( resid 56   and name N    )
       ( resid 56   and name HN   )   4.39    0.966
assign ( resid 57   and name N    )
       ( resid 57   and name HN   )   4.59    1.936
assign ( resid 58   and name N    )
       ( resid 58   and name HN   )   3.94    1.845
assign ( resid 59   and name N    )
       ( resid 59   and name HN   )   3.87    1.473
assign ( resid 60   and name N    )
       ( resid 60   and name HN   )   4.71    1.752
assign ( resid 61   and name N    )
       ( resid 61   and name HN   )   4.92    2.115
assign ( resid 62   and name N    )
```

```
       ( resid 62    and name HN  )    5.42     0.775
assign ( resid 63    and name N   )
       ( resid 63    and name HN  )    4.2      1.134
assign ( resid 65    and name N   )
       ( resid 65    and name HN  )    4.82     1.187
assign ( resid 76    and name N   )
       ( resid 76    and name HN  )    4.29     1.488
assign ( resid 77    and name N   )
       ( resid 77    and name HN  )    4.8      1.926
assign ( resid 78    and name N   )
       ( resid 78    and name HN  )    4.95     3.052
assign ( resid 80    and name N   )
       ( resid 80    and name HN  )    4.03     1.938
assign ( resid 82    and name N   )
       ( resid 82    and name HN  )    3.8      1.03
assign ( resid 87    and name N   )
       ( resid 87    and name HN  )    4.77     1.682
```

APPENDIX 5A
Structure calculations protocols – PARALLHDG

- generating *.psf file without SS bridges (initial structures)
f4f5_psf.inp

```
topology @TOPPAR:nilges/dg_version5/topallhdg.pro end

segment
name=""
chain
link pepp head - * tail + pro end
link pept head - * tail + * end
first prop tail + pro end
first nter tail + * end
last cter head - * end
sequence ALA GLU LYS CYS PHE ASP HIS ALA ALA GLY THR SER TYR VAL
VAL GLY GLU THR TRP GLU LYS PRO TYR GLN GLY TRP MET MET
VAL ASP CYS THR CYS LEU GLY GLU GLY SER GLY ARG ILE THR
CYS THR SER ARG ASN ARG CYS ASN ASP GLN ASP THR ARG THR
SER TYR ARG ILE GLY ASP THR TRP SER LYS LYS ASP ASN ARG
GLY ASN LEU LEU GLN CYS ILE CYS THR GLY ASN GLY ARG GLY
GLU TRP LYS CYS GLU ARG HIS THR SER END

end
end
!patch disu reference=1=(resid 4) reference=2=(resid 33) end
!patch disu reference=1=(resid 31) reference=2=(resid 43) end
!patch disu reference=1=(resid 49) reference=2=(resid 78) end
!patch disu reference=1=(resid 76) reference=2=(resid 88) end

patch disn reference=1=(resid 4) reference=2=(resid 33) end
patch disn reference=1=(resid 31) reference=2=(resid 43) end
patch disn reference=1=(resid 49) reference=2=(resid 78) end
patch disn reference=1=(resid 76) reference=2=(resid 88) end


write psf output=f4f5_without_ss.psf
end

stop
```

- generating template file
f4f5_generate_template.inp

```
remarks  file  nmr/generate_template.inp
remarks  generates a "template" coordinate set.  This produces
remarks  an arbitrary extended conformation with ideal geometry.
remarks  author: Axel T. Brunger

{====>}
structure @f4f5_without_ss.psf end                {* read structure file *}


parameter
evaluate ($par_nonbonded = PARALLHDG)
@TOPPAR:nilges/dg_version5/parallhdg5.1.pro end

topology
   presidue NDIS
      delete bond 1SG 2SG
      delete angle 1CB 1SG 2SG
      delete angle 1SG 2SG 2CB
   end
end

{====>}
 {* if your protein contains S-S bridges appropriately modify and   *}
```

```
 {* then uncomment the following lines.                                    *}
!patch ndis reference=1=( residue 4 ) reference=2=( residue 33 ) end
!patch ndis reference=1=( residue 31 ) reference=2=( residue 43 ) end
!patch ndis reference=1=( residue 49 ) reference=2=( residue 78 ) end
!patch ndis reference=1=( residue 76 ) reference=2=( residue 88 ) end

vector ident (x) ( all )
vector do (x=x/10.) ( all )
vector do (y=random(0.5) ) ( all )
vector do (z=random(0.5) ) ( all )

vector do (fbeta=50) (all)          {* friction coefficient, in 1/ps *}
vector do (mass=100) (all)          {* heavy masses, in amu          *}

parameter
   nbonds
      cutnb=5.5 rcon=20. nbxmod=-2 repel=0.9  wmin=0.1 tolerance=1.
      rexp=2 irexp=2 inhibit=0.25
   end
end

flags exclude * include bond angle vdw end

minimize powell nstep=50  nprint=10 end

flags include impr end

minimize powell nstep=50 nprint=10 end

dynamics  verlet
   nstep=50  timestep=0.001 iasvel=maxwell  firsttemp= 300.
   tcoupling = true  tbath = 300.   nprint=50  iprfrq=0
end

parameter
   nbonds
      rcon=2. nbxmod=-3 repel=0.75
   end
end

minimize powell nstep=100 nprint=25 end

dynamics  verlet
   nstep=500  timestep=0.005 iasvel=maxwell  firsttemp= 300.
   tcoupling = true  tbath = 300.   nprint=100  iprfrq=0
end

flags exclude vdw elec end
vector do (mass=1.) ( name h* )
hbuild selection=( name h* ) phistep=360 end
flags include vdw elec end

minimize powell nstep=200 nprint=50 end
                                                {* write coordinates *}
remarks produced by nmr/f4f5_generate_template.inp
write coordinates output=f4f5_template.pdb end

stop
```

- simulated annealing

f4f5_sa.inp

```
remarks sa_1.inp
remarks Author: Michael Nilges

evaluate ($ini_count = 0)
evaluate ($end_count = 100)

evaluate ($iniseed = 245613)
```

```
evaluate ($init_t = 2000 )  {* initial simulated annealing temperature *}
evaluate ($high_steps = 75000)      {*Total number of steps at high temp.*}
evaluate ($cool1_steps = 60000)     {*Total number of steps during cooling.*}
evaluate ($cool2_steps = 30000)

evaluate ($fileroot = "f4f5_complete_all_")
evaluate ($template = "f4f5_template.pdb")

structure @@f4f5_without_ss.psf end

parameter
evaluate ($par_nonbonded = PARALLHDG)
@TOPPAR:nilges/dg_version5/parallhdg5.1.pro end

parameter  nbfix S  S  462  13.6  462  13.6 end
parameter
   bond (name sg) (name sg) 0.0 TOKEN
   angle (all) (name sg) (name sg)  0.0 TOKEN
end


noe                                     {*Parameters for NOE effective energy term.*}
        reset
        nrestraints = 5000                   ! allocate space for NOEs
        ceiling 100                     {* Maximum energy constant that is allowed.*}

        set echo on message on end
        class       dist @f4f5_d2o_100ms_final.tbl
                         @f4f5_h2o_100ms.tbl
                         @f4_additional.tbl
                         @f5_additional.tbl

!        class       ambi @f4_ambi.tbl
!                         @f5_ambi.tbl

        class       hbon @f4_hbonds.tbl
                         @f5_hbonds.tbl

        set echo off message off end

        averaging  * sum
        potential  * soft
        scale      * 1.0                {* This is the energy constant. *}
        sqconstant * 1.0
        sqexponent * 2
        soexponent * 1
        rswitch    * 1.0        {* This scales the observed intensities.*}
        sqoffset   * 0.0
        asymptote  * 2.0
end


restraints
 dihedral
  reset
@f4_phi.tbl
@f5_phi.tbl
end

noe
  assign (resid  4 and name sg) (resid 33 and name sg) 2.02 0.1 0.1
  assign (resid  31 and name sg) (resid 43 and name sg) 2.02 0.1 0.1
  assign (resid  49 and name sg) (resid 78 and name sg) 2.02 0.1 0.1
  assign (resid  76 and name sg) (resid 88 and name sg) 2.02 0.1 0.1

end


flags exclude * include bonds angle impr vdw noe cdih end
```

```
@setup_swap_45.xplor
set echo on message on end
vector ident (store2) (store1)
parameter
   improper (store2) (store2) (all) (all) 0.0 TOKEN TOKEN
   improper (all) (all) (store2) (store2) 0.0 TOKEN TOKEN
end
set echo false message false end



vector do (fbeta=10) (all)     {* friction coefficient for MD heatbath, in 1/ps *}
vector do (mass=100) (all)     {*Uniform heavy masses to speed molecular dynamics.*}


evaluate ($kcdih = 5)
restraints dihedral
      scale=$kcdih
end

evaluate ($count = $ini_count)
while ($count < $end_count ) loop main

   evaluate ($count=$count+1)
   evaluate ($nreassign = 0)
   coor init end
   coor @@$template
   evaluate ($seed = $count*$iniseed)
   set seed $seed end
   @sa_1_randomchain.xplor

   evaluate ($cpu1 = $cpu)

   evaluate ($final1_t = 1000)       { K }
   evaluate ($final2_t = 100)        { K }
   evaluate ($tempstep = 50)        { K }

   evaluate ($ncycle = ($init_t-$final1_t)/$tempstep)
   evaluate ($nstep = int($cool1_steps/$ncycle))

   @@sa_1_initial_values.xplor
   parameter
     angle (store2) (all) (store2) $ini_flt TOKEN
     angle (all)    (all) (store2) $ini_flt TOKEN
   end

   parameter nbonds
      atom cutnb 12 tolerance 3.5 repel=1.2 wmin 0.5
      rexp=2 irexp=2 rcon=1. nbxmod 4
   end end

   @@sa_1_reduced.xplor  { defines store1 }

   constraints
      interaction (all) (not store1) weights * 1 angl 1.0 impr 1.0 vdw 0.0 elec 0 end
      interaction (store1) (store1)  weights * 1 angl 1.0 impr 1.0 vdw 0.1 elec 0 end
   end

   {* 1 ================================================ initial minimization  *}
   restraints dihedral   scale=5.   end
   noe potential * soft scale * 1.0 asymptote * 2.0  end
   minimize powell nstep=50 drop=10.  nprint=25 end


   {* 2 ================================================ high temperature dynamics *}

