ABSTRACT

N.M.R. Structural Studies and Modelling of Modular Proteins

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This thesis focuses on structural studies of the fibronectin type 1 module (F1) of tissue-type plasminogen activator (t-PA) and the N-terminal src homology 2 (SH2) module of the p85α subunit of phosphatidylinositol-3-OH kinase (PI3-kinase). t-PA plays a key role in the dissolution of blood clots. It is the only serine protease that binds to the fibrin network of a thrombus and localises its activity to the site of a clot. The t-PA F1 module is critical to t-PA fibrin binding. This module was produced for structural studies using a yeast alpha secretion system. N.m.r. data collected on the purified protein were used to calculate a high resolution structure of the module.

The structures of the t-PA finger and the seventh type 1 module from human fibronectin were compared and found to be strikingly similar. Topological differences between the two molecules suggested that one face of the t-PA finger engages in domain-domain interactions in the intact protein. A model of the intact t-PA was constructed and used to explain differences in the biological activity of the two naturally abundant glycosylated variants of the molecule.

The SH2 module binds sequence-specifically to intracellular phosphorylated tyrosines on receptor tyrosine kinases and thus mediates cell signalling pathways which lead ultimately to changes in cell growth and differentiation. The 3-dimensional structure of the N-terminal SH2 of the p85α subunit of PI3-kinase was computed and compared to the *v-src* SH2 complexed with low affinity phosphopeptides in order to assess the conformational changes associated with phosphopeptide binding. Finally, the structures of two homologous SH2 modules were modelled to identify possible high affinity protein-peptide interactions responsible for the variation in phosphopeptide affinity of SH2 modules. The predicted interactions were subsequently observed in two high affinity SH2-peptide complexes.

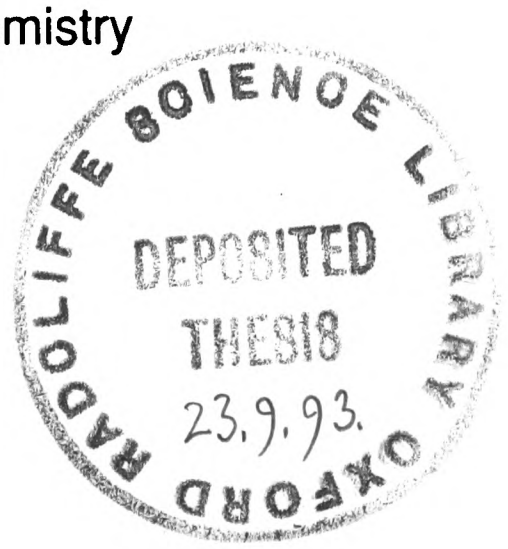
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by

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To my parents

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Abbreviations and Symbols

The 20 standard three-letter abbreviations and one-letter codes for the amino acids are given in Table 1.1.

n.m.r.	nuclear magnetic resonance spectroscopy
F1	fibronectin type 1 or finger (module)
t-PA	tissue-type plasminogen activator
SH2	src homology 2 (module)
PI3-kinase	phosphatidylinositol-3-OH kinase
sc-tPA	single chain t-PA
tc-tPA	two chain t-PA
p85	regulatory subunit of PI3-kinase
DNA	deoxyribonucleic acid
HPLC	high pressure liquid chromatography
ddH_2O	double distilled H_2O
MW	molecular weight
COSY	correlated spectroscopy
NOESY	nuclear Overhauser enhancement spectroscopy
HOHAHA	Hartman-Hahn spectroscopy
FID	free induction decay
DQF	double quantum filtration
ppm	parts per million
NOE	nuclear Overhauser effect
TPPI	time proportional phase incrementation
DQF-COSY	double quantum filtered correlated spectroscopy
PE-COSY	primitive exclusive correlated spectroscopy
SA	simulated annealing
r.m.s.	root mean square

G	EGF-like module
K1, K2	kringle modules
SP	serine protease domain
p85α-N SH2	N-terminal SH2 module of the p85α subunit of PI3-kinase
GAP-N SH2	N-terminal SH2 of the GTPase-activating protein of p21-ras
p85α-C SH2	C-terminal SH2 module of the p85α subunit of PI3-kinase
βPDGFR	platelet-derived growth factor β-subunit
δ	chemical shift
J	spin-spin coupling constant
τ_m	mixing time
τ_c	molecular correlation time
r	distance separating two spin-1/2 nuclei
W_1, W_0, W_2	single, zero and double quantum transition probabilities
η	nuclear Overhauser enhancement factor
B_1	radiofrequency field

Chapter 1

Introduction to Structural Studies of Modular Proteins

1.1 Elements of Protein Structure

Protein 3-dimensional structure arises because particular sequences of amino acids in polypeptide chains fold to generate compact, energetically favourable conformations in solution. There exists a hierarchy of the structural features of a protein. For a single protein chain the complete covalent structure, including covalent intrastrand cross-links, is known as the *primary* structure. Different regions of the linear polypeptide chain form local *secondary* structure, for example, α-helices and β-strands. *Tertiary* structure is formed by packing such structural elements into one or more globular units called domains. Some proteins contain several polypeptide chains arranged in a *quaternary* structure. A brief introduction to the chemistry of the amino acids and how it contributes to the formation of the levels of structure will now be reviewed briefly. More detailed discussion can be found in

Biophysical Chemistry Part I: The Conformation of Biological Macromolecules (Cantor & Schimmel, 1980) and *Introduction to Protein Structure* (Branden and Tooze, 1991).

The names and commonly used three-letter and one-letter abbreviations of the 20 standard amino acids are given in Table 1.1. All of the amino acids have a central carbon atom (C^{α}) which is attached to an amino group and a carboxy group. However their side chains, which are shown in Figure 1.1, differ in size and display a wide range of chemical properties. The variation in size and polarity of amino acids imparts each with a unique preferred local environment which significantly influences protein folding. The 20 amino acids are commonly grouped into three classes according to their side chain properties. The first class includes amino acids with hydrophobic side chains: ile, val, leu, phe, met, ala and pro. A second class is comprised of the residues with polar side chains: cys, thr, ser, trp, tyr, his, gln and asn. The four amino acids with charged side chains, glu, asp, lys and arg, form a third class. Glycine, the smallest of the amino acids, is usually considered to belong to the first class or to form a fourth class. Three-dimensional protein structures are usually stabilised by the packing of hydrophobic side chains to form a hydrophobic core, while hydrophilic residues are often found on the surface. To gradate the division of amino acid side chains, within each class the amino acids have been ranked in order of decreasing hydropathy, combined hydrophobic and hydrophilic tendencies (Kyte & Doolittle, 1982). In general the greater the hydropathy of a side chain, the more likely it is to be found at the interior of a 3-dimensional protein structure.

Two adjacent cysteine residues in a 3-dimensional protein structure can be oxidised to form a disulphide bridge. Such covalent cross-links are not usually found in intracellular proteins which normally

Table 1.1

Names and Abbreviations of the 20 Standard Amino Acids

Amino Acid	Three Letter	One Letter	Amino Acid	Three Letter	One Letter
glycine	gly	G	proline	pro	P
alanine	ala	A	aspartate	asp	D
cysteine	cys	C	glutamate	glu	E
tyrosine	tyr	Y	lysine	lys	K
tryptophan	trp	W	arginine	arg	R
phenylalanine	phe	F	histidine	his	H
isoleucine	ile	I	glutamine	gln	Q
leucine	leu	L	asparagine	asn	N
methionine	met	M	serine	ser	S
valine	val	V	threonine	thr	T

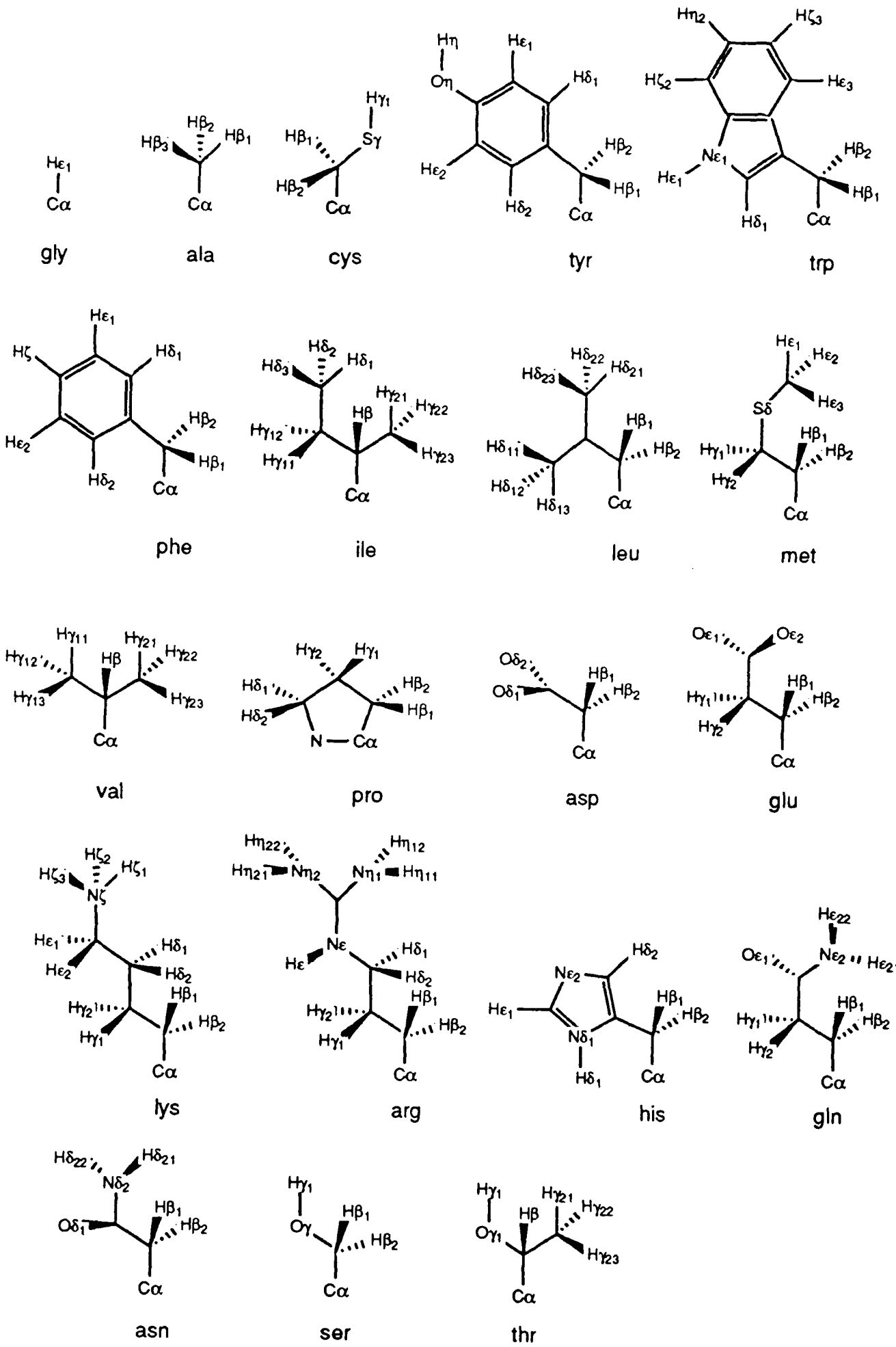


Figure 1.1

The 20 standard amino acid side chains. The atom names are consistent with those used by the protein structure calculation program XPLOR (Brünger, 1990).

exist in a reductive environment, however they occur frequently in extracellular proteins. Disulphide bridges help to stabilise protein structure and sometimes hold together different polypeptide chains.

During protein synthesis amino acids are joined by the formation of peptide bonds. Figure 1.2 illustrates the geometry of the peptide bond and the definition of the torsion angles. Protein backbone conformation is described principally by the angles ϕ and ψ because ω rotation is inhibited. The χ^1 torsion angle describes rotation about the side chain C^{α}-$C\beta$ bond. The amino acid side chains restrict main chain flexability. As a result specific regions of a (ϕ, ψ) plot, known as a Ramachandran plot (Ramachandran *et al.*, 1963), are not sterically allowed. The small side chain of glycine allows it to adopt a wider range of conformations than the other residues. Proline residues, on the other hand, have less conformational freedom because the proline side chain is fixed to the main chain by a covalent bond.

Most protein structures are built up from combinations of the two most common secondary structure elements, α-helices and β-strands, which are connected by loop regions. Both α-helices and β-strands are stabilised by regular hydrogen bonds (Figure 1.3). The α-helix includes hydrogen bonds between the carbonyl and peptide amide groups of residues i and i+4, and the side chains project out from the helix. Adjacent β-strands hydrogen bond as shown in Figure 1.3 to form parallel and antiparallel β-sheets. While the α-helix is formed from one continuous region of the polypeptide chain, β-sheets are built from a combination of two or more regions of the polypeptide chain.

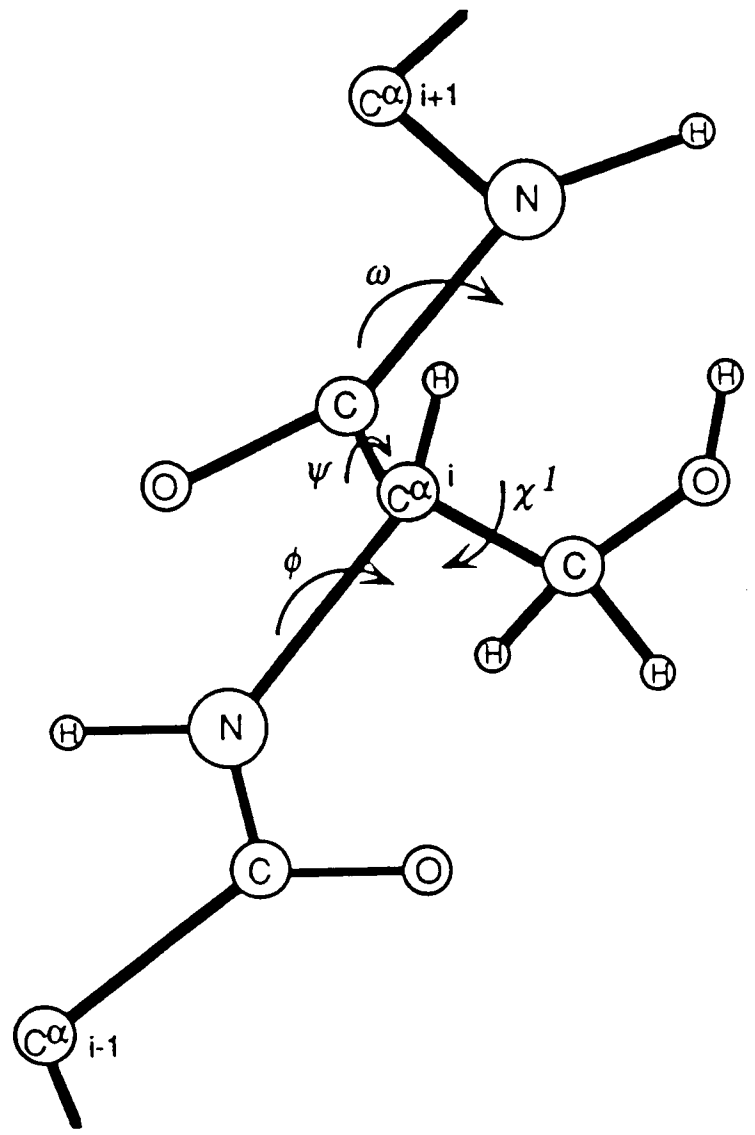
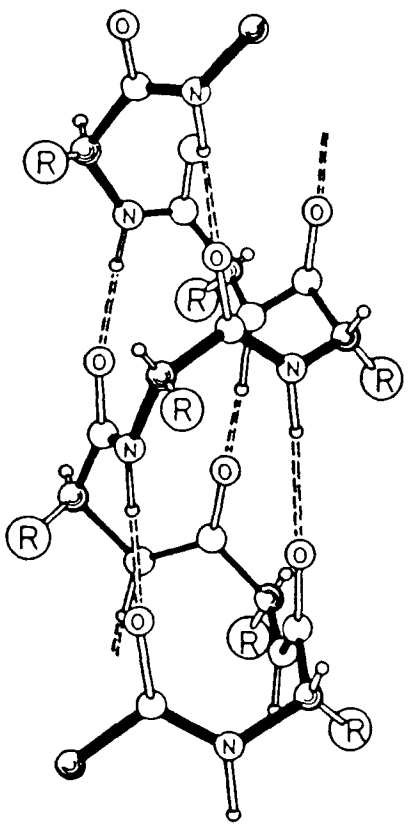
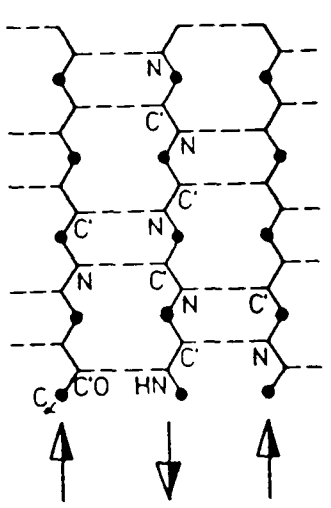


Figure 1.2

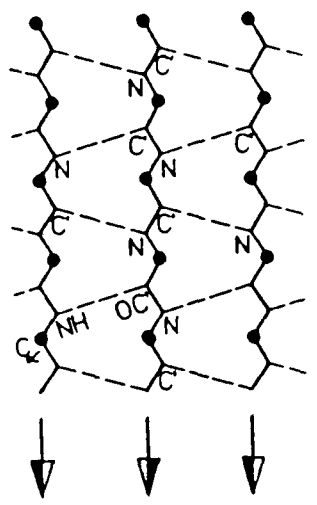
The peptide bond and definition of the dihedral angles in a polypeptide chain. The bond is shown in a *trans* conformation because *cis* peptide bonds are rarely observed.



α-helix



antiparallel
β-sheet



parallel
β-sheet

Figure 1.3

Common protein patterns of folding. The right-handed α-helix is the most abundant secondary structure in proteins, while left-handed helices are very rarely observed. The hydrogen bonds which stabilise the sheet structures are indicated by dashed lines. These illustrations have been reproduced from Schulz & Schirmer, 1979.

1.2 Techniques for High Resolution 3-Dimensional Structure Determination

Knowledge of the high resolution structure of a protein is critical to understanding of its function and mechanism of action. Presently only two techniques are available for structural studies at atomic resolution: x-ray crystallography and nuclear magnetic resonance spectroscopy (n.m.r.). A brief overview of these two techniques follows for the purpose of their comparison. A more detailed introduction to these methods is found, for example, in *Protein Structure: New Approaches to Disease and Therapy* (Perutz, 1992) and references therein. In later chapters it will be assumed that the reader of this thesis is familiar with n.m.r. parameters and the elementary features of n.m.r. experiments. Newcomers to n.m.r. and those readers wishing to review these subjects are advised to consult *Modern NMR Techniques for Chemistry Research* (Derome, 1987), *A Handbook of Nuclear Magnetic Resonance* (Freeman, 1988) and *NMR of Proteins and Nucleic Acids* (Wüthrich, 1986).

X-ray crystallography is a well-established technique in which the structure of the protein is calculated from its diffraction pattern, but usually not without difficulties. The most problematic aspect of this technique is often crystallisation of the protein. Once this step has been successfully accomplished, the x-ray diffraction pattern of the crystal is measured. This pattern is a Fourier transform of the electron density of the protein that scattered it. If it were possible to measure both the phase and intensity of scattered radiation one could simply perform an inverse Fourier transform to derive the protein structure. However in practice it is only possible to measure the intensity. The intensity pattern contains sufficient information to calculate the structure, but it is difficult to interpret.

This complication, which is known as the "phase problem", is usually resolved in one of three ways. If the protein under study is very similar to one for which a crystal structure is already known then the phases of the known structure may be used. When the structure of the protein is unknown but is thought to be homologous to a known structure then the phases may be obtained by the technique of molecular replacement. In order to determine the structure of a novel protein fold, heavy-metal derivatives of the parent crystal must be prepared, and the structure is solved by the technique of multiple isomorphous replacement. Using this technique the scattered intensities of the parent and derivatives are compared in order to perform a preliminary calculation of the heavy-atom positions. These positions and differences in the diffraction patterns of the parent and derivative crystals are used to estimate the phase of the scattered radiation. The estimate allows more precise localisation of the heavy-metal atoms, and the determination of the phase is refined iteratively.

The difficulties associated with protein structure determination by x-ray crystallography have motivated the development of n.m.r. methodology as an alternative over the past 10 years. N.m.r. is a solution technique which probes the behaviour of the nuclear spins using polarisation of the sample magnetisation. Advantages of n.m.r. over x-ray crystallography include the fact that there are no crystallisation or phase problems associated with this method. Furthermore n.m.r. can be used to study dynamic processes on the picosecond to second time scale, e.g., amide proton exchange rates. In addition the conditions of the solution sample (pH, temperature, concentration) can be varied for comparative studies. However, n.m.r. also has its share of disadvantages. The protein being studied must be soluble up to a concentration of at least 1 mM and remain folded. Also, the resolution of

n.m.r. data decreases with increasing molecular weight which limits the size of proteins which may be studied. Using the current methodology high resolution structures can be determined for proteins of up to ~200 amino acids.

1.3 Modular Proteins

The eukaryotic gene structure is mosaic in nature with short, coding regions called exons separated by long, noncoding regions called introns (Breathnach *et al.*, 1977; Breathnach & Chambon, 1981). The exons often code for independently folding protein subunits which are referred to as domains or modules (Blake, 1978; Blake, 1979). This observation led to the exon-shuffling hypothesis (Gilbert, 1978; Blake, 1978; Blake, 1979), which postulates that the substructures encoded by exons have been duplicated and shuffled during the course of evolution to create novel protein molecules. This theory is illustrated schematically in Figure 1.4.