   @sa_1_hightemp.xplor

   {* 3 ================================================ cooling 1 *}
```

```
@sa_1_cool1.xplor

{* 4 ============================================ cooling 2 *}

@sa_1_cool2.xplor

{* 5 ================================================== final minimization *}

evaluate ($swap = 1.001)
flags exclude * include noe end
@swap15v.xplor
flags include bond angl impr vdw noe cdih end

minimize powell nstep=200 drop=10.0 nprint=25 end



{* 6 ================================== write out the final structure(s) *}


evaluate ($filename=$fileroot+ encode($count)+ ".pdb")
print threshold=0.2 noe
evaluate ($rms_noe=$result)
evaluate ($violations_noe=$violations)
print threshold=2. cdih
evaluate ($rms_cdih=$result)
evaluate ($violations_cdih=$violations)
print thres=0.05 bonds
evaluate ($rms_bonds=$result)
print thres=5. angles
evaluate ($rms_angles=$result)
print thres=5. impropers
evaluate ($rms_impropers=$result)
remarks initial random number seed: $seed
remarks ============================================================
remarks            overall,bonds,angles,improper,vdw,noe,cdih
remarks energies: $ener, $bond, $angl, $impr, $vdw, $noe, $cdih
remarks ============================================================
remarks            bonds,angles,impropers,noe,cdih
remarks rms-dev.: $rms_bonds,$rms_angles,$rms_impropers,$rms_noe,$rms_cdih
remarks ============================================================
remarks               noe,  cdih
remarks violations.: $violations_noe, $violations_cdih
remarks ============================================================
write coordinates sele= (all) output =$filename end
evaluate ($fname=$fileroot+encode($count)+".prt")
set print = $fname end
noe print thresh = 0.1 end {* for .prt files *}
close $fname end


end loop main


stop
```

- generating *.psf file with SS bridges (refinement)

f4f5_ss_psf.inp

```
topology @TOPPAR:nilges/dg_version5/topallhdg.pro end

segment
name=""
chain
link pepp head - * tail + pro end
link pept head - * tail + * end
first prop tail + pro end
first nter tail + * end
last cter head - * end
sequence ALA GLU LYS CYS PHE ASP HIS ALA ALA GLY THR SER TYR VAL
```

```
VAL GLY GLU THR TRP GLU LYS PRO TYR GLN GLY TRP MET MET
VAL ASP CYS THR CYS LEU GLY GLU GLY SER GLY ARG ILE THR
CYS THR SER ARG ASN ARG CYS ASN ASP GLN ASP THR ARG THR
SER TYR ARG ILE GLY ASP THR TRP SER LYS LYS ASP ASN ARG
GLY ASN LEU LEU GLN CYS ILE CYS THR GLY ASN GLY ARG GLY
GLU TRP LYS CYS GLU ARG HIS THR SER END

end
end
patch disu reference=1=(resid 4) reference=2=(resid 33) end
patch disu reference=1=(resid 31) reference=2=(resid 43) end
patch disu reference=1=(resid 49) reference=2=(resid 78) end
patch disu reference=1=(resid 76) reference=2=(resid 88) end

!patch disn reference=1=(resid 4) reference=2=(resid 33) end
!patch disn reference=1=(resid 31) reference=2=(resid 43) end
!patch disn reference=1=(resid 49) reference=2=(resid 78) end
!patch disn reference=1=(resid 76) reference=2=(resid 88) end


write psf output=f4f5_ss.psf
end

stop
```

- NOE/CDIH refinement
refine_f4f5.inp

```
remarks file nmr/refine.inp  -- Simulated annealing refinement
remarks                         for NMR structure determination
remarks  Authors: Michael Nilges, John Kuszewski, and Axel T. Brunger

{====>}
evaluate ($init_t = 2000)           {*Initial annealing temperature, in K.*}
{====>}
evaluate ($cool_steps = 60000 )     {*Total number of steps during cooling.*}

parameter                                          {*Read the parameter file.*}
evaluate ($par_nonbonded = PARALLHDG)
@TOPPAR:nilges/dg_version5/parallhdg5.1.pro
end

{====>} structure @f4f5_ss.psf end                  {*The structure file.*}

        @setup_swap_45.xplor
        vector ident (store2) (store1)
        parameter
           improper (store2) (store2) (all) (all) 0.0 TOKEN TOKEN
           improper (all) (all) (store2) (store2) 0.0 TOKEN TOKEN
        end

!set echo = off message off end
noe
{====>}
   nres=5000             {*Estimate greater than the actual number of NOEs.*}


class dist
   @f4f5_d2o_100ms_final.tbl
   @f4f5_h2o_100ms.tbl
   @f4_additional.tbl
   @f5_additional.tbl
!class ambi
!   @f4_ambi.tbl
!   @f5_ambi.tbl
class hbond
   @f4_hbonds.tbl
   @f5_hbonds.tbl
end
set echo = on end
```

```
{====>}

restraints
  dihedral
      reset
@f4_phi.tbl
@f5_phi.tbl
end


                              {*Friction coefficient for MD heatbath, in 1/ps.*}
vector do (fbeta=10) (all)
vector do (mass=100) (all)       {*Heavy masses to speed molecular dynamics.*}

noe                              {*Parameters for NOE effective energy term.*}
   ceiling=1000
   averaging  * sum
   potential  * square
   sqconstant * 1.
   sqexponent * 2
   scale      * 50.            {*Constant NOE scale throughout the protocol.*}
end

parameter                        {*Parameters for the repulsive energy term.*}
   nbonds
      repel=0.5                   {*Initial value for repel--modified later.*}
      rexp=2 irexp=2 rcon=1.
      nbxmod=3
      wmin=0.01
      cutnb=4.5 ctonnb=2.99 ctofnb=3.
      tolerance=0.5
   end
end

restraints dihedral
       scale=200.
end

{====>}
evaluate ($end_count= 81)          {*Loop through a family of 10 structures.*}

evaluate ($count = 0)
while ($count < $end_count ) loop main

   evaluate ($count=$count+1)

{====>}                                {*Filename(s) for embedded coordinates.*}
   evaluate ($filename="rnk_f4f5_complete_all_"+encode($count)+".pdb")

   coor init end
   coor @@$filename

   flags exclude * include bond angl impr vdw noe cdih end

   minimize powell tolg=0.1 drop=10 nprint=10 nstep=1000 end

   vector do (vx=maxwell($init_t)) ( all )
   vector do (vy=maxwell($init_t)) ( all )
   vector do (vz=maxwell($init_t)) ( all )

   evaluate ($final_t = 100)     { K }
   evaluate ($tempstep = 25)     { K }

   evaluate ($ncycle = ($init_t-$final_t)/$tempstep)
   evaluate ($nstep = int($cool_steps/$ncycle))

   evaluate ($ini_rad  = 0.9)        evaluate ($fin_rad  = 0.75)
   evaluate ($ini_con=  0.003)       evaluate ($fin_con=  4.0)

   evaluate ($bath  = $init_t)
```

```
   evaluate ($k_vdw = $ini_con)
   evaluate ($k_vdwfact = ($fin_con/$ini_con)^(1/$ncycle))
   evaluate ($radius=    $ini_rad)
   evaluate ($radfact = ($fin_rad/$ini_rad)^(1/$ncycle))

   evaluate ($i_cool = 0)
   while ($i_cool < $ncycle) loop cool
      evaluate ($i_cool=$i_cool+1)

      evaluate ($bath  = $bath  - $tempstep)
      evaluate ($k_vdw=min($fin_con,$k_vdw*$k_vdwfact))
      evaluate ($radius=max($fin_rad,$radius*$radfact))

      parameter  nbonds repel=$radius   end end
      constraints interaction (all) (all) weights * 1. vdw $k_vdw end end

      dynamics  verlet
         nstep=$nstep time=0.003 iasvel=current firstt=$bath
         tcoup=true tbath=$bath nprint=$nstep iprfrq=0
      end


{====>}                                                    {*Abort condition.*}
      evaluate ($critical=$temp/$bath)
      if ($critical >  10. ) then
         display  ****&&&& rerun job with smaller timestep (i.e., 0.003)
         stop
      end if
   end loop cool

   {* ================================================ Final minimization.*}
   minimize powell nstep= 200 nprint=25 end

   {* ================================== Write out the final structure(s).*}
   evaluate ($fname="4f15f1_final_"+encode($count)+".prt")
   set print = $fname end
   noe print thresh = 0.2
   print thresh = 2.0 cdih end
   close $fname end
   print threshold=0.1 noe
   evaluate ($rms_noe=$result)
   evaluate ($violations_noe=$violations)
   print threshold=2. cdih
   evaluate ($rms_cdih=$result)
   evaluate ($violations_cdih=$violations)
   print thres=0.05 bonds
   evaluate ($rms_bonds=$result)
   print thres=2. angles
   evaluate ($rms_angles=$result)
   print thres=2. impropers
   evaluate ($rms_impropers=$result)
   remarks ============================================================
   remarks            overall,bonds,angles,improper,vdw,noe,cdih
   remarks energies: $ener, $bond, $angl, $impr, $vdw, $noe, $cdih
   remarks ============================================================
   remarks            bonds,angles,impropers,noe,cdih
   remarks rms-d: $rms_bonds,$rms_angles,$rms_impropers,$rms_noe,$rms_cdih
   remarks ============================================================
   remarks               noe,  cdih
   remarks violations.: $violations_noe, $violations_cdih
   remarks ============================================================

{====>}                          {*Name(s) of the family of final structures.*}
   evaluate ($filename="4f15f1_final_"+encode($count)+".pdb")

   write coordinates output =$filename end

end loop main

stop
```

- tenso/DANI refinement

refine_f4f5_tenso_dani.inp

```
remarks file nmr/refine.inp  -- Simulated annealing refinement
remarks                         for NMR structure determination
remarks  Authors: Michael Nilges, John Kuszewski, and Axel T. Brunger
remarks modified by Ewa Pilka


parameter                                                {*Read the parameter file.*}
{====>}
evaluate ($par_nonbonded = PARALLHDG)
@TOPPAR:nilges/dg_version5/parallhdg5.1.pro
@par_axis.pro
end

{====>} structure @f4f5_ss.psf                           {*The structure file.*}
                  @axis.psf
                  end

        @setup_swap_45.xplor
        vector ident (store2) (store1)
        parameter
           improper (store2) (store2) (all) (all) 0.0 TOKEN TOKEN
           improper (all) (all) (store2) (store2) 0.0 TOKEN TOKEN
        end

evaluate ($kintra = 50)
evaluate ($kinter = 1.0)

!set echo = off message off end
noe
{====>}
   nres=5000              {*Estimate greater than the actual number of NOEs.*}

class intra
    @f4f5_d2o_100ms_intra.tbl
    @f4f5_h2o_100ms_intra.tbl
    @f4_additional.tbl
    @f5_additional.tbl
    @f4_hbonds.tbl
    @f5_hbonds.tbl
class inter
    @f4f5_inter.tbl
end
set echo = on end

{====>}

restraints
  dihedral
      reset
@f4_phi.tbl
@f5_phi.tbl
end


                              {*Friction coefficient for MD heatbath, in 1/ps.*}
vector do (fbeta=10) (all)
vector do (mass=100) (all)       {*Heavy masses to speed molecular dynamics.*}

noe                              {*Parameters for NOE effective energy term.*}
   ceiling=1000
   averaging  * sum
   potential  * square
   sqconstant * 1.
   sqexponent * 2
   scale      intra $kintra
   scale      inter $kinter
```