Support for the exon-shuffling hypothesis was provided by the discovery that proteases of blood coagulation and fibrinolysis display a complex distribution of similar domains (Bányai *et al.*, 1983; Patthy *et al.*, 1984; Patthy, 1985) as shown in Figure 1.5. Subsequent examination of the intron-exon organisations of these domains revealed that, as a rule, the introns separating them split the reading frame in phase 1 (Patthy, 1987). This observation led Patthy (1991) to define a module as an independently folding protein substructure flanked by introns of identical phase. Since domains without well-defined intron-exon boundaries are often referred to modules in the literature, in this thesis the word domain will be used to refer to an independently folded protein substructure, and

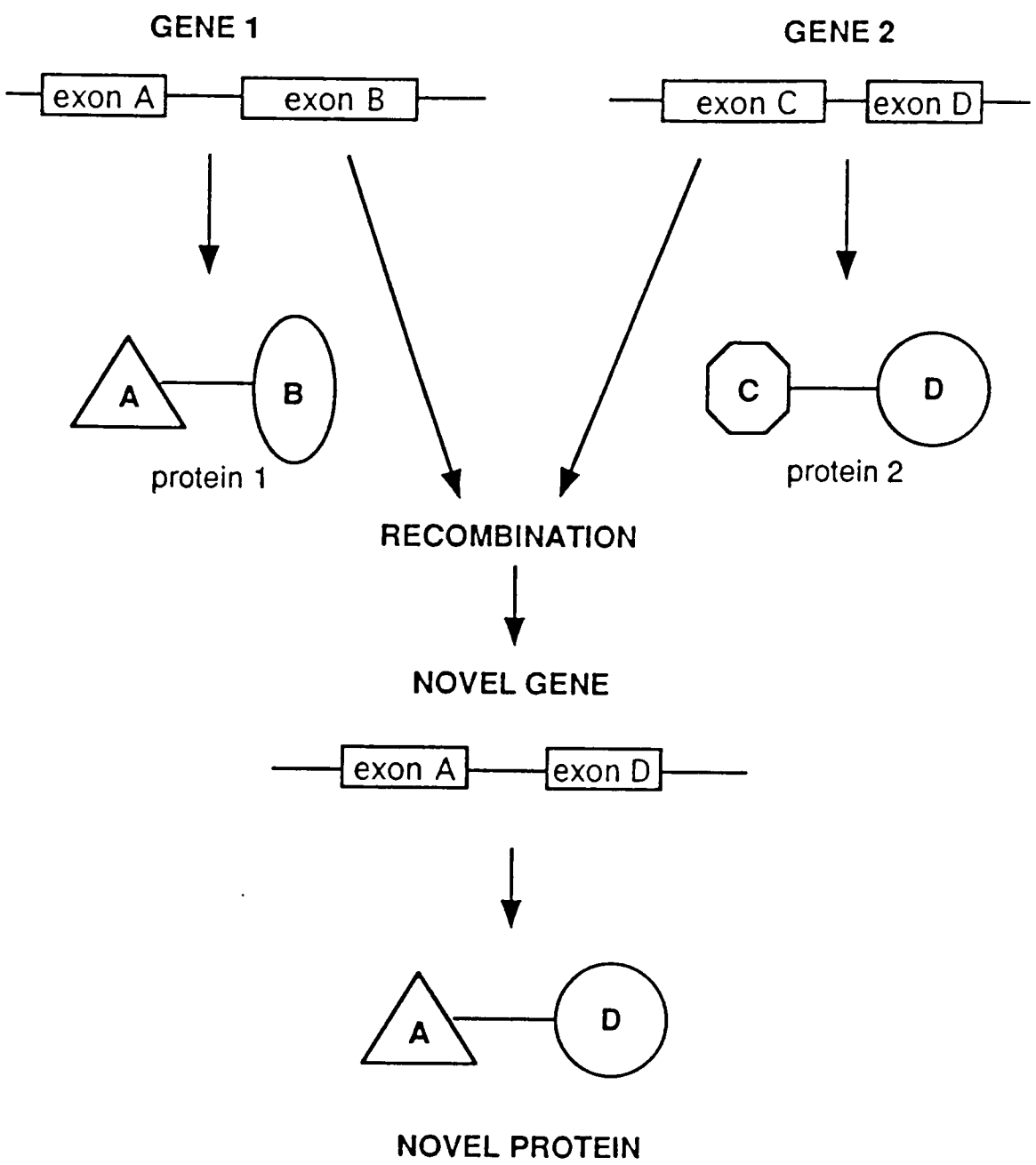


Figure 1.4

Schematic representation of the Gilbert-Blake exon-shuffling hypothesis.

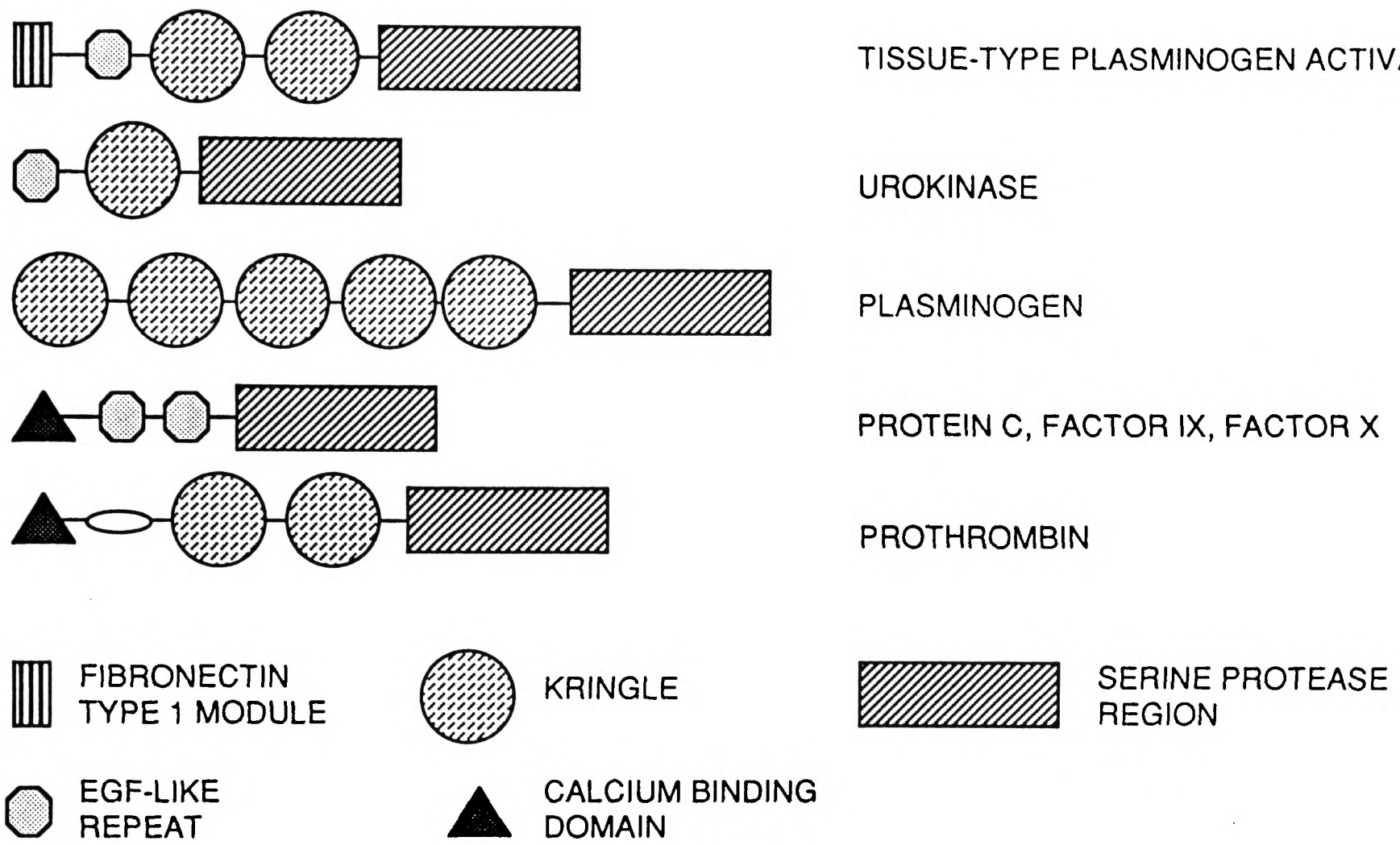


Figure 1.5

Modular structures of proteases of blood coagulation and fibrinolysis including tissue-type plasminogen activator, urokinase, plasminogen, protein C, factor IX, factor X and prothrombin. The presence of homologous units in these otherwise unrelated proteins has been attributed to exon-shuffling (Bányai *et al.*, 1983, Patthy *et al.*, 1994).

a module will be defined as a domain that is clearly associated with exon shuffling and duplication.

Observation of the sequence similarity of the protease modules led to the idea that a correlation exists between highly conserved amino acids and a generalised consensus structure for each module (Campbell & Baron, 1991). In other words, highly conserved residues define a structural framework. This being so, it might also be concluded that specific mutations impart functional specificity. This hypothesis is substantiated by the observation that nonsubstituted residues are principally those known to be structurally important including nonpolar residues, which usually comprise the hydrophobic core of a protein molecule, and those which have unique conformational properties (gly and pro).

Structure determination of protein modules is motivated principally by the desire to interpret functional data in terms of protein structure. Furthermore, it provides support for the theory of exon-shuffling, and comparison of homologous modules from different proteins gives a measure of the general value of consensus structures.

1.4 Selection of Modules for Study

Often protein subunits of less than 200 amino acids fold independently and are ideal candidates for study using n.m.r. techniques. Of course, one must be able to isolate an adequate amount of the domain of interest, and it must be soluble and folded at a concentration of at least 1 mM. Aside from these technical requirements the most important criterion of protein selection is its scientific interest. This thesis focuses on n.m.r. structural studies of the fibronectin type 1 or finger module (F1) from tissue-type plasminogen activator (t-PA) and the

N-terminal src homology 2 (SH2) module of the p85α subunit of phosphatidylinositol-3-OH kinase (PI3-kinase).

A homologous fibronectin type 1 structure had been reported prior to selection of the t-PA F1 module for n.m.r. study (Baron *et al.*, 1990a). However, only ~50% of the residues which are highly conserved in fibronectin type 1 modules are also conserved in the t-PA F1 (Baron *et al.*, 1990a). Therefore study of the t-PA F1 module enabled a comparison of the two structures to derive an indication of the degree of correlation that exists between consensus sequences and structures. Experimental evidence has accumulated which contradicts the previously held belief that the t-PA finger module functions autonomously in the context of the intact molecule (Bennett *et al.*, 1991; Novokhatny *et al.*, 1991). Numerous attempts to crystallise intact t-PA have proven unsuccessful, and a high resolution structure of the F1 module also provided clues to its interaction with other domains in t-PA.

Unlike the F1 module, the 3-dimensional structure of the SH2 module was unknown prior to its selection for study. Biological data suggested that this module functions independently in a large number of proteins, including PI3-kinase (Koch *et al.*, 1991). Thus while interest in the t-PA F1 module stemmed primarily from a desire to understand its role with respect to the intact protein, the structure of the N-terminal SH2 from PI3-kinase provided insight into the general mechanism of SH2 module action. Detailed studies of the biology of tissue-type plasminogen activator have been extensively reviewed (van Zonneveld *et al.*, 1986a; Harris, 1987; Pannekoek *et al.*, 1988), and the functional role of the SH2 module has been described in depth (Ullrich & Schlessinger, 1990; Cantley *et al.*, 1991; Schlessinger & Ullrich, 1992; Koch *et al.*, 1991; Pawson & Gish, 1992; Pawson, 1992). A brief

overview of the highly significant functional properties of the t-PA F1 and SH2 modules will now be presented.

1.4.1 Biology of tissue-type plasminogen activator: role of the finger module

t-PA is a serine protease that has a critical role in the fibrinolytic process. Specifically, it catalyses the conversion of the zymogen glu-plasminogen to its active form, plasmin, which is the enzyme responsible for degrading the fibrin network of a blood clot. t-PA is synthesised as a single chain protein (sc-tPA), but can be converted by plasmin into a two-chain form (tc-tPA) in which the chains are connected by a disulphide bond. t-PA is an atypical serine protease because it cannot be classified as a zymogen: the single chain and two chain forms are equally active under physiological conditions. Unlike other thrombolytic agents, the action of t-PA is localised to the site of a clot, forming a ternary complex with plasminogen and fibrin, thus avoiding systemic fibrinolytic activation (Weimer *et al.*, 1981; Van de Werf *et al.*, 1984). In addition, the low rate of plasminogen activation by t-PA is substantially accelerated in the presence of fibrin or fibrinogen degradation products. Consequently, considerable attention has been directed towards the investigation of the structural interaction between t-PA and fibrin.

t-PA is composed of five discrete structural domains (Figure 1.5) which share homology with regions of other proteins (Ny *et al.*, 1984). The results of domain addition and deletion experiments have identified the finger and second kringle modules as critical for fibrin binding (van Zonneveld *et al.*, 1986b; Verheijen *et al.*, 1986; Gething *et al.*, 1988). Specifically, van Zonneveld *et al.* (1986b) have proposed that the finger module governs the initial interaction between t-PA and fibrin. A high

resolution structure-function analysis of t-PA has reinforced this idea by showing that mutations in the finger module affect fibrin binding (Bennett *et al.*, 1991). The association of t-PA with fibrin and plasminogen leads to localised generation of plasmin in the vicinity of a thrombus. Hence structural and functional studies of the finger module which shed light on the mechanism of t-PA fibrin binding are of intense biological interest.

1.4.2 Biological role of the src homology 2 module

The SH2 module plays a critical role in control of growth factor signalling by receptor tyrosine kinases which ultimately leads to changes in cell growth and proliferation. Most receptor tyrosine kinases are composed of three structural regions: an extracellular ligand binding domain, a single membrane-spanning domain and a cytoplasmic domain. The signalling cascade is initiated by binding to the receptor of an external ligand as illustrated schematically in Figure 1.6. The receptor dimerises and autophosphorylates tyrosine residues by an intermolecular reaction (Pawson & Bernstein, 1990; Ullrich & Schlessinger, 1990). The phosphorylated tyrosines then act as high affinity binding sites for cellular proteins such as phospholipase C-γ, the GTPase-activating protein of p21-ras and the regulatory subunit (p85) of PI3-kinase (Vogel *et al.*, 1988; Meisenhelder *et al.*, 1989; Margolis *et al.*, 1990; Burgess *et al.*, 1990; Escobedo *et al.*, 1991; Hu *et al.*, 1992; McGlade *et al.*, 1992).

The association of these and other signalling proteins with growth factor receptors is mediated by SH2 modules which recognise and tightly bind to phosphorylated tyrosines (Matsuda *et al.*, 1990). Furthermore, as first proposed by Cantley *et al.* (1991), high affinity binding of an SH2 module is dependent on the sequence surrounding the phosphotyrosine.

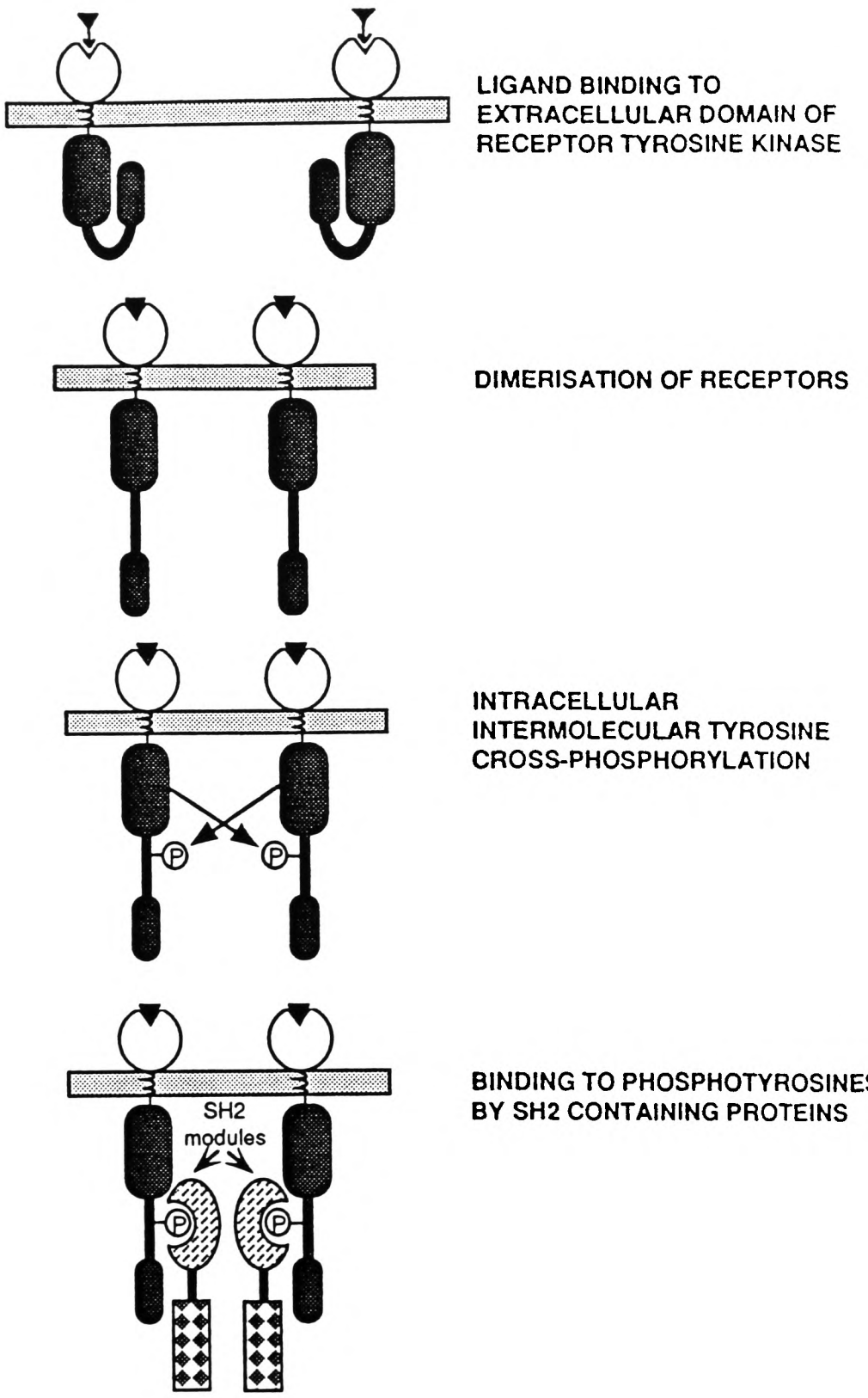


Figure 1.6

Schematic representation of the dimerisation model for receptor tyrosine kinase activation. The SH2 module binds specifically and with high affinity to phosphorylated tyrosines on the activated receptor.

The pathways leading ultimately to cell proliferation and differentiation are not yet completely understood. However, it is clear that protein-protein interactions mediated by the SH2 module play an integral role in controlling these processes, and knowledge of the SH2 structure is fundamental to comprehension of its phosphotyrosine binding mechanism.

Chapter 2

Expression, Purification and N.M.R. Sample Preparation of the t-PA F1 Module

2.1 Introduction

Three steps were involved in the preparation of the t-PA F1 sample for n.m.r. studies. First the module was expressed in sufficient quantity, then it was purified, and finally the sample conditions for experimentation were optimised. The methods used for expression and purification of the t-PA F1 module were developed by B. O. Smith and T. J. Dudgeon, hence discussion of these steps will be limited to a brief account of their work in section 2.2. First, however, the considerations involved in the optimisation of n.m.r. sample conditions will be introduced.

2.1.1 Optimisation of n.m.r. sample conditions

The n.m.r. experiments used in structure determination of small proteins are dependent on observation of ^1H proton signals. Both the temperature and pH at which an experiment is conducted directly affect the quality of n.m.r. data. A maximum number of HN resonances are observed at acidic pH due to slower proton exchange rate which typically reaches its minimum value at pH ~ 4. However, many small proteins are not stable and folded at low pH. Spectral resolution improves at higher temperature due to more rapid molecular tumbling, yet protein structure is sometimes destabilised at high temperatures causing sample denaturation. In addition, pH and temperature affect the rate of proton chemical exchange which can lead to line broadening or sharpening. Spectral assignment requires the comparison of spectra obtained at different temperatures in order to resolve ambiguous assignments due to chemical shift degeneracy. Therefore a range of temperatures must be identified over which the protein is stable and spectral resolution and sensitivity are optimised. pH and temperature effects on these parameters are usually monitored by inspection of peak height and resolution in 1-dimensional n.m.r. spectra.

Another important consideration is the concentration of the sample. The number of proton signals observed usually increases with increasing concentration, however protein solubility is often pH dependent. Also proteins are sometimes observed to aggregate at high concentration. Taking all of these factors into consideration, the strategy commonly adopted in the determination of n.m.r. sample conditions is to first optimise the sample pH at low concentration (1mM to 3 mM) while ascertaining that the structural integrity of the module is conserved over a

range of temperatures. Then the sample concentration is adjusted for maximum signal to noise ratio.

2.2 Expression of the t-PA F1 Module

Two microbial expression systems commonly used in the expression of heterologous genes are prokaryotic and yeast cells. While the protein yield using yeast cells is usually not as high as one would expect using prokaryotic cells, Baron *et al.* (1990b) reported significant advantages afforded by using yeast as a host system. Proteins are expressed by yeast with correctly formed disulphide bonds and in adequate quantity for n.m.r. research (Kingsman *et al.,* 1985). Furthermore, purification is facilitated because protein expression can be directed into the culture medium using the alpha factor leader sequence (Kurjan & Herskowitz, 1982). The only drawback associated with use of yeast as an expression system is occasional limited proteolysis of the product. These considerations led to the selection of a yeast host system for the expression of the t-PA finger module.

The design for the plasmid to express the t-PA finger module was based on the expression of the alpha-factor mating pheromone by *S. Cerevisiae.* The 165 residue precursor of the yeast alpha-factor mating pheromone consists of a 22 amino acid pre-sequence which directs secretion, a pro-sequence and four copies of the alpha factor separated by spacer sequences (Figure 2.1). These spacer sequences include lys-arg sequences, which are sites for proteolytic processing by the KEX 2 protease, and a series of glu-ala or asp-ala spacer dipeptides, which are removed by the STE 13 protease. It has been shown by Brake *et al.* (1984) that the lys-arg sequence alone is sufficient for correct N-terminal processing of the secreted protein. Therefore the construction of the t-PA