```
end

evaluate ($ktenso = 0.0001)

tenso
        reset
        nres=300
        class JNH
        force $ktenso
        potential harmonic
        coef 0.0000
        @4f15f1_rdc_tenso_afttalk.tbl
end

evaluate ($kdani = 0.0001)

dani
      reset
      nres=400
      class relax
      potential harmonic
      coef 6.2 1.2 0 500.10237 50.68065  {*tc,Dratio,rhombicity,freqs.*}
      @f4f5_dani_afttalk.tbl
end

vector do (refx=x) (all)
vector do (refy=y) (all)
vector do (refz=z) (all)

constraints fix (resid 500 and name OO) end


parameter                          {*Parameters for the repulsive energy term.*}
   nbonds
      repel=0.5                     {*Initial value for repel--modified later.*}
      rexp=2 irexp=2 rcon=1
      nbxmod=3
      wmin=0.01
      cutnb=4.5 ctonnb=2.99 ctofnb=3.
      tolerance=0.5
   end
end

restraints dihedral
        scale=200.
end

{====>}
evaluate ($end_count= 40)          {*Loop through a family of 10 structures.*}

evaluate ($count = 20)
while ($count < $end_count ) loop main

   evaluate ($count=$count+1)

{====>}                                {*Filename(s) for embedded coordinates.*}
   evaluate ($filename="final/rnk_4f15f1_final_"+encode($count)+".pdb")

   coor init end
   coor @@$filename
   coor @@axis.pdb

{============================1st phase============================}

   evaluate ($init_t = 1000)

   vector do (vx=maxwell($init_t)) ( all )
   vector do (vy=maxwell($init_t)) ( all )
   vector do (vz=maxwell($init_t)) ( all )

   evaluate ($ini_con = 1.0)
```

```
   evaluate ($k_vdw = $ini_con)

   flags exclude * include bond angl impr vdw noe cdih dani tenso end
   constraints interaction (all) (all) weights * 1. vdw $k_vdw end end

 noe
   scale intra $kintra
   scale inter $kinter
 end

   dani class relax force $kdani end
   tenso class JNH force $ktenso end

   dynamics  verlet
         nstep=4000 time=0.003 iasvel=current firstt=$init_t
         tcoup=true tbath=$init_t nprint=200 iprfrq=0
  end

{============================2nd phase============================}

   evaluate ($init_t = 1000)
   evaluate ($final_t = 500)      { K }
   evaluate ($tempstep = 25)      { K }
   evaluate ($cool_steps = 5000)

   flags exclude * include bond angl impr vdw noe cdih dani tenso end

   evaluate ($ncycle = ($init_t-$final_t)/$tempstep)
   evaluate ($nstep = int($cool_steps/$ncycle))

   evaluate ($ini_rad  = 1.0)       evaluate ($fin_rad  = 0.9)
   evaluate ($ini_con=  1.0)        evaluate ($fin_con=  3.0)

   evaluate ($ini_inter = 1.0)      evaluate ($fin_inter = 15.0)
   evaluate ($kinter = $ini_inter)

noe
  scale intra $kintra
  scale inter $kinter
end

  evaluate ($ini_dani = 0.0001)         evaluate ($fin_dani = 0.1)
  evaluate ($kdani= $ini_dani)

  dani class relax force $kdani end

  evaluate ($ini_tenso = 0.0001)      evaluate ($fin_tenso = 0.1)
  evaluate ($ktenso  = $ini_tenso)

  tenso class JNH force $ktenso end

  evaluate ($bath  = $init_t)
  evaluate ($k_vdw = $ini_con)
  evaluate ($k_vdwfact = ($fin_con/$ini_con)^(1/$ncycle))
  evaluate ($radius= $ini_rad)
  evaluate ($radfact = ($fin_rad/$ini_rad)^(1/$ncycle))

  evaluate ($inter_fac = ($fin_inter/$ini_inter)^(1/$ncycle))
  evaluate ($kinter = $ini_inter)

  evaluate ($dani_fac = ($fin_dani/$ini_dani)^(1/$ncycle))
  evaluate ($kdani = $ini_dani)
  evaluate ($tenso_fac = ($fin_tenso/$ini_tenso)^(1/$ncycle))
  evaluate ($ktenso = $ini_tenso)

  evaluate ($i_cool = 0)
  while ($i_cool < $ncycle) loop cool
     evaluate ($i_cool=$i_cool+1)

     evaluate ($bath  = $bath  - $tempstep)
     evaluate ($k_vdw=min($fin_con,$k_vdw*$k_vdwfact))
```

```
      evaluate ($radius=max($fin_rad,$radius*$radfact))
      evaluate ($kinter  = $kinter*$inter_fac)
      evaluate ($kdani   = $kdani*$dani_fac)
      evaluate ($ktenso  = $ktenso*$tenso_fac)

      parameter  nbonds repel=$radius   end end
      constraints interaction (all) (all) weights * 1. vdw $k_vdw end end

 noe
   scale intra $kintra
   scale inter $kinter
 end

      dani class relax force $kdani end
      tenso class JNH force $ktenso end

      dynamics  verlet
         nstep=$nstep time=0.003 iasvel=current firstt=$bath
         tcoup=true tbath=$bath nprint=$nstep iprfrq=0
      end


{====>}                                                  {*Abort condition.*}
      evaluate ($critical=$temp/$bath)
      if ($critical >  10. ) then
         display  ****&&&& rerun job with smaller timestep (i.e., 0.003)
         stop
      end if
   end loop cool

{=========================3rd phase=========================}

   evaluate ($init_t = 500)
   evaluate ($final_t = 10)      { K }
   evaluate ($tempstep = 25)      { K }
   evaluate ($cool_steps = 20000)

   flags exclude * include bond angl impr vdw noe cdih dani tenso end

   evaluate ($ncycle = ($init_t-$final_t)/$tempstep)
   evaluate ($nstep = int($cool_steps/$ncycle))

   evaluate ($ini_rad  = 0.9)       evaluate ($fin_rad  = 0.75)
   evaluate ($ini_con=  3.0)        evaluate ($fin_con=  4.0)

   evaluate ($ini_inter = 15.0)      evaluate ($fin_inter = 50.0)
   evaluate ($kinter = $ini_inter)

noe
   scale intra $kintra
   scale inter $kinter
end

   evaluate ($ini_dani = 0.1)         evaluate ($fin_dani = 0.6)
   evaluate ($kdani= $ini_dani)

   dani class relax force $kdani end

   evaluate ($ini_tenso = 0.1)      evaluate ($fin_tenso = 0.6)
   evaluate ($ktenso  = $ini_tenso)

   tenso class JNH force $ktenso end

   evaluate ($bath  = $init_t)
   evaluate ($k_vdw = $ini_con)
   evaluate ($k_vdwfact = ($fin_con/$ini_con)^(1/$ncycle))
   evaluate ($radius= $ini_rad)
   evaluate ($radfact = ($fin_rad/$ini_rad)^(1/$ncycle))

   evaluate ($inter_fac = ($fin_inter/$ini_inter)^(1/$ncycle))
   evaluate ($kinter = $ini_inter)
```

```
   evaluate ($dani_fac = ($fin_dani/$ini_dani)^(1/$ncycle))
   evaluate ($kdani = $ini_dani)
   evaluate ($tenso_fac = ($fin_tenso/$ini_tenso)^(1/$ncycle))
   evaluate ($ktenso = $ini_tenso)

   evaluate ($i_cool = 0)
   while ($i_cool < $ncycle) loop cool
      evaluate ($i_cool=$i_cool+1)

      evaluate ($bath  = $bath  - $tempstep)
      evaluate ($k_vdw=min($fin_con,$k_vdw*$k_vdwfact))
      evaluate ($radius=max($fin_rad,$radius*$radfact))
      evaluate ($kinter  = $kinter*$inter_fac)
      evaluate ($kdani  = $kdani*$dani_fac)
      evaluate ($ktenso  = $ktenso*$tenso_fac)

      parameter  nbonds repel=$radius   end end
      constraints interaction (all) (all) weights * 1. vdw $k_vdw end end

noe
  scale intra $kintra
  scale inter $kinter
end

      dani class relax force $kdani end
      tenso class JNH force $ktenso end

      dynamics  verlet
         nstep=$nstep time=0.003 iasvel=current firstt=$bath
         tcoup=true tbath=$bath nprint=$nstep iprfrq=0
      end


{====>}                                          {*Abort condition.*}
      evaluate ($critical=$temp/$bath)
   if ($critical >  10. ) then
      display  ****&&&& rerun job with smaller timestep (i.e., 0.003)
      stop
   end if
end loop cool



{* ================================================ Final minimization.*}
minimize powell nstep= 200 nprint=25 end

{* ================================== Write out the final structure(s).*}
evaluate ($fname="trials/rdc/4f15f1_tenso_dani_yas_2_"+encode($count)+".prt")
set print = $fname end
noe print thresh = 0.1
cdih print thresh = 2.0 all end
close $fname end
print threshold=0.1 noe
evaluate ($rms_noe=$result)
evaluate ($violations_noe=$violations)
print threshold=2. cdih
evaluate ($rms_cdih=$result)
evaluate ($violations_cdih=$violations)
evaluate ($fname="trials/rdc/4f15f1_tenso_dani_yas_2_"+encode($count)+".tens")
set print = $fname end
tenso print threshold=0.5 all end
evaluate ($rms_tenso=$result)
evaluate ($violations_tenso=$violations)
close $fname end
tenso print threshold=1.0 all end
evaluate ($rms_tenso=$result)
evaluate ($violations_tenso=$violations)
evaluate ($fname="trials/rdc/4f15f1_tenso_dani_yas_2_"+encode($count)+".dani")
set print = $fname end
dani print threshold=0.5 all end
```

```
   evaluate ($rms_dani=$result)
   evaluate ($violations_dani=$violations)
   close $fname end
   dani print threshold=1.0 all end
   evaluate ($rms_dani=$result)
   evaluate ($violations_dani=$violations)
   print thres=0.05 bonds
   evaluate ($rms_bonds=$result)
   print thres=2. angles
   evaluate ($rms_angles=$result)
   print thres=2. impropers
   evaluate ($rms_impropers=$result)

   remarks ============================================================
   remarks            overall,bonds,angles,improper,vdw,noe,cdih,dani,tenso
   remarks energies: $ener, $bond, $angl, $impr, $vdw, $noe, $cdih, $dani, $tens
   remarks ============================================================
   remarks            bonds,angles,impropers,noe,cdih,dani,tenso
   remarks rms-d:
$rms_bonds,$rms_angles,$rms_impropers,$rms_noe,$rms_cdih,$rms_dani,$rms_tenso
   remarks ============================================================
   remarks               noe,  cdih,  dani,  tenso
   remarks violations.: $violations_noe, $violations_cdih, $violations_dani,
$violations_tenso
   remarks ============================================================

{====>}                          {*Name(s) of the family of final structures.*}
   evaluate ($filename="trials/rdc/4f15f1_tenso_dani_yas_2_"+encode($count)+".pdb")

   write coordinates output =$filename end

end loop main

stop
```

APPENDIX 5B
Structure calculations protocols – PROLSQ

- generating *.psf file without SS bridges (initial structures)
f4f5_psf.inp