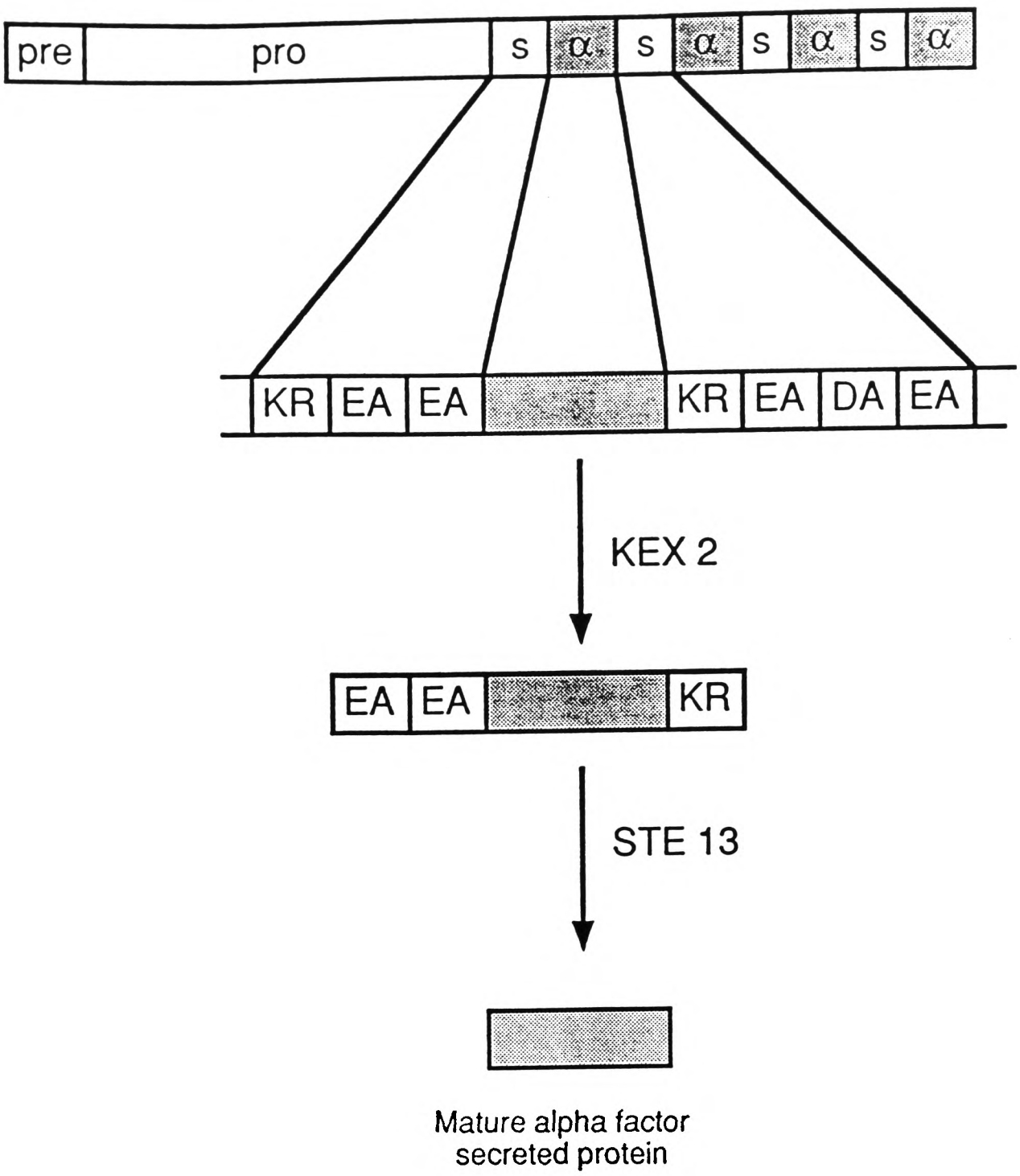


Figure 2.1

Schematic representation of the 165 residue precursor of the yeast alpha-factor mating pheromone upon which the design of the plasmid for expression of the t-PA finger module was based. The 22 amino acid pre-sequence which directs secretion is followed by a pro-sequence and four copies of the alpha factor separated by spacer sequences. These spacer sequences contain the necessary lys-arg sequences required for proteolytic processing by the KEX 2 protease as well as glu-ala or asp-ala dipeptides which are removed by the STE 13 protease.

F1 expression vector required introduction of the alpha factor prepro-sequence and a lys-arg cleavage point at the N-terminus of the finger sequence and two stop codons at its C-terminus.

The plasmid pTD4-43 used in expression of the t-PA finger module was made by replacing the *HindIII - BamHI* fragment of pTD4-18 with a *HindIII - BamHI* fragment containing the sequence coding for the finger module. The complete strategy adopted in the construction of the yeast expression vector for the finger module is outlined in Figure 2.2. Site directed mutagenesis was carried out on a M13mp19 clone containing the *HindIII - XmaI* restriction endonuclease fragment of t-PA according to the method of Kunkel *et al.* (1987). A *HindIII* restriction site followed by a lys-arg cleavage site was introduced at the N terminus of the sequence coding for the finger module. At the C terminus two stop codons followed by a *BamHI* site were incorporated. A portion of the DNA and amino acid sequences of t-PA and the oligonucleotides B2204 and B2201 used in these manipulations is shown in Figure 2.3. Correctly altered clones were screened by ^{35}S-dATP labelled dideoxy sequencing using Sequenase (United States Biochemicals). Sequences were read after running the reaction mixture on thin polyacrylamide gels which were dried onto Whatman 3MM and autoradiographed.

The double-stranded replicative form of the plaque-purified clones were grown up in *E. coli* strain JM103, harvested by the alkaline lysis method and purified by ultracentrifugation on a caesium chloride gradient. The *HindIII - BamHI* fragments were excised and purified by agarose gel electrophoresis. These fragments were ligated into the *HindIII - BamHI* linearised expression vector pTD4-18 to create pTD4-43, shown in Figure 2.4.

The yeast cells were grown in sterilised media consisting of 1.7 g yeast nitrogen base without amino acids (Difco), 5.0 g ammonium

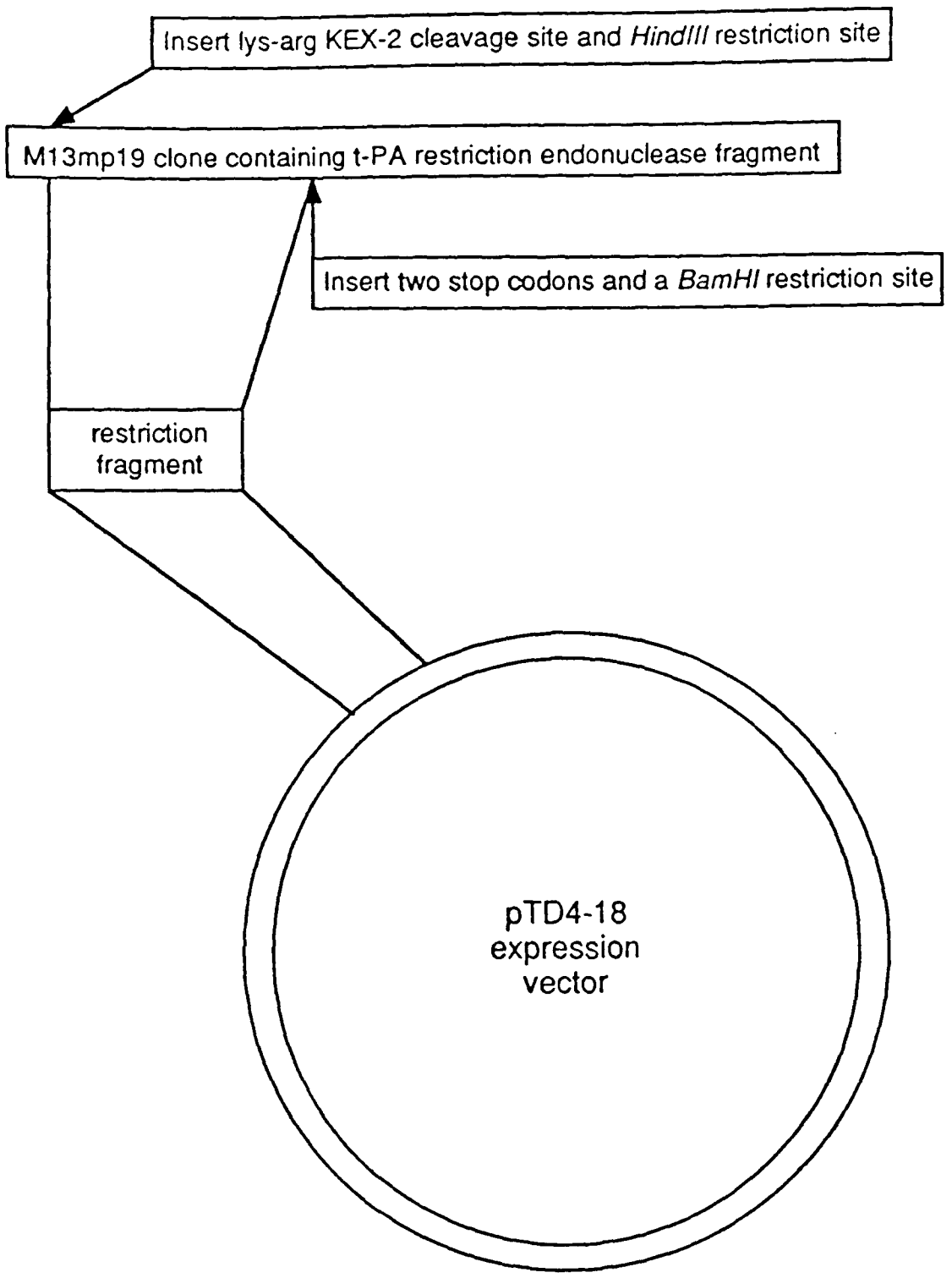


Figure 2.2

Strategy for the construction of the yeast expression vector for the t-PA finger module. Site directed mutagenesis performed on a M13mp19 clone containing a t-PA restriction endonuclease fragment introduced a lys-arg KEX 2 cleavage site and a *HindIII* restriction site at the N terminus of the sequence coding for the F1 module and two stop codons followed by a *BamHI* restriction site at its C terminus. Subsequently purified *HindIII -BamHI* fragments were ligated into the *HindIII-BamHI* linearised expression vector pTD4-18 to create the expression vector for the t-PA finger module.

```
                                M   D   A   M   K   R   G   L   C   C   L

TGT GAA GCA ATC ATG GAT GCA ATG AAG AGA GGG CTC TGC TGT GCT

 L   L   C   G   A   V   F   V   S   P   S   Q   E   I   H

GCT GCT TGT GGA GCA GTC TTC GTT TCG CCC AGC CAG GAA ATC CAT
                                                    TAG GTA
                                                    B2204

 A   R   F   R   R   G   A   R  S   Y   Q   V   I   C

GCC CGA TTC AGA AGA GGA GCC AGA TCT TAC CAA GTG ATC TGC
CGG GCT AAT TCG AAC CTA TTT TCT AGA ATG GTT CAC TAG
          HindIII

 R   D   E   K   T   Q   M   I   Y   Q   Q   H   Q

AGA GAT GAA AAA ACG CAG ATG ATA TAC CAG CAA CAT CAG

 S   W   L   R   P   V   L   R   S   N   R   V   E

TCA TGG CTG CGC CCT GTG CTC AGA AGC AAC CGG GTG GAA

 Y   C   W   C   N   S   G   R   A   Q   C   H   S

TAT TGC TGG TGC AAC TCC GGA AGG GCA CAG TGC CAT TCA

 V   P   V   K   S   C   S   E   P   R   C   F   N   G

GTG CCT GTC AAA AGT TGC AGT GAG CCA AGG TGT TTC AAC GGG
CAC GGA CAG TTT TCA ATT ATT CTT AGG TCC ACA AAG TTG CCC
B2201                       *   *   BamHI

 G   T   C   Q   Q   A   L   Y

GGC ACC TGC CAG CAG GCC CTG TAC
```

Figure 2.3

A portion of the DNA and amino acid sequences of t-PA and the oligonucleotides B2204 and B2201 which were used in site directed mutagensis of the M13mp19 clone containing the *HindIII - XmaI* restriction endonuclease fragment of t-PA. The B2204 and B2201 oligonucleotides were used to introduce the *HindIII* and the *BamHI* restriction sites respectively as shown.