```
topology @TOPPAR:nilges/dg_version5/topallhdg.pro end

segment
name=""
chain
link pepp head - * tail + pro end
link pept head - * tail + * end
first prop tail + pro end
first nter tail + * end
last cter head - * end
sequence ALA GLU LYS CYS PHE ASP HIS ALA ALA GLY THR SER TYR VAL
VAL GLY GLU THR TRP GLU LYS PRO TYR GLN GLY TRP MET MET
VAL ASP CYS THR CYS LEU GLY GLU GLY SER GLY ARG ILE THR
CYS THR SER ARG ASN ARG CYS ASN ASP GLN ASP THR ARG THR
SER TYR ARG ILE GLY ASP THR TRP SER LYS LYS ASP ASN ARG
GLY ASN LEU LEU GLN CYS ILE CYS THR GLY ASN GLY ARG GLY
GLU TRP LYS CYS GLU ARG HIS THR SER END

end
end
!patch disu reference=1=(resid 4) reference=2=(resid 33) end
!patch disu reference=1=(resid 31) reference=2=(resid 43) end
!patch disu reference=1=(resid 49) reference=2=(resid 78) end
!patch disu reference=1=(resid 76) reference=2=(resid 88) end


patch disn reference=1=(resid 4) reference=2=(resid 33) end
patch disn reference=1=(resid 31) reference=2=(resid 43) end
patch disn reference=1=(resid 49) reference=2=(resid 78) end
patch disn reference=1=(resid 76) reference=2=(resid 88) end


write psf output=f4f5_without_ss.psf
end

stop
```

- generating template file
f4f5_generate_template_bound.inp

```
remarks  file  nmr/generate_template.inp
remarks  generates a "template" coordinate set.  This produces
remarks  an arbitrary extended conformation with ideal geometry.
remarks  author: Axel T. Brunger

{====>}
structure @f4f5_without_ss.psf end                    {* read structure file *}


parameter
evaluate ($par_nonbonded = PROLSQ)
@TOPPAR:nilges/dg_version5/parallhdg5.1.pro end

topology
   presidue NDIS
      delete bond 1SG 2SG
      delete angle 1CB 1SG 2SG
      delete angle 1SG 2SG 2CB
   end
end

{====>}
 {* if your protein contains S-S bridges appropriately modify and   *}
```

```
 {* then uncomment the following lines.                                    *}
!patch ndis reference=1=( residue 4 ) reference=2=( residue 33 ) end
!patch ndis reference=1=( residue 31 ) reference=2=( residue 43 ) end
!patch ndis reference=1=( residue 49 ) reference=2=( residue 78 ) end
!patch ndis reference=1=( residue 76 ) reference=2=( residue 88 ) end

vector ident (x) ( all )
vector do (x=x/10.) ( all )
vector do (y=random(0.5) ) ( all )
vector do (z=random(0.5) ) ( all )

vector do (fbeta=50) (all)          {* friction coefficient, in 1/ps *}
vector do (mass=100) (all)          {* heavy masses, in amu          *}

parameter
   nbonds
      cutnb=5.5 rcon=20. nbxmod=-2 repel=0.9  wmin=0.1 tolerance=1.
      rexp=2 irexp=2 inhibit=0.25
   end
end

flags exclude * include bond angle vdw end

minimize powell nstep=50  nprint=10 end

flags include impr end

minimize powell nstep=50 nprint=10 end

dynamics  verlet
   nstep=50  timestep=0.001 iasvel=maxwell  firsttemp= 300.
   tcoupling = true  tbath = 300.   nprint=50  iprfrq=0
end

parameter
   nbonds
      rcon=2. nbxmod=-3 repel=0.75
   end
end

minimize powell nstep=100 nprint=25 end

dynamics  verlet
   nstep=500  timestep=0.005 iasvel=maxwell  firsttemp= 300.
   tcoupling = true  tbath = 300.   nprint=100  iprfrq=0
end

flags exclude vdw elec end
vector do (mass=1.) ( name h* )
hbuild selection=( name h* ) phistep=360 end
flags include vdw elec end

minimize powell nstep=200 nprint=50 end
                                                {* write coordinates *}
remarks produced by nmr/f4f5_generate_template.inp
write coordinates output=f4f5_template_pro.pdb end

stop
```

- simulated annealing

f4f5_bound_sa.inp

```
remarks sa_1.inp
remarks Author: Michael Nilges

evaluate ($ini_count = 60)
evaluate ($end_count = 100)

evaluate ($iniseed = 19760304)
```

```
evaluate ($init_t = 2000 )  {* initial simulated annealing temperature *}
evaluate ($high_steps = 30000)      {*Total number of steps at high temp.*}
evaluate ($cool1_steps = 40000)     {*Total number of steps during cooling.*}
evaluate ($cool2_steps = 30000)

evaluate ($fileroot = "f4f5_bound_")
evaluate ($template = "f4f5_template_pro.pdb")

structure @@f4f5_without_ss.psf end

parameter
evaluate ($par_nonbonded = PROLSQ)
@TOPPAR:nilges/dg_version5/parallhdg5.1.pro end

parameter  nbfix S  S  462  13.6  462  13.6 end
parameter
   bond (name sg) (name sg) 0.0 TOKEN
   angle (all) (name sg) (name sg)  0.0 TOKEN
end


noe                                     {*Parameters for NOE effective energy term.*}
        reset
        nrestraints = 5000                   ! allocate space for NOEs
        ceiling 100                     {* Maximum energy constant that is allowed.*}

        set echo on message on end
        class       dist @f4f5_bound_d2o_100ms_final_pro.tbl
                         @f4f5_bound_h2o_100ms_final_pro.tbl
                         @f4f5_bound_stripplot_100ms_pro.tbl
                         @f4f5_bound_additional_final.tbl
                         @f4f5_bound_additional_new.tbl


        class       ambi @f4f5_bound_ambi.tbl
       class       hbon @f4f5_bound_hbonds.tbl

        set echo off message off end

        averaging  * sum
        potential  * soft
        scale      * 1.0                {* This is the energy constant. *}
        sqconstant * 1.0
        sqexponent * 2
        soexponent * 1
        rswitch    * 1.0        {* This scales the observed intensities.*}
        sqoffset   * 0.0
        asymptote  * 2.0
end


restraints
 dihedral
  reset
@f4f5_bound_phi.tbl

end

noe
  assign (resid  4 and name sg) (resid 33 and name sg) 2.02 0.1 0.1
  assign (resid  31 and name sg) (resid 43 and name sg) 2.02 0.1 0.1
  assign (resid  49 and name sg) (resid 78 and name sg) 2.02 0.1 0.1
  assign (resid  76 and name sg) (resid 88 and name sg) 2.02 0.1 0.1

end


flags exclude * include bonds angle impr vdw noe cdih end
```

```
@setup_swap_45.xplor
set echo on message on end
vector ident (store2) (store1)
parameter
   improper (store2) (store2) (all) (all) 0.0 TOKEN TOKEN
   improper (all) (all) (store2) (store2) 0.0 TOKEN TOKEN
end
set echo false message false end


vector do (fbeta=10) (all)     {* friction coefficient for MD heatbath, in 1/ps *}
vector do (mass=100) (all)     {*Uniform heavy masses to speed molecular dynamics.*}


evaluate ($kcdih = 5)
restraints dihedral
      scale=$kcdih
end

evaluate ($count = $ini_count)
while ($count < $end_count ) loop main

   evaluate ($count=$count+1)
   evaluate ($nreassign = 0)
   coor init end
   coor @@$template
   evaluate ($seed = $count*$iniseed)
   set seed $seed end
   @sa_1_randomchain.xplor

   evaluate ($cpu1 = $cpu)

   evaluate ($final1_t = 1000)       { K }
   evaluate ($final2_t = 100)       { K }
   evaluate ($tempstep = 50)       { K }

evaluate ($ncycle = ($init_t-$final1_t)/$tempstep)
evaluate ($nstep = int($cool1_steps/$ncycle))

@@sa_1_initial_values.xplor
parameter
  angle (store2) (all) (store2) $ini_flt TOKEN
  angle (all)    (all) (store2) $ini_flt TOKEN
end

parameter nbonds
   atom cutnb 12 tolerance 3.5 repel=1.6 wmin 0.5
   rexp=4 irexp=1 rcon=16 nbxmod 5
end end

@@sa_1_reduced.xplor  { defines store1 }

constraints
   interaction (all) (not store1) weights * 1 angl 1.0 impr 1.0 vdw 0.0 elec 0 end
   interaction (store1) (store1)  weights * 1 angl 1.0 impr 1.0 vdw 0.1 elec 0 end
end

{* 1 ================================================ initial minimization  *}
restraints dihedral   scale=5.   end
noe potential * soft scale * 1.0 asymptote * 2.0  end
minimize powell nstep=50 drop=10.  nprint=25 end


{* 2 ================================================ high temperature dynamics *}

@sa_1_hightemp.xplor

{* 3 ================================================ cooling 1 *}

@sa_1_cool1.xplor
```

```
{* 4 ========================================== cooling 2 *}

@sa_1_cool2.xplor

{* 5 ================================================ final minimization *}

evaluate ($swap = 1.001)
flags exclude * include noe end
@swap15v.xplor
flags include bond angl impr vdw noe cdih end

minimize powell nstep=200 drop=10.0 nprint=25 end



{* 6 ================================== write out the final structure(s) *}


evaluate ($filename=$fileroot+ encode($count)+ ".pdb")
print threshold=0.2 noe
evaluate ($rms_noe=$result)
evaluate ($violations_noe=$violations)
print threshold=2. cdih
evaluate ($rms_cdih=$result)
evaluate ($violations_cdih=$violations)
print thres=0.05 bonds
evaluate ($rms_bonds=$result)
print thres=5. angles
evaluate ($rms_angles=$result)
print thres=5. impropers
evaluate ($rms_impropers=$result)
remarks initial random number seed: $seed
remarks ============================================================
remarks            overall,bonds,angles,improper,vdw,noe,cdih
remarks energies: $ener, $bond, $angl, $impr, $vdw, $noe, $cdih
remarks ============================================================
remarks             bonds,angles,impropers,noe,cdih
remarks rms-dev.: $rms_bonds,$rms_angles,$rms_impropers,$rms_noe,$rms_cdih
remarks ============================================================
remarks               noe,  cdih
remarks violations.: $violations_noe, $violations_cdih
remarks ============================================================
write coordinates sele= (all) output =$filename end
evaluate ($fname=$fileroot+encode($count)+".prt")
set print = $fname end
noe print thresh = 0.1 end {* for .prt files *}
close $fname end


end loop main

stop
```

- generating *.psf file with SS bridges (refinement)

f4f5_ss_psf.inp

```
topology @TOPPAR:nilges/dg_version5/topallhdg.pro end

segment
name=""
chain
link pepp head - * tail + pro end
link pept head - * tail + * end
first prop tail + pro end
first nter tail + * end
last cter head - * end
sequence ALA GLU LYS CYS PHE ASP HIS ALA ALA GLY THR SER TYR VAL
VAL GLY GLU THR TRP GLU LYS PRO TYR GLN GLY TRP MET MET
VAL ASP CYS THR CYS LEU GLY GLU GLY SER GLY ARG ILE THR
```

```
CYS THR SER ARG ASN ARG CYS ASN ASP GLN ASP THR ARG THR
SER TYR ARG ILE GLY ASP THR TRP SER LYS LYS ASP ASN ARG
GLY ASN LEU LEU GLN CYS ILE CYS THR GLY ASN GLY ARG GLY
GLU TRP LYS CYS GLU ARG HIS THR SER END

end
end
patch disu reference=1=(resid 4) reference=2=(resid 33) end
patch disu reference=1=(resid 31) reference=2=(resid 43) end
patch disu reference=1=(resid 49) reference=2=(resid 78) end
patch disu reference=1=(resid 76) reference=2=(resid 88) end

!patch disn reference=1=(resid 4) reference=2=(resid 33) end
!patch disn reference=1=(resid 31) reference=2=(resid 43) end
!patch disn reference=1=(resid 49) reference=2=(resid 78) end
!patch disn reference=1=(resid 76) reference=2=(resid 88) end


write psf output=f4f5_ss.psf
end

stop
```

- NOE/CDIH refinement

refine_f4f5_bound.inp

```
remarks file nmr/refine.inp  -- Simulated annealing refinement
remarks                         for NMR structure determination
remarks  Authors: Michael Nilges, John Kuszewski, and Axel T. Brunger

{====>}
evaluate ($init_t = 1000)            {*Initial annealing temperature, in K.*}
{====>}
evaluate ($cool_steps = 25000 )      {*Total number of steps during cooling.*}

parameter                                          {*Read the parameter file.*}
evaluate ($par_nonbonded = PROLSQ)
@TOPPAR:nilges/dg_version5/parallhdg5.1.pro
end

{====>} structure @f4f5_ss.psf end                    {*The structure file.*}

        @setup_swap_45.xplor
        vector ident (store2) (store1)
        parameter
           improper (store2) (store2) (all) (all) 0.0 TOKEN TOKEN
           improper (all) (all) (store2) (store2) 0.0 TOKEN TOKEN
        end

!set echo = off message off end
noe
{====>}
   nres=5000              {*Estimate greater than the actual number of NOEs.*}


class dist
    @f4f5_bound_d2o_100ms_final_pro.tbl
    @f4f5_bound_h2o_100ms_final_pro.tbl
    @f4f5_bound_stripplot_100ms_pro.tbl
    @f4f5_bound_additional_final.tbl
    @f4f5_bound_additional_new.tbl
class ambi
    @f4f5_bound_ambi.tbl

class hbond
    @f4f5_bound_hbonds.tbl
end
set echo = on end

{====>}
```

```
restraints
  dihedral
      reset
@f4f5_bound_phi.tbl
end


                              {*Friction coefficient for MD heatbath, in 1/ps.*}
vector do (fbeta=10) (all)
vector do (mass=100) (all)       {*Heavy masses to speed molecular dynamics.*}

noe                              {*Parameters for NOE effective energy term.*}
   ceiling=1000
   averaging  * sum
   potential  * square
   sqconstant * 1.
   sqexponent * 2
   scale      * 50.            {*Constant NOE scale throughout the protocol.*}
end

parameter                        {*Parameters for the repulsive energy term.*}
   nbonds
      repel=0.5                   {*Initial value for repel--modified later.*}
      rexp=4 irexp=1 rcon=16
      nbxmod=5
      wmin=0.01
      cutnb=4.5 ctonnb=2.99 ctofnb=3.
      tolerance=0.5
   end
end

restraints dihedral
        scale=200.
end

{====>}
evaluate ($end_count= 90)          {*Loop through a family of 10 structures.*}

evaluate ($count = 72)
while ($count < $end_count ) loop main

   evaluate ($count=$count+1)

{====>}                                {*Filename(s) for embedded coordinates.*}
   evaluate ($filename="rnk_f4f5_bound_"+encode($count)+".pdb")

   coor init end
   coor @@$filename

   flags exclude * include bond angl impr vdw noe cdih end

   minimize powell tolg=0.1 drop=10 nprint=10 nstep=1000 end

   vector do (vx=maxwell($init_t)) ( all )
   vector do (vy=maxwell($init_t)) ( all )
   vector do (vz=maxwell($init_t)) ( all )

   evaluate ($final_t = 0)      { K }
   evaluate ($tempstep = 25)      { K }

   evaluate ($ncycle = ($init_t-$final_t)/$tempstep)
   evaluate ($nstep = int($cool_steps/$ncycle))

   evaluate ($ini_rad  = 1.2)          evaluate ($fin_rad  = 1.0)
   evaluate ($ini_con=  0.003)         evaluate ($fin_con=  4.0)

   evaluate ($bath  = $init_t)
   evaluate ($k_vdw = $ini_con)
   evaluate ($k_vdwfact = ($fin_con/$ini_con)^(1/$ncycle))
   evaluate ($radius=    $ini_rad)
```

```
   evaluate ($radfact = ($fin_rad/$ini_rad)^(1/$ncycle))

   evaluate ($i_cool = 0)
   while ($i_cool < $ncycle) loop cool
      evaluate ($i_cool=$i_cool+1)

      evaluate ($bath  = $bath  - $tempstep)
      evaluate ($k_vdw=min($fin_con,$k_vdw*$k_vdwfact))
      evaluate ($radius=max($fin_rad,$radius*$radfact))

      parameter  nbonds repel=$radius   end end
      constraints interaction (all) (all) weights * 1. vdw $k_vdw end end

      dynamics  verlet
         nstep=$nstep time=0.003 iasvel=current firstt=$bath
         tcoup=true tbath=$bath nprint=$nstep iprfrq=0
      end


{====>}                                                    {*Abort condition.*}
      evaluate ($critical=$temp/$bath)
      if ($critical >  10. ) then
         display  ****&&&& rerun job with smaller timestep (i.e., 0.003)
         stop
      end if
   end loop cool

   {* ================================================ Final minimization.*}
   minimize powell nstep= 200 nprint=25 end

   {* ================================== Write out the final structure(s).*}
   evaluate ($fname="4f15f1_bound_new_"+encode($count)+".prt")
   set print = $fname end
   noe print thresh = 0.1 end
   cdih print thresh = 2.0 end
   close $fname end
   print threshold=0.1 noe
   evaluate ($rms_noe=$result)
   evaluate ($violations_noe=$violations)
   print threshold=2. cdih
   evaluate ($rms_cdih=$result)
   evaluate ($violations_cdih=$violations)
   print thres=0.05 bonds
   evaluate ($rms_bonds=$result)
   print thres=2. angles
   evaluate ($rms_angles=$result)
   print thres=2. impropers
   evaluate ($rms_impropers=$result)
   remarks ============================================================
   remarks            overall,bonds,angles,improper,vdw,noe,cdih
   remarks energies: $ener, $bond, $angl, $impr, $vdw, $noe, $cdih
   remarks ============================================================
   remarks            bonds,angles,impropers,noe,cdih
   remarks rms-d: $rms_bonds,$rms_angles,$rms_impropers,$rms_noe,$rms_cdih
   remarks ============================================================
   remarks               noe,  cdih
   remarks violations.: $violations_noe, $violations_cdih
   remarks ============================================================

{====>}                          {*Name(s) of the family of final structures.*}
   evaluate ($filename="4f15f1_bound_new"+encode($count)+".pdb")

   write coordinates output =$filename end

end loop main

stop
```

- tenso/DANI refinement

refine_f4f5_bound_tenso_dani.inp

```
remarks file nmr/refine.inp  -- Simulated annealing refinement
remarks                         for NMR structure determination
remarks  Authors: Michael Nilges, John Kuszewski, and Axel T. Brunger
remarks modified by Ewa Pilka


parameter                                          {*Read the parameter file.*}
{====>}
evaluate ($par_nonbonded = PROLSQ)
@TOPPAR:nilges/dg_version5/parallhdg5.1.pro
@par_axis.pro
end

{====>} structure @f4f5_ss.psf                     {*The structure file.*}
                  @axis.psf
                  end

        @setup_swap_45.xplor
        vector ident (store2) (store1)
        parameter
           improper (store2) (store2) (all) (all) 0.0 TOKEN TOKEN
           improper (all) (all) (store2) (store2) 0.0 TOKEN TOKEN
        end

evaluate ($kintra = 50)
evaluate ($kinter = 1.0)

!set echo = off message off end
noe
{====>}
   nres=5000              {*Estimate greater than the actual number of NOEs.*}

class intra
    @f4f5_bound_d2o_100ms_intra.tbl
    @f4f5_bound_h2o_100ms_intra.tbl
    @f4f5_bound_stripplot_100ms_pro.tbl
    @f4f5_bound_additional_intra.tbl
    @f4f5_bound_ambi.tbl
    @f4f5_bound_hbonds.tbl
class inter
    @f4f5_bound_inter.tbl
end
set echo = on end

{====>}

restraints
  dihedral
      reset
@f4f5_bound_phi.tbl
end


                                {*Friction coefficient for MD heatbath, in 1/ps.*}
vector do (fbeta=10) (all)
vector do (mass=100) (all)      {*Heavy masses to speed molecular dynamics.*}

                                {*Parameters for NOE effective energy term.*}
noe
   ceiling=1000
   averaging  * sum
   potential  * square
   sqconstant * 1.
   sqexponent * 2
   scale      intra $kintra
   scale      inter $kinter
end
```

```
evaluate ($ktenso = 0.0001)

tenso
        nres=300
        class JNH
        force $ktenso
        potential harmonic
        coef 0.0000
        @4f15f1_bound_rdc_tenso_afttalk.tbl
end

evaluate ($kdani = 0.0001)

dani
      reset
      nres=400
      class relax
      potential harmonic
      coef 7.4 1.4 0 500.10237 50.68065  {*tc,Dratio,rhombicity,freqs.*}
      @f4f5_dani_bound_afttalk.tbl
end

vector do (refx=x) (all)
vector do (refy=y) (all)
vector do (refz=z) (all)

constraints fix (resid 500 and name OO) end


parameter                          {*Parameters for the repulsive energy term.*}
   nbonds
      repel=0.5                     {*Initial value for repel--modified later.*}
      rexp=4 irexp=1 rcon=16
      nbxmod=5
      wmin=0.01
      cutnb=4.5 ctonnb=2.99 ctofnb=3.
      tolerance=0.5
   end
end

restraints dihedral
       scale=200.
end

{====>}
evaluate ($end_count= 50)          {*Loop through a family of 10 structures.*}

evaluate ($count = 48)
while ($count < $end_count ) loop main

   evaluate ($count=$count+1)