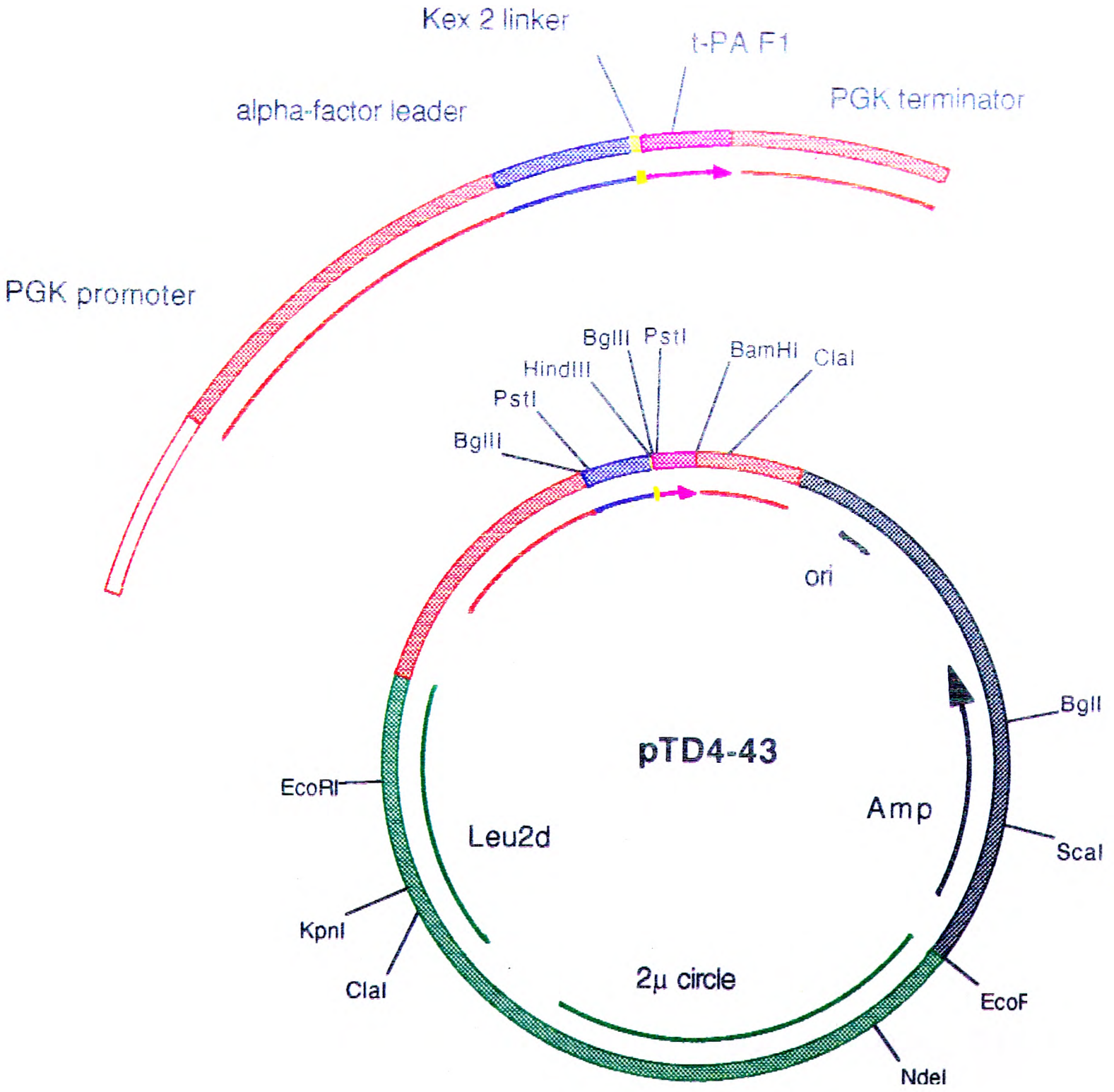


Figure 2.4

The pTD4-43 plasmid used to express the t-PA F1 module. pTD4-43 was constructed by ligating the *HindIII-BamHI* restriction fragment containing the sequence encoding the t-PA finger module into the linearised expression vector pTD4-18.

sulphate and 15 g glucose/l water. To this medium was added 1% sterile amino acid stock solution (without leucine or ammonium sulphate) immediately prior to use. Initially 10 ml of medium was inoculated and incubated without shaking at 30° C overnight. Typically after 24 h cells had visibly precipitated to the bottom of the tube. These cells were resuspended by shaking, then the preculture was diluted to 1 l and incubated with shaking at 30° C in a 2.5 l baffle flask for 24 h. This litre of media was subsequently diluted to 20 l, and each litre was incubated with shaking in a 2.5 l baffle flask at 30° C for 60 h. Cells were filtered from the protein-containing supernatant using an Amicon DC 10 l cell concentrator.

2.3 Purification of the t-PA F1 Module

Upon expression of the protein into the culture medium its concentration was on the order of µgs/ml. Hence it was desirable to concentrate the protein prior to purification. Concentration and partial purification was achieved by Baron *et al.* (1990b) by adding C18 derivatised silica beads to culture medium during cell growth. Afterwards these beads were filtered from the supernatant and eluted. The product was freeze-dried and subjected to successive rounds of ion exchange and reverse phase high pressure liquid chromatography (HPLC). Product yield is optimised when the purification process is achieved in the fewest number of steps. Therefore, in the purification of the t-PA finger module, concentration and ion exchange purification were combined into a single procedure by batch absorbing the cell-free culture supernatant onto an ion exchange cartridge.

The pH of the supernatant was adjusted to 4.5, approximately 2 pH units below the calculated pI of the protein. A Zeta Prep SP-60 ion-

exchange cartridge (CUNO) was pre-equilibrated at pH 4.5 with 100 ml 50 mM sodium acetate buffer at 5° C at a flow rate of 10 ml/min. Batch absorption of the supernatant onto the cartridge followed at a flow rate of 15 ml/min The cartridge was washed with 100 ml of 50 mM Tris buffer (pH 7.5) before eluting the protein using 50 mM Tris buffer, 2 M sodium chloride (pH 7.5). Ten 25 ml fractions collected at a flow rate of 10 ml/min were analysed by HPLC using a reverse phase column (Beckman C8, 250 mm x 100 mm) with a gradient running from 21% to 26% acetonitrile in 0.1% aqueous trifluoroacetic acid over a time course of 20 minutes Fractions containing protein (typically 1 to 6) were pooled and dialysed overnight against ddH_2O using Spectrapore dialysis tubing (3000 MW cutoff) to remove the sodium chloride. After dialysis the crude reaction mixture was freeze dried to a white powder and resuspended in 10 ml 0.1% aqueous trifluoroacetic acid prior to HPLC purification.

The finger module was purified in two successive reverse phase HPLC passages using the same column and gradient as in analysis of the fractions. Chromatograms illustrating the purification process are shown in Figure 2.5. The finger module is the main component in the crude reaction mixture, and the protein is pure and uncontaminated at the conclusion of the process. Typically 0.5 mg of pure protein was obtained/l cells. The identity of the purified protein was confirmed by low-resolution mass spectrometric analysis (Figure 2.6).

2.4 Optimisation of t-PA F1 N.M.R. Sample Conditions

A sample containing 3 mM t-PA F1 in 95% H_2O/5% D_2O was prepared for use in pH optimisation experiments. Figure 2.7 shows 1-dimensional n.m.r. experiments performed at varying pH. The pulse sequence used in these experiments is found in Appendix I. The 1-

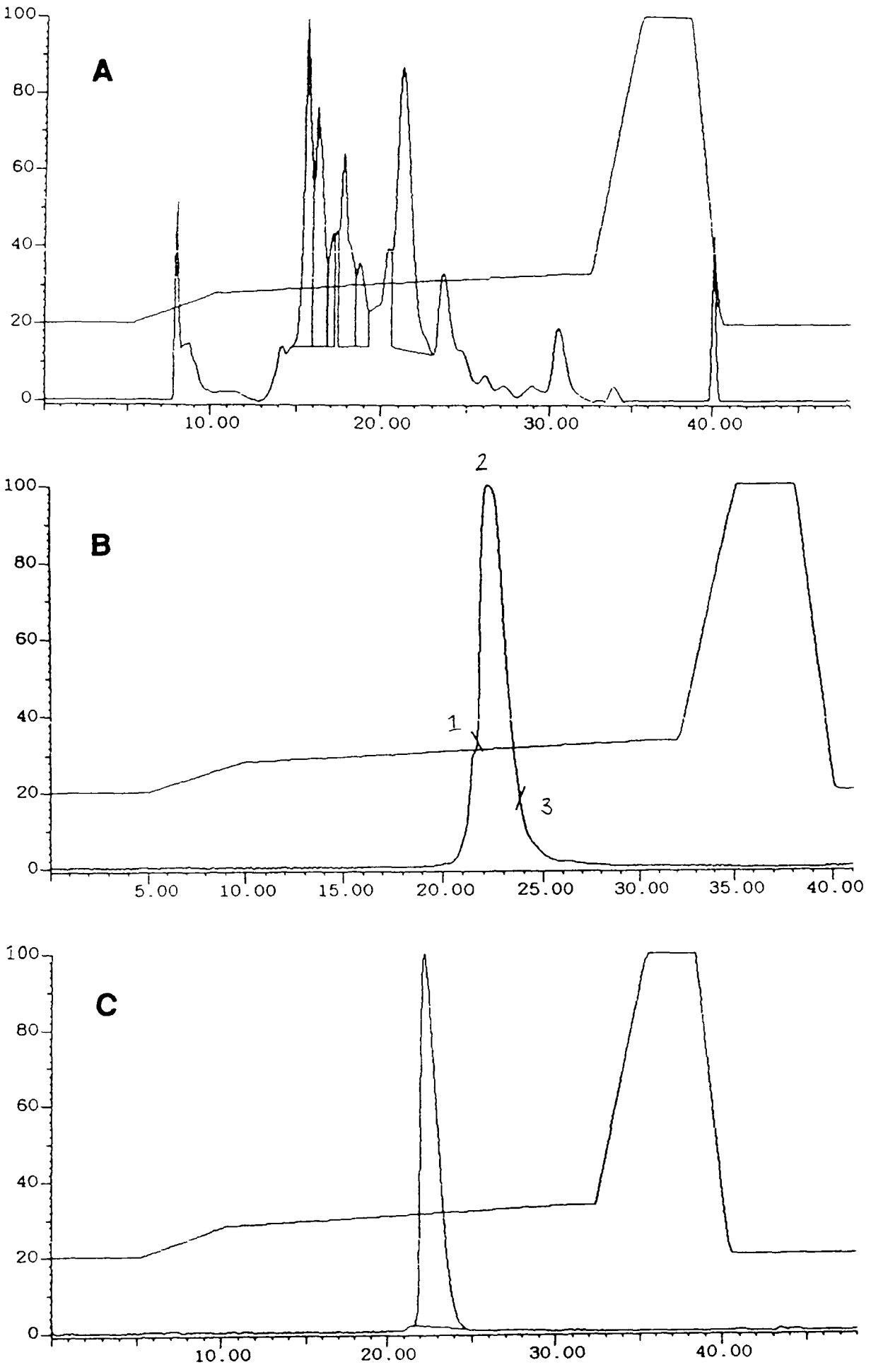


Figure 2.5

HPLC chromatograms illustrating reverse phase purification of the t-PA finger module. The main component of the crude reaction mixture shown in A was collected and subjected to a second round of HPLC purification (B). Three fractions were collected at this stage as indicated, of which only the second was retained. The HPLC chromatogram of the purified material (C) indicates that the final product was free of contamination.