{====>}                                {*Filename(s) for embedded coordinates.*}
   evaluate ($filename="rnk_4f15f1_bound_new_"+encode($count)+".pdb")

   coor init end
   coor @@$filename
   coor @@axis.pdb

{============================1st phase============================}

   evaluate ($init_t = 1000)

   vector do (vx=maxwell($init_t)) ( all )
   vector do (vy=maxwell($init_t)) ( all )
   vector do (vz=maxwell($init_t)) ( all )

   evaluate ($ini_con = 1.0)
   evaluate ($k_vdw = $ini_con)

   flags exclude * include bond angl impr vdw noe cdih dani tenso end
```

```
  constraints interaction (all) (all) weights * 1. vdw $k_vdw end end

noe
  scale intra $kintra
  scale inter $kinter
end

  dani class relax force $kdani end
  tenso class JNH force $ktenso end

  dynamics  verlet
        nstep=4000 time=0.003 iasvel=current firstt=$init_t
        tcoup=true tbath=$init_t nprint=200 iprfrq=0
 end

{=========================2nd phase=========================}

  evaluate ($init_t = 1000)
  evaluate ($final_t = 500)      { K }
  evaluate ($tempstep = 25)      { K }
  evaluate ($cool_steps = 5000)

  flags exclude * include bond angl impr vdw noe cdih dani tenso end

  evaluate ($ncycle = ($init_t-$final_t)/$tempstep)
  evaluate ($nstep = int($cool_steps/$ncycle))

  evaluate ($ini_rad  = 1.2)       evaluate ($fin_rad  = 1.1)
  evaluate ($ini_con=  1.0)        evaluate ($fin_con=  3.0)

  evaluate ($ini_inter = 1.0)      evaluate ($fin_inter = 15.0)
  evaluate ($kinter = $ini_inter)

noe
  scale intra $kintra
  scale inter $kinter
end

  evaluate ($ini_dani = 0.0001)        evaluate ($fin_dani = 0.1)
  evaluate ($kdani= $ini_dani)

  dani class relax force $kdani end

  evaluate ($ini_tenso = 0.0001)     evaluate ($fin_tenso = 0.1)
  evaluate ($ktenso  = $ini_tenso)

  tenso class JNH force $ktenso end

  evaluate ($bath  = $init_t)
  evaluate ($k_vdw = $ini_con)
  evaluate ($k_vdwfact = ($fin_con/$ini_con)^(1/$ncycle))
  evaluate ($radius= $ini_rad)
  evaluate ($radfact = ($fin_rad/$ini_rad)^(1/$ncycle))

  evaluate ($inter_fac = ($fin_inter/$ini_inter)^(1/$ncycle))
  evaluate ($kinter = $ini_inter)

  evaluate ($dani_fac = ($fin_dani/$ini_dani)^(1/$ncycle))
  evaluate ($kdani = $ini_dani)
  evaluate ($tenso_fac = ($fin_tenso/$ini_tenso)^(1/$ncycle))
  evaluate ($ktenso = $ini_tenso)

  evaluate ($i_cool = 0)
  while ($i_cool < $ncycle) loop cool
     evaluate ($i_cool=$i_cool+1)

     evaluate ($bath  = $bath  - $tempstep)
     evaluate ($k_vdw=min($fin_con,$k_vdw*$k_vdwfact))
     evaluate ($radius=max($fin_rad,$radius*$radfact))
     evaluate ($kinter  = $kinter*$inter_fac)
     evaluate ($kdani  = $kdani*$dani_fac)
```

```
      evaluate ($ktenso  = $ktenso*$tenso_fac)

      parameter  nbonds repel=$radius   end end
      constraints interaction (all) (all) weights * 1. vdw $k_vdw end end

 noe
   scale intra $kintra
   scale inter $kinter
 end

      dani class relax force $kdani end
      tenso class JNH force $ktenso end

      dynamics  verlet
         nstep=$nstep time=0.003 iasvel=current firstt=$bath
         tcoup=true tbath=$bath nprint=$nstep iprfrq=0
      end


{====>}                                                {*Abort condition.*}
      evaluate ($critical=$temp/$bath)
      if ($critical >  10. ) then
         display  ****&&&& rerun job with smaller timestep (i.e., 0.003)
         stop
      end if
   end loop cool

{==========================3rd phase==========================}

   evaluate ($init_t = 500)
   evaluate ($final_t = 10)      { K }
   evaluate ($tempstep = 25)      { K }
   evaluate ($cool_steps = 20000)

   flags exclude * include bond angl impr vdw noe cdih dani tenso end

   evaluate ($ncycle = ($init_t-$final_t)/$tempstep)
   evaluate ($nstep = int($cool_steps/$ncycle))

   evaluate ($ini_rad  = 1.1)       evaluate ($fin_rad  = 1.0)
   evaluate ($ini_con=  3.0)        evaluate ($fin_con=  4.0)

   evaluate ($ini_inter = 15.0)      evaluate ($fin_inter = 50.0)
   evaluate ($kinter = $ini_inter)

noe
   scale intra $kintra
   scale inter $kinter
end

   evaluate ($ini_dani = 0.1)         evaluate ($fin_dani = 0.6)
   evaluate ($kdani= $ini_dani)

   dani class relax force $kdani end

   evaluate ($ini_tenso = 0.1)      evaluate ($fin_tenso = 0.6)
   evaluate ($ktenso  = $ini_tenso)

   tenso class JNH force $ktenso end

   evaluate ($bath  = $init_t)
   evaluate ($k_vdw = $ini_con)
   evaluate ($k_vdwfact = ($fin_con/$ini_con)^(1/$ncycle))
   evaluate ($radius= $ini_rad)
   evaluate ($radfact = ($fin_rad/$ini_rad)^(1/$ncycle))

   evaluate ($inter_fac = ($fin_inter/$ini_inter)^(1/$ncycle))
   evaluate ($kinter = $ini_inter)

   evaluate ($dani_fac = ($fin_dani/$ini_dani)^(1/$ncycle))
   evaluate ($kdani = $ini_dani)
```

```
  evaluate ($tenso_fac = ($fin_tenso/$ini_tenso)^(1/$ncycle))
  evaluate ($ktenso = $ini_tenso)

  evaluate ($i_cool = 0)
  while ($i_cool < $ncycle) loop cool
     evaluate ($i_cool=$i_cool+1)

     evaluate ($bath  = $bath  - $tempstep)
     evaluate ($k_vdw=min($fin_con,$k_vdw*$k_vdwfact))
     evaluate ($radius=max($fin_rad,$radius*$radfact))
     evaluate ($kinter  = $kinter*$inter_fac)
     evaluate ($kdani  = $kdani*$dani_fac)
     evaluate ($ktenso  = $ktenso*$tenso_fac)

     parameter  nbonds repel=$radius   end end
     constraints interaction (all) (all) weights * 1. vdw $k_vdw end end

noe
  scale intra $kintra
  scale inter $kinter
end

     dani class relax force $kdani end
     tenso class JNH force $ktenso end

     dynamics  verlet
        nstep=$nstep time=0.003 iasvel=current firstt=$bath
        tcoup=true tbath=$bath nprint=$nstep iprfrq=0
     end


{====>}                                          {*Abort condition.*}
     evaluate ($critical=$temp/$bath)
     if ($critical >  10. ) then
        display  ****&&&& rerun job with smaller timestep (i.e., 0.003)
        stop
   end if
end loop cool


{* ================================================ Final minimization.*}
minimize powell nstep= 200 nprint=25 end

{* ================================== Write out the final structure(s).*}
evaluate ($fname="trials/rdc/4f15f1_bound_tenso_dani_yas_fin_"+encode($count)+".prt")
set print = $fname end
noe print thresh = 0.1
cdih print thresh = 2.0 all end
close $fname end
print threshold=0.1 noe
evaluate ($rms_noe=$result)
evaluate ($violations_noe=$violations)
print threshold=2. cdih
evaluate ($rms_cdih=$result)
evaluate ($violations_cdih=$violations)
evaluate ($fname="trials/rdc/4f15f1_bound_tenso_dani_yas_fin_"+encode($count)+".tens")
set print = $fname end
tenso print threshold=0.5 all end
evaluate ($rms_tenso=$result)
evaluate ($violations_tenso=$violations)
close $fname end
tenso print threshold=1.0 all end
evaluate ($rms_tenso=$result)
evaluate ($violations_tenso=$violations)
evaluate ($fname="trials/rdc/4f15f1_bound_tenso_dani_yas_fin_"+encode($count)+".dani")
set print = $fname end
dani print threshold=0.5 all end
evaluate ($rms_dani=$result)
evaluate ($violations_dani=$violations)
close $fname end
```

```
   dani print threshold=1.0 all end
   evaluate ($rms_dani=$result)
   evaluate ($violations_dani=$violations)
   print thres=0.05 bonds
   evaluate ($rms_bonds=$result)
   print thres=2. angles
   evaluate ($rms_angles=$result)
   print thres=2. impropers
   evaluate ($rms_impropers=$result)

   remarks ============================================================
   remarks            overall,bonds,angles,improper,vdw,noe,cdih,dani,tenso
   remarks energies: $ener, $bond, $angl, $impr, $vdw, $noe, $cdih, $dani, $tens
   remarks ============================================================
   remarks            bonds,angles,impropers,noe,cdih,dani,tenso
   remarks rms-d:
$rms_bonds,$rms_angles,$rms_impropers,$rms_noe,$rms_cdih,$rms_dani,$rms_tenso
   remarks ============================================================
   remarks               noe,  cdih,  dani,  tenso
   remarks violations.: $violations_noe, $violations_cdih, $violations_dani,
$violations_tenso
   remarks ============================================================

{====>}                          {*Name(s) of the family of final structures.*}
   evaluate
($filename="trials/rdc/4f15f1_bound_tenso_dani_yas_fin_"+encode($count)+".pdb")

   write coordinates output =$filename end

end loop main

stop
```

APPENDIX 5C
Examples of input file formats

NOE restraints

```
assign ( resid   26 and name  hz3 ) ( resid   26 and name  hz2 ) 0.0 0.0 5.0 ! 1.89500
assign ( resid   26 and name  hh2 ) ( resid   26 and name  he3 ) 0.0 0.0 5.0 ! 1.75550
assign ( resid   31 and name   ha ) ( resid   43 and name   ha ) 0.0 0.0 2.8 ! 37.14940
assign ( resid   30 and name   ha ) ( resid   20 and name   ha ) 0.0 0.0 2.8 ! 19.38430
```

H-bond restraints

```
!32-42
assign (resid  32 and   name O    )(resid   42 and   name N    ) 0.0 0.0 3.2
assign (resid  32 and   name O    )(resid   42 and   name HN   ) 0.0 0.0 2.3
```

CDIH restraints

```
! f5,  8.924/9.087
assign  (resid  4 and name c ) (resid  5 and name n)
        (resid  5 and name ca) (resid  5 and name c) 1 -130.0 40 2
```

SANI restraints