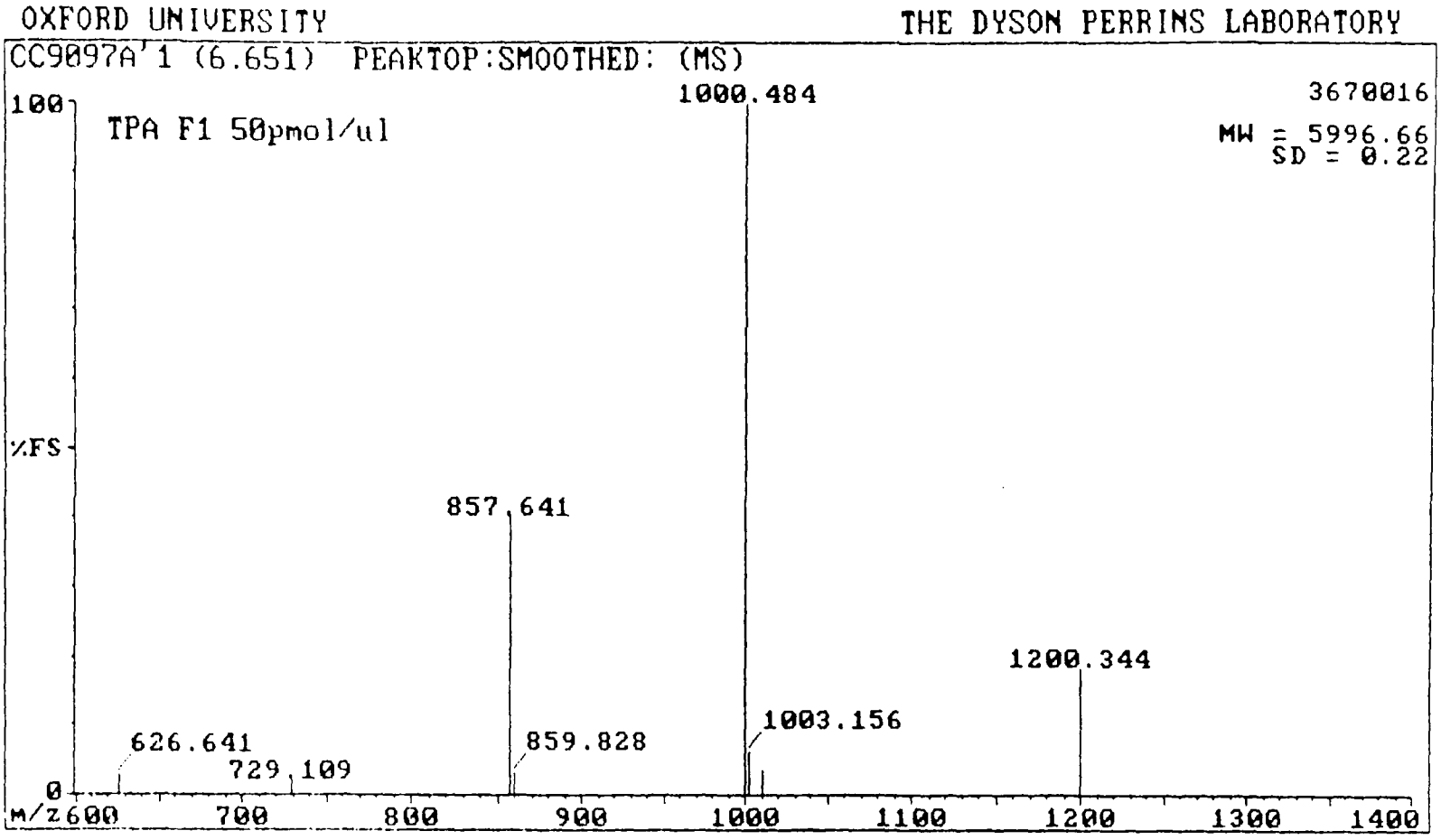
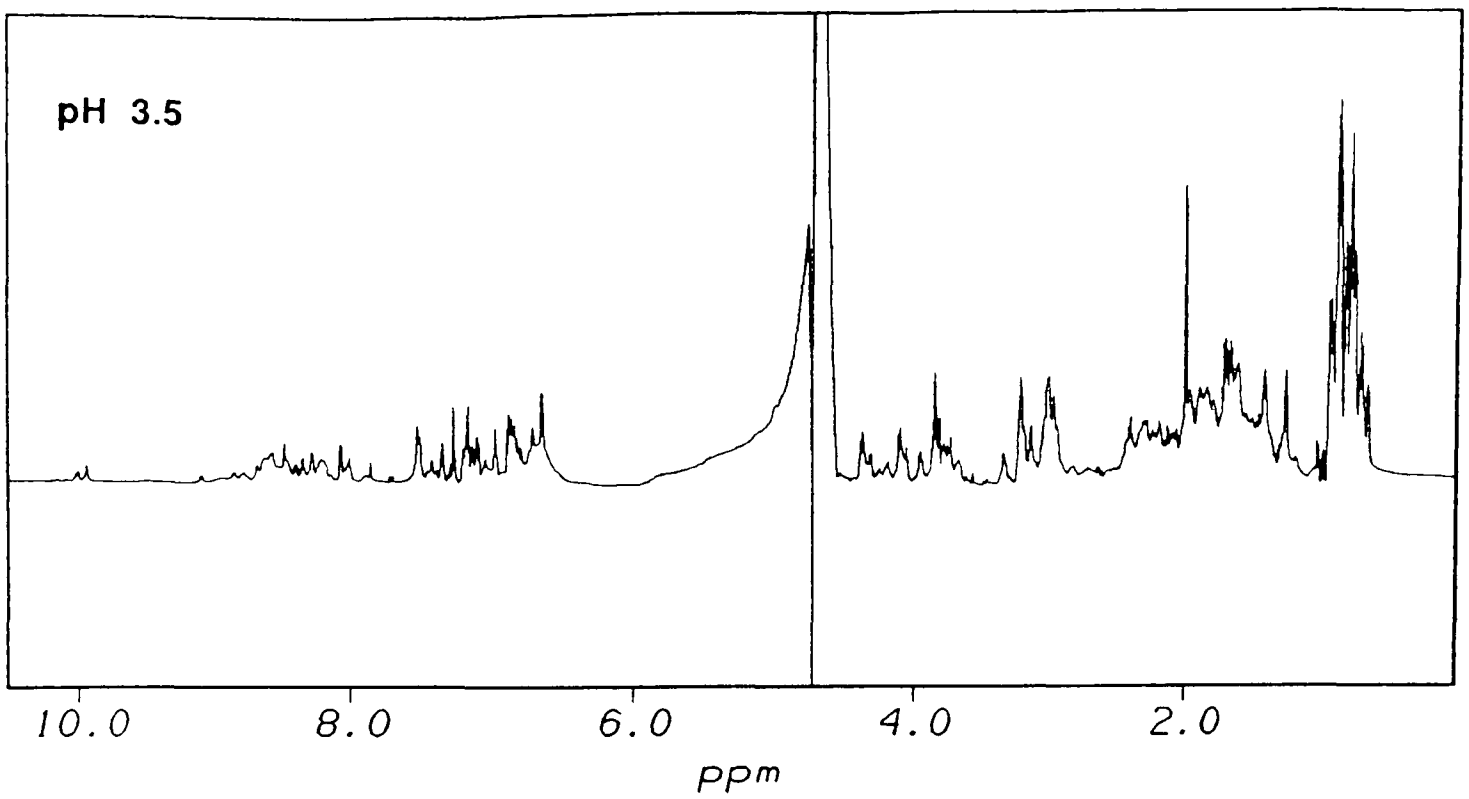
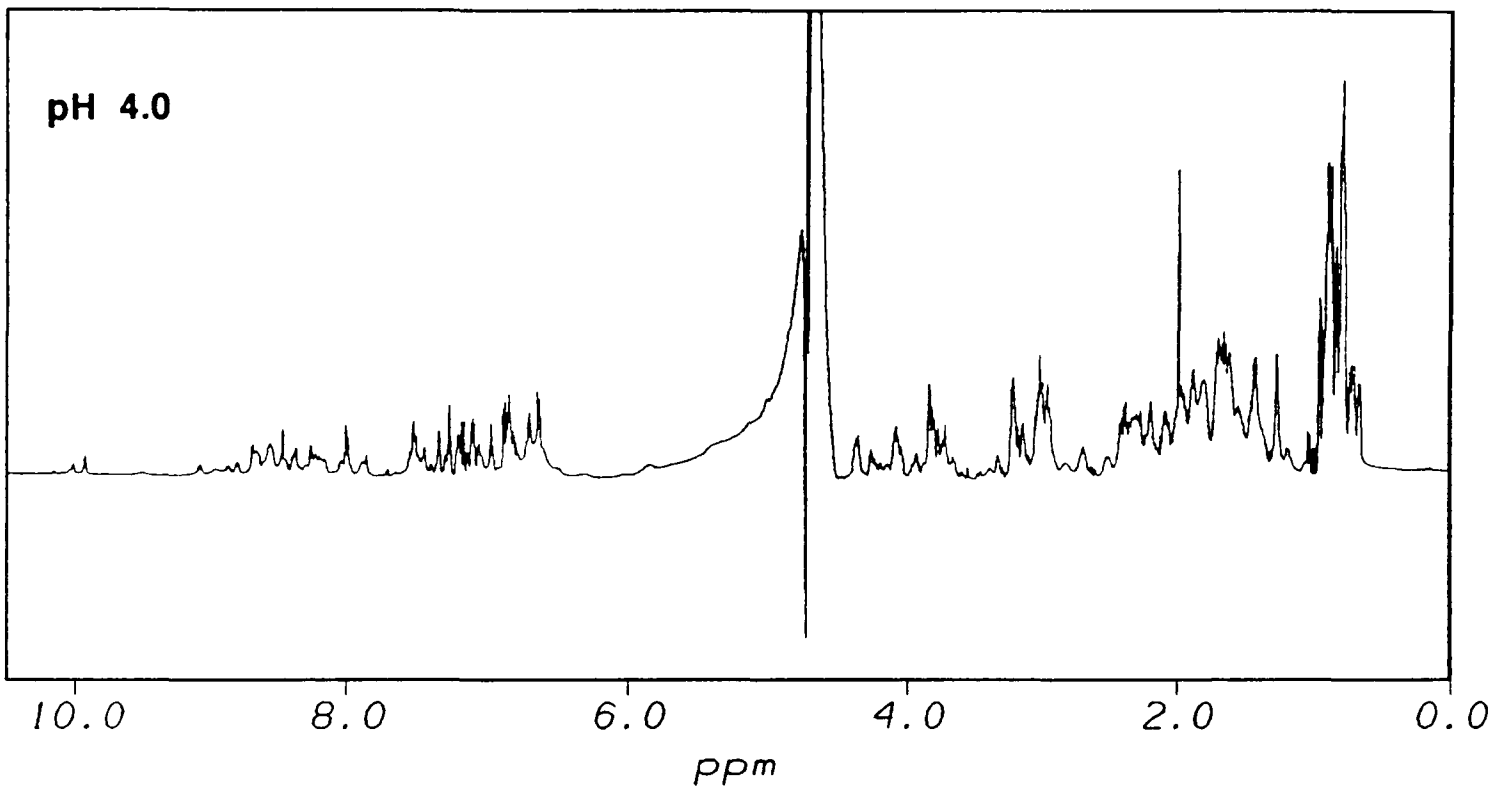


Figure 2.6

Low resolution mass spectrometric analysis of the purified t-PA finger module. The mass of the protein determined from this spectrum is consistent with the calculated mass for the F1 module and serves as confirmation of the identity of the purified protein.