```
assign ( resid 500  and name OO  )
       ( resid 500  and name Z   )
       ( resid 500  and name X   )
       ( resid 500  and name Y   )
       ( resid 4    and name N   )
       ( resid 4    and name HN  )   3.8    1.0
```

ISAC restraints

```
assign ( resid 4    and name N   )
       ( resid 4    and name HN  )   3.8    0.5
```

DANI restraints

```
assign ( resid 500  and name OO  )
       ( resid 500  and name Z   )
       ( resid 500  and name X   )
       ( resid 500  and name Y   )
       ( resid 6    and name N   )
       ( resid 6    and name HN  )   3.43    0.328
```

MODULE data file

```
4 N     4 HN    3.8     1.0
5 N     5 HN    11.3     1.0
10 N    10 HN   13.6     1.0
13 N    13 HN   3.7     1.0
```

TENSOR2 data file

```
3       1.637   0.233   9.171   0.472
4       1.992   0.437   9.684   0.837
5       2.170   0.540   7.879   0.760
6       2.139   0.409   8.886   0.656
```

APPENDIX 5D
X-PLOR files

sa_l_randomchain.xplor

```
REMARK randomchain.inp
REMARK randomize a structure by random rotations around the N-Ca and Ca-C bonds.
REMARK
REMARK input:  a coordinate set obtained with generate_template.inp or any
REMARK         other pdb file with good geometry
REMARK output: the coordinates randomized around phi and psi. chi_i are not
REMARK         affected.
REMARK
REMARK the input file can be used also to generate the randomized coordinates
REMARK "on the fly" as part of the sa.inp protocol. the lines setting the seed,
REMARK reading the structure and the template coordinates at the beginning,
REMARK and writing out the randomized coordinates and the stop command at the end,
REMARK should then be deleted.
REMARK this file should be read after the "coor swap" and "coor copy" statements
REMARK at the beginning of the main loop in sa.inp.
REMARK
REMARK Author: M. Nilges, EMBL, Oct. 1993

set message off echo off end

! The vector store1 is set to 1 for all atoms that have already been
! rotated.
vector do (store1=0) (all)
vector do (store3=0) (all)

! loop through all ca atoms.
for $id in id (name ca) loop pept

  vector show element (resid) (id $id)
  evaluate ($res = $result)
  vector show element (segid) (id $id)
  evaluate ($seg = $result)
  vector do (store3=1) (byres (id $id))

  evaluate ($phi = random()*360-180)
  evaluate ($psi = random()*360-180)

! all atoms up to the present ca are held fixed for phi rotation
  vector do (store1=1)
    ((store3 and (name n or name hn or name ht* or name ca)))

! rotate phi
  coor rotate sele= (attribute store1=0)
      center (head (store3 and name ca))
      axis   (head (store3 and name ca)
              tail (store3 and name n)) $phi
  end

! fix the sidechain of the current residue for psi rotation
  vector do (store1=1)
    ((store3 and not (name c or name o or name ot#)))

! rotate psi
  coor rotate sele= (attribute store1=0 and segid $seg)
      center (head (store3 and name c) )
      axis   (head (store3 and name c)
              tail (store3 and name ca)) $psi
  end

! fix all of current residue
  vector do (store1=1) (store3)
  vector do (store3=0) (byres (id $id))

end loop pept
```

```
!!coor translate selection (segid B) vector (head (segid A) tail (segid B)) end

evaluate ($filename="random"+encode($count)+".pdb")

!!!write coordinates output =$filename end

set message on echo on end
```

sa_l_initial_values.xplor

```
   evaluate ($ini_rad  = 1.2)          evaluate ($fin_rad  = 1.0)
   evaluate ($ini_con=  0.003)         evaluate ($fin_con=  4.0)
   evaluate ($ini_flt = 25)            evaluate ($fin_flt = 500)
   evaluate ($ini_ang = 1.0)           evaluate ($fin_ang = 1.0)
   evaluate ($ini_imp = 1.0)           evaluate ($fin_imp = 1.0)
   evaluate ($ini_amb = 1.0)           evaluate ($fin_amb = 50.0)

   evaluate ($bath  = $init_t)
   evaluate ($k_vdw = $ini_con)
   evaluate ($k_vdwfact = ($fin_con/$ini_con)^(1/$ncycle))
   evaluate ($radius=    $ini_rad)
   evaluate ($radfact = ($fin_rad/$ini_rad)^(1/$ncycle))
   evaluate ($k_flt = $ini_flt)
   evaluate ($flt_fac = ($fin_flt/$ini_flt)^(1/$ncycle))
   evaluate ($k_ang = $ini_ang)
   evaluate ($ang_fac = ($fin_ang/$ini_ang)^(1/$ncycle))
   evaluate ($k_imp = $ini_imp)
   evaluate ($imp_fac = ($fin_imp/$ini_imp)^(1/$ncycle))
   evaluate ($k_amb = $ini_amb)
   evaluate ($amb_fac = ($fin_amb/$ini_amb)^(1/$ncycle))
```

sa_l_reduced.xplor

```
vector ident (store1)
((resn Gly  and name CA) or
 (resn Ala  and (name CA or name CB  )) or
 (resn Val  and (name CA or name CG1 )) or
 (resn Ile  and (name CA or name CG1 )) or
 (resn Leu  and (name CA or name CD1 )) or
 (resn Phe  and (name CA or name CD1 )) or
 (resn Pro  and (name CA or name CG  )) or
 (resn Met  and (name CA or name SD  )) or
 (resn Trp  and (name CA or name CE2 )) or
 (resn Cys  and (name CA or name SG  )) or
 (resn Ser  and (name CA or name CB  )) or
 (resn Thr  and (name CA or name CG2 )) or
 (resn Asn  and (name CA or name CG  )) or
 (resn Gln  and (name CA or name CD  )) or
 (resn Tyr  and (name CA or name CD1 )) or
 (resn His  and (name CA or name CD2 )) or
 (resn Asp  and (name CA or name CG  )) or
 (resn Glu  and (name CA or name CD  )) or
 (resn Lys  and (name CA or name CD  )) or
 (resn Arg  and (name CA or name CD  )))
```

setup_swap_45.xplor

```
REMARK setup_swap.inp
REMARK this file defines explicitely all methylene and propyl
REMARK groups for which floating assignment is to be performed.
REMARK Author: Michael Nilges, EMBL
REMARK modified by A.K. Downing, Oxford

set message on echo on end
vector do (q = 0) (all)
vector do (store1 = 0) (all)
vector do (store1 = store1 + 1)
          ((resn leu and name cd*) or (resn val and name cg*))

for $resid in ( 10 16 25 35 37 39 61 71 80 82 84 )      !all Gly
loop meth
!  eval ($name = "HA%")
```

```
!  coor sele= (resid $resid and name $name) end
  coor sele= (resid $resid and name HA%) end
  if ($select = 2) then
!    vector do (store1 = store1 + 1) (resid $resid and name $name)
    vector do (store1 = store1 + 1) (resid $resid and name HA%)
  end if
end loop meth
for $resid in ( 2 3 4 5 6 7 12 13 17 19 20 21 22 23 24 26 27 28 30 31 )
loop meth
!  eval ($name = "HB%")
!  coor sele= (resid $resid and name $name) end
  coor sele= (resid $resid and name HB%) end
  if ($select = 2) then
!    vector do (store1 = store1 + 1) (resid $resid and name $name)
   vector do (store1 = store1 + 1) (resid $resid and name HB%)
  end if
end loop meth
for $resid in ( 33 34 36 38 40 43 45 46 47 48 49 50 51 52 53 55 )
loop meth
!  eval ($name = "HB%")
!  coor sele= (resid $resid and name $name) end
  coor sele= (resid $resid and name HB%) end
  if ($select = 2) then
!    vector do (store1 = store1 + 1) (resid $resid and name $name)
   vector do (store1 = store1 + 1) (resid $resid and name HB%)
  end if
end loop meth
for $resid in ( 57 58 59 62 64 65 66 67 68 69 70 )
loop meth
!  eval ($name = "HB%")
!  coor sele= (resid $resid and name $name) end
  coor sele= (resid $resid and name HB%) end
  if ($select = 2) then
!    vector do (store1 = store1 + 1) (resid $resid and name $name)
   vector do (store1 = store1 + 1) (resid $resid and name HB%)
  end if
end loop meth
for $resid in ( 72 73 74 75 76 78 81 83 85 86 87 88 89 90 91 93 )
loop meth
!  eval ($name = "HB%")
!  coor sele= (resid $resid and name $name) end
  coor sele= (resid $resid and name HB%) end
  if ($select = 2) then
!    vector do (store1 = store1 + 1) (resid $resid and name $name)
   vector do (store1 = store1 + 1) (resid $resid and name HB%)
  end if
end loop meth
for $resid in ( 2 3 17 20 21 22 24 27 28 36 40 46 )
loop meth
!  eval ($name = "HG%") !for Gln Glu Lys Arg Pro Met
!  coor sele= (resid $resid and name $name) end
  coor sele= (resid $resid and name HG%) end
  if ($select = 2) then
!    vector do (store1 = store1 + 1) (resid $resid and name $name)
   vector do (store1 = store1 + 1) (resid $resid and name HG%)
  end if
end loop meth
for $resid in ( 48 52 55 59 66 67 70 75 83 85 87 89 90 )
loop meth
!  eval ($name = "HG%") !for Gln Glu Lys Arg Pro Met
!  coor sele= (resid $resid and name $name) end
  coor sele= (resid $resid and name HG%) end
  if ($select = 2) then
!    vector do (store1 = store1 + 1) (resid $resid and name $name)
   vector do (store1 = store1 + 1) (resid $resid and name HG%)
  end if
end loop meth
for $resid in ( 41 60 77 ) !for Ile
loop meth
!  eval ($name = "HG1%")
!  coor sele= (resid $resid and name $name) end
```