Figure 2.7


pH 3.5
10.0
8.0
6.0
4.0
2.0
ppm


pH 4.0
10.0
8.0
6.0
4.0
2.0
0.0
ppm

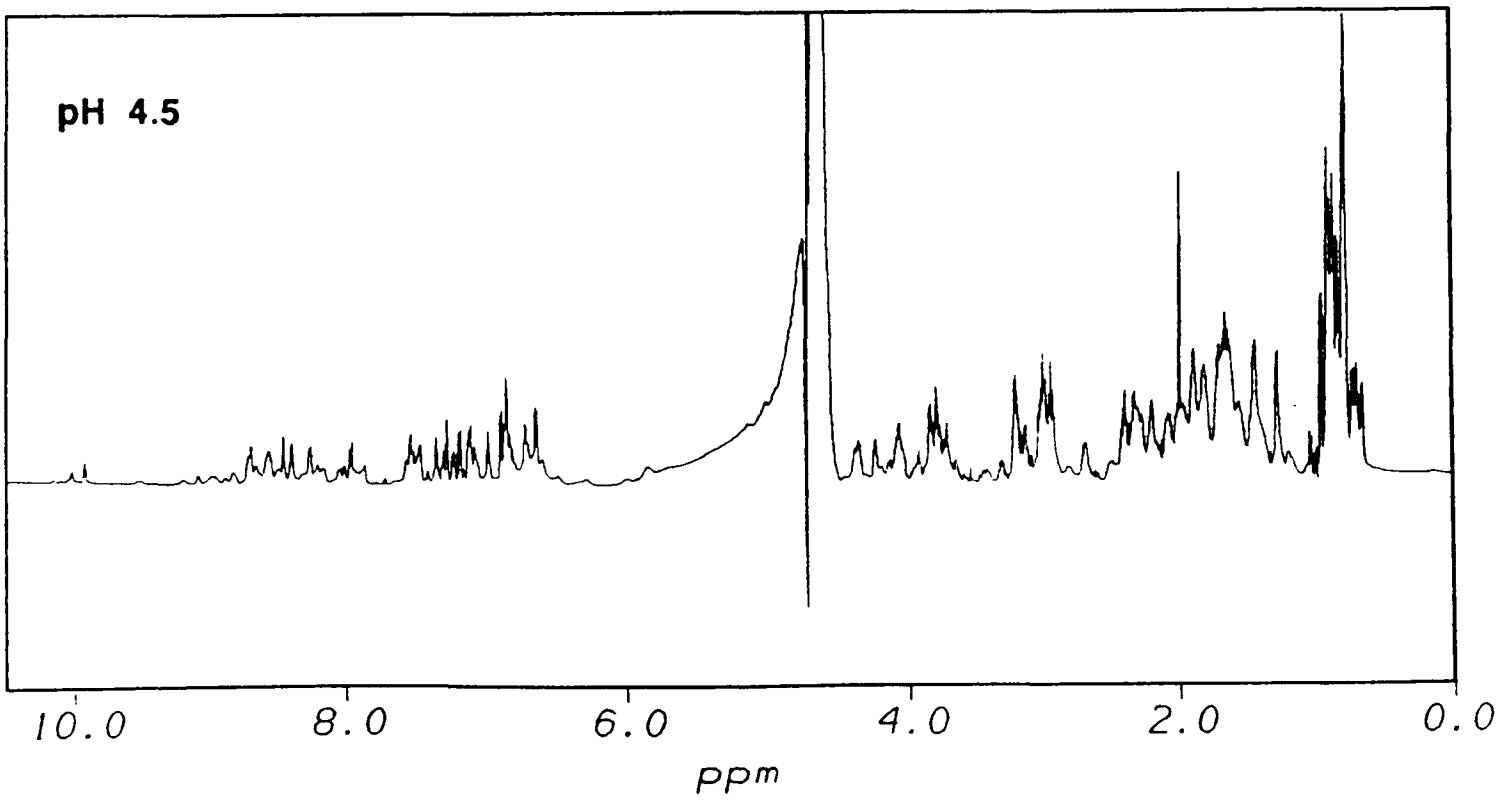
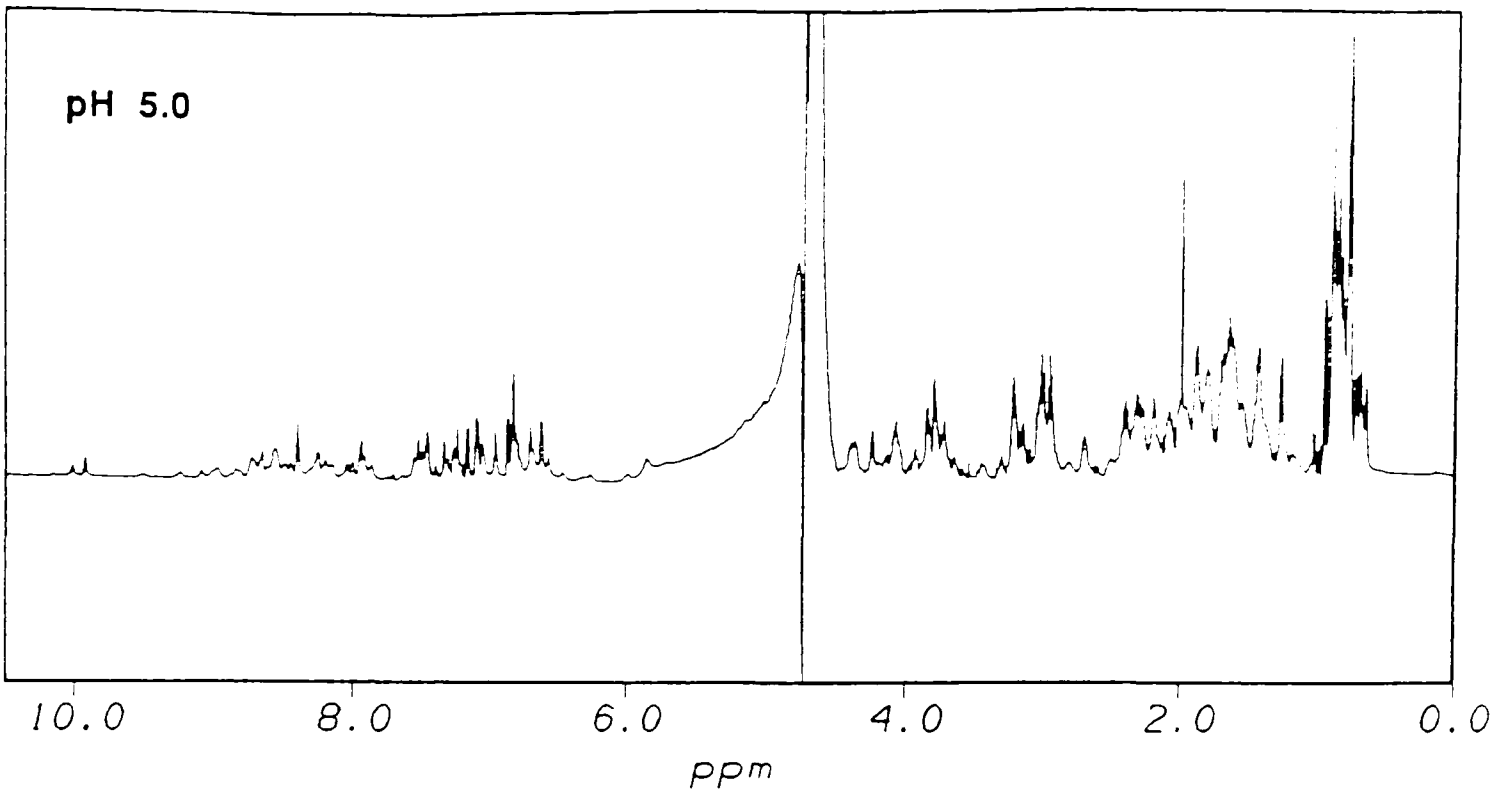
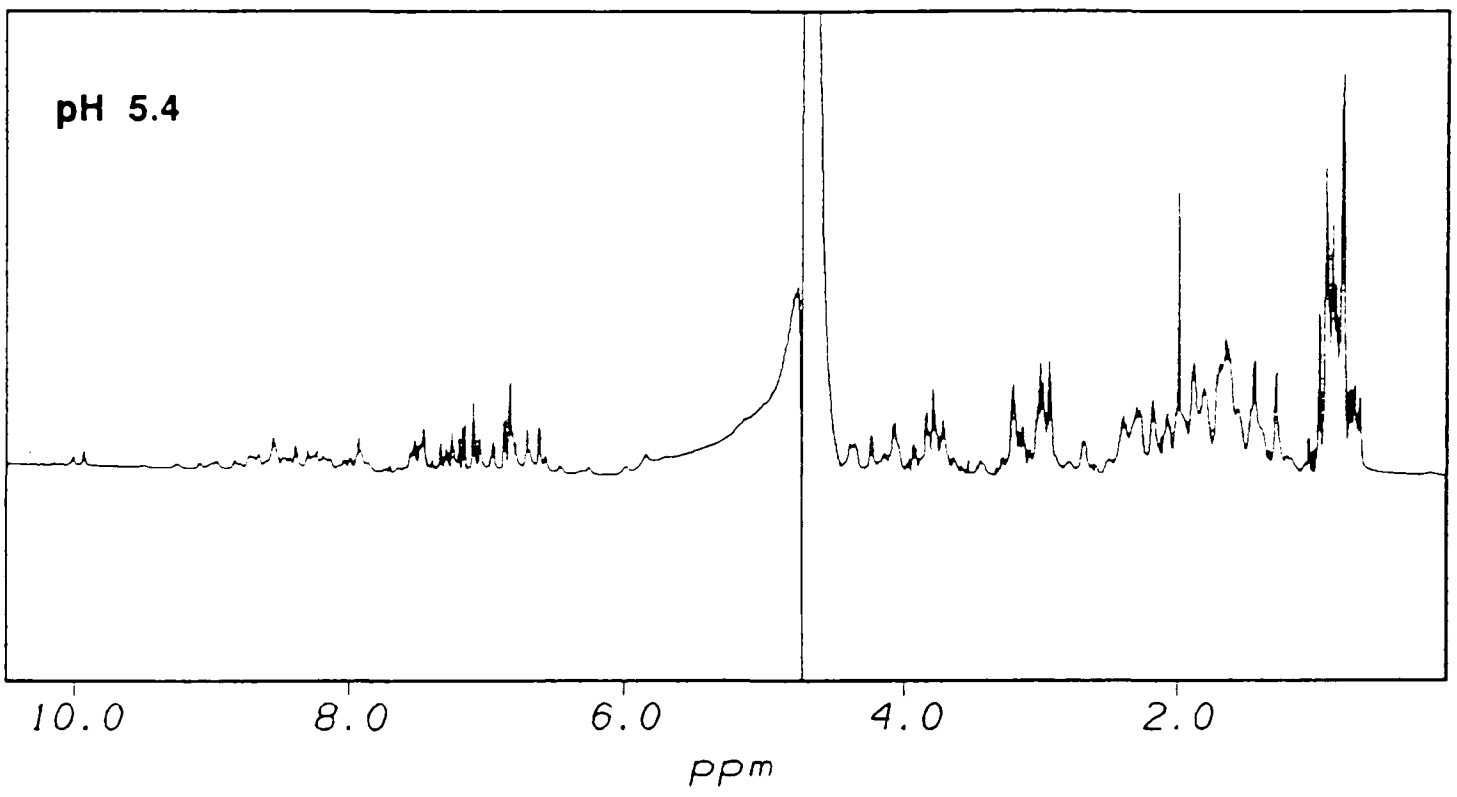