```
  coor sele= (resid $resid and name HG1%) end
  if ($select = 2) then
!    vector do (store1 = store1 + 1) (resid $resid and name $name)
  vector do (store1 = store1 + 1) (resid $resid and name HG1%)
  end if
end loop meth
for $resid in ( 3 21 22 40 46 48 55 ) !for Arg, Lys and Pro
loop meth
!  eval ($name = "HD%")
!  coor sele= (resid $resid and name $name) end
  coor sele= (resid $resid and name HD%) end
  if ($select = 2) then
!    vector do (store1 = store1 + 1) (resid $resid and name $name)
   vector do (store1 = store1 + 1) (resid $resid and name HD%)
  end if
end loop meth
for $resid in ( 59 66 67 70 83 87 90 ) !for Arg, Lys and Pro
loop meth
!  eval ($name = "HD%")
!  coor sele= (resid $resid and name $name) end
  coor sele= (resid $resid and name HD%) end
  if ($select = 2) then
!    vector do (store1 = store1 + 1) (resid $resid and name $name)
  vector do (store1 = store1 + 1) (resid $resid and name HD%)
  end if
end loop meth
for $resid in ( 3 21 66 67 87 )  !for Lys
loop meth
!  eval ($name = "HE%")
!  coor sele= (resid $resid and name $name) end
  coor sele= (resid $resid and name HE%) end
  if ($select = 2) then
!    vector do (store1 = store1 + 1) (resid $resid and name $name)
   vector do (store1 = store1 + 1) (resid $resid and name HE%)
  end if
end loop meth
for $resid in ( 24 52 75 )  !for Gln
loop meth
!  eval ($name = "HE2%")
!  coor sele= (resid $resid and name $name) end
  coor sele= (resid $resid and name HE2%) end
  if ($select = 2) then
!    vector do (store1 = store1 + 1) (resid $resid and name $name)
   vector do (store1 = store1 + 1) (resid $resid and name HE2%)
  end if
end loop meth
for $resid in ( 47 50 69 72 81 )
loop meth
!  eval ($name = "HD2%")  !for Asn
!  coor sele= (resid $resid and name $name) end
  coor sele= (resid $resid and name HD2%) end
  if ($select = 2) then
!    vector do (store1 = store1 + 1) (resid $resid and name $name)
   vector do (store1 = store1 + 1) (resid $resid and name HD2%)
  end if
end loop meth
set echo on message on end
```

swap15v.xplor

```
REMARK swaps systematically randomly selected methylene protons
REMARK   accepts the conformation with lower noe energy
REMARK   can be used in conjunction with floating assignment
REMARK
REMARK the floating groups have to be identified in store2
REMARK (with setup_swap.xplor and vector ident (store2) (store1) )
REMARK
REMARK uses the new "coor flip" and "coor vflip" commands
REMARK
REMARK   Author: Michael Nilges, EMBL.
```

```
set echo off message off end

evaluate ($reject = 0)
evaluate ($tries = 0)
evaluate ($newnoe=0)
energy end
evaluate ($oldnoe=$noe)

if ($swap > 0) then
   vector do (q = q * ran()) (attr q > -0.5)
end if

for $id in id (store2 and name h*1 and attr q < $swap and attr q > -0.5) loop meth
  vector show element (resid) (id $id)
  eval ($resid = $result)
  vector show element (name) (id $id)
  eval ($name1 = $result)
  eval ($name2 = $result - "1" + "2")
  coor flip sele= (resid $resid and (name $name1 or name $name2)) end

  energy end
  evaluate ($newnoe=$noe)
  evaluate ($tries = $tries + 1)

  if ($newnoe >= $oldnoe) then
    coor flip sele= (resid $resid and (name $name1 or name $name2)) end
    evaluate ($reject = $reject+1)
  else
    evaluate ($oldnoe = $newnoe)
    coor vflip sele= (resid $resid and (name $name1 or name $name2)) end
  end if
end loop meth

for $id in id (store2 and ((resn val and name cg1) or (resn leu and name cd1))
               and attr q < $swap and attr q > -0.5) loop meth
  coor copy end

  vector show element (resid) (id $id)
  eval ($resid = $result)
  vector show element (name) (id $id)
  eval ($name1 = $result)
  eval ($name2 = $name1 - "1" + "2")

  coor flip sele= (resid $resid and (name $name1 or name $name2)) end

  for $hycount in (1 2 3) loop myle
      eval ($hname1 = "H" + $name1 - "C" + encode($hycount))
      eval ($hname2 = "H" + $name2 - "C" + encode($hycount))
      coor flip sele= (resid $resid and (name $hname1 or name $hname2)) end
  end loop myle

  energy end
  evaluate ($newnoe=$noe)
  evaluate ($tries = $tries + 1)

  if ($newnoe >= $oldnoe) then
    coor swap end
    evaluate ($reject = $reject+1)
  else
    evaluate ($oldnoe = $newnoe)
    coor vflip sele= (resid $resid and (name $name1 or name $name2)) end
    for $hycount in (1 2 3) loop myle
      eval ($hname1 = "H" + $name1 - "C" + encode($hycount))
      eval ($hname2 = "H" + $name2 - "C" + encode($hycount))
      coor vflip sele= (resid $resid and (name $hname1 or name $hname2)) end
    end loop myle
  end if
end loop meth

if ($tries > 0) then evaluate ($reject = 100 * $reject / $tries) end if
```

```
vector do (q=1) (attr q < $swap and attr q > -0.5)

display  swaps tried: $tries   swaps rejected: $reject percent

set echo on message on end
```

sa_l_hightemp.xplor

```
   evaluate ($nstep1=int($high_steps / 5.0 ) )

   vector do (vx = maxwell($init_t)) (all)
   vector do (vy = maxwell($init_t)) (all)
   vector do (vz = maxwell($init_t)) (all)


   for $knoe in (10 20 30 40 50) loop anne
   !evaluate ($filename=$fileroot+ encode($count)+"_"+encode($knoe)+"a.pdb")
   !write coor output=$filename end
     noe scale dist $knoe scale ambi $ini_amb scale hbon $ini_amb end

      flags exclude * include noe end
      evaluate ($swap = 1.001)
      @swap15v.xplor
      flags include bond angl impr vdw noe cdih end

     evaluate ($kvdw = $knoe * 0.002)
     constraints
        interaction (all) (not store1) weights * 1 angl 1.0 impr 1.0 vdw   0.0 elec 0 end
        interaction (store1) (store1)  weights * 1 angl 1.0 impr 1.0 vdw $kvdw elec 0 end
     end


     dynamics  verlet
        nstep=$nstep1   timestep=0.002   iasvel=current   firstt=$init_t
        tcoupling=true  tbath=$init_t  nprint=50  iprfrq=0
        ntrfrq=9999
     end

     evaluate ($critical=$temp/$init_t)
     if ($critical > 1.2) then
        evaluate ($nreassign = $nreassign + 1)
        mini powell nstep 100 end
        vector do (vx=maxwell($init_t)) ( all )
        vector do (vy=maxwell($init_t)) ( all )
        vector do (vz=maxwell($init_t)) ( all )
     end if
   end loop anne
```

sa_l_cool1.xplor

```
   vector do (vx=maxwell($init_t)) ( all )
   vector do (vy=maxwell($init_t)) ( all )
   vector do (vz=maxwell($init_t)) ( all )

   {* 4 ================================================== cool the system *}



   evaluate ($swap = 0.05)

   restraints dihedral   scale=200.   end

   parameter                          {* parameters for the repulsive energy term *}
    nbonds
      repel=1.                      {* initial value for repel - modified later *}
      rexp=4 irexp=1 rcon=1.
      nbxmod=5
      wmin=0.01
```

```
   cutnb=4.5 ctonnb=2.99 ctofnb=3.
   tolerance=0.5
end
end

evaluate ($ncycle = ($init_t-$final1_t)/$tempstep)
evaluate ($nstep = int($cool1_steps/$ncycle))


evaluate ($i_cool = 0)
while ($i_cool < $ncycle) loop cool
   evaluate ($i_cool=$i_cool+1)

   evaluate ($bath  = $bath  - $tempstep)
   evaluate ($k_vdw=min($fin_con,$k_vdw*$k_vdwfact))
   evaluate ($radius=max($fin_rad,$radius*$radfact))
   evaluate ($k_flt = $k_flt*$flt_fac)
   evaluate ($k_ang = $k_ang*$ang_fac)
   evaluate ($k_imp = $k_imp*$imp_fac)
   evaluate ($k_amb = $k_amb*$amb_fac)


   flags exclude * include noe end
   @swap15v.xplor
   flags include bond angl impr vdw noe cdih end

   parameter
     bond  (name sg) (name sg) $k_flt TOKEN
     angle (all) (name sg) (name sg)  $k_flt TOKEN
     angle (store2) (all) (store2) $k_flt TOKEN
     angle (all)    (all) (store2) $k_flt TOKEN
     nbonds repel=$radius end
   end

   constraints
     interaction  (all) (all) weights * 1 vdw $k_vdw end
     end
     noe scale ambi $k_amb scale hbon $k_amb end

     dynamics  verlet
        nstep=$nstep time=0.001 iasvel=current firstt=$bath
        tcoup=true tbath=$bath nprint=$nstep iprfrq=0
        ntrfrq=99999
     end

     evaluate ($critical=$temp/$bath)
     if ($critical > 1.2) then
        evaluate ($nreassign = $nreassign + 1)
        mini powell nstep 100 end
        vector do (vx=maxwell($bath)) ( all )
        vector do (vy=maxwell($bath)) ( all )
        vector do (vz=maxwell($bath)) ( all )
     end if

end loop cool
```

sa_l_cool2.xplor

```
constraints interaction (all) (all) weights * 1. vdw 4. end end

   evaluate ($ncycle = ($bath-$final2_t)/$tempstep)
   evaluate ($nstep = int($cool2_steps/$ncycle))


   evaluate ($i_cool = 0)
   while ($i_cool < $ncycle) loop cool
      evaluate ($i_cool=$i_cool+1)
      evaluate ($bath = $bath - $tempstep)

      flags exclude * include noe end
      @swap15v.xplor
```

```
      flags include bond angl impr vdw noe cdih end


      dynamics  verlet
         nstep=$nstep time=0.001 iasvel=current firstt=$bath
         tcoup=true tbath=$bath nprint=$nstep iprfrq=0
        ntrfrq=9999
     end

{====>}                                                    {* abort condition *}
      evaluate ($critical=$temp/$bath)
      if ($critical > 1.2) then
         evaluate ($nreassign = $nreassign + 1)
         mini powell nstep 100 end
         vector do (vx=maxwell($bath)) ( all )
         vector do (vy=maxwell($bath)) ( all )
         vector do (vz=maxwell($bath)) ( all )
      end if

   end loop cool
```

axis.psf

```
PSF

       4 !NTITLE
 REMARKS FILENAME="test2.psf"
 REMARKS   para_anis.pro
 REMARKS molecule for anisotropy
 REMARKS DATE:03-Jun-98  09:46:57       created by user: lsmith

       4 !NATOM
       1       500  ANI  X    XXX    0.000000E+00   10.0000          0
       2       500  ANI  Y    YYY    0.000000E+00   10.0000          0
       3       500  ANI  Z    ZZZ    0.000000E+00   10.0000          0
       4       500  ANI  OO   OOO    0.000000E+00   10.0000          0

       3 !NBOND: bonds
       4       1       4       2       4       3

       3 !NTHETA: angles
       1       4       2       1       4       3       2       4       3

       0 !NPHI: dihedrals


       0 !NIMPHI: impropers


       0 !NDON: donors


       0 !NACC: acceptors


       0 !NNB

       0       0       0       0

       1       0 !NGRP
       0       0       0
```

axis.pdb

```
ATOM   1232  X   ANI   500      159.637   6.049   7.058  1.00  3.06
ATOM   1233  Y   ANI   500      155.838   4.216   6.602  1.00  4.37
ATOM   1234  Z   ANI   500      159.007   2.095   8.462  1.00  4.35
ATOM   1235  OO  ANI   500      158.647   3.496   5.834  1.00  4.04
END
```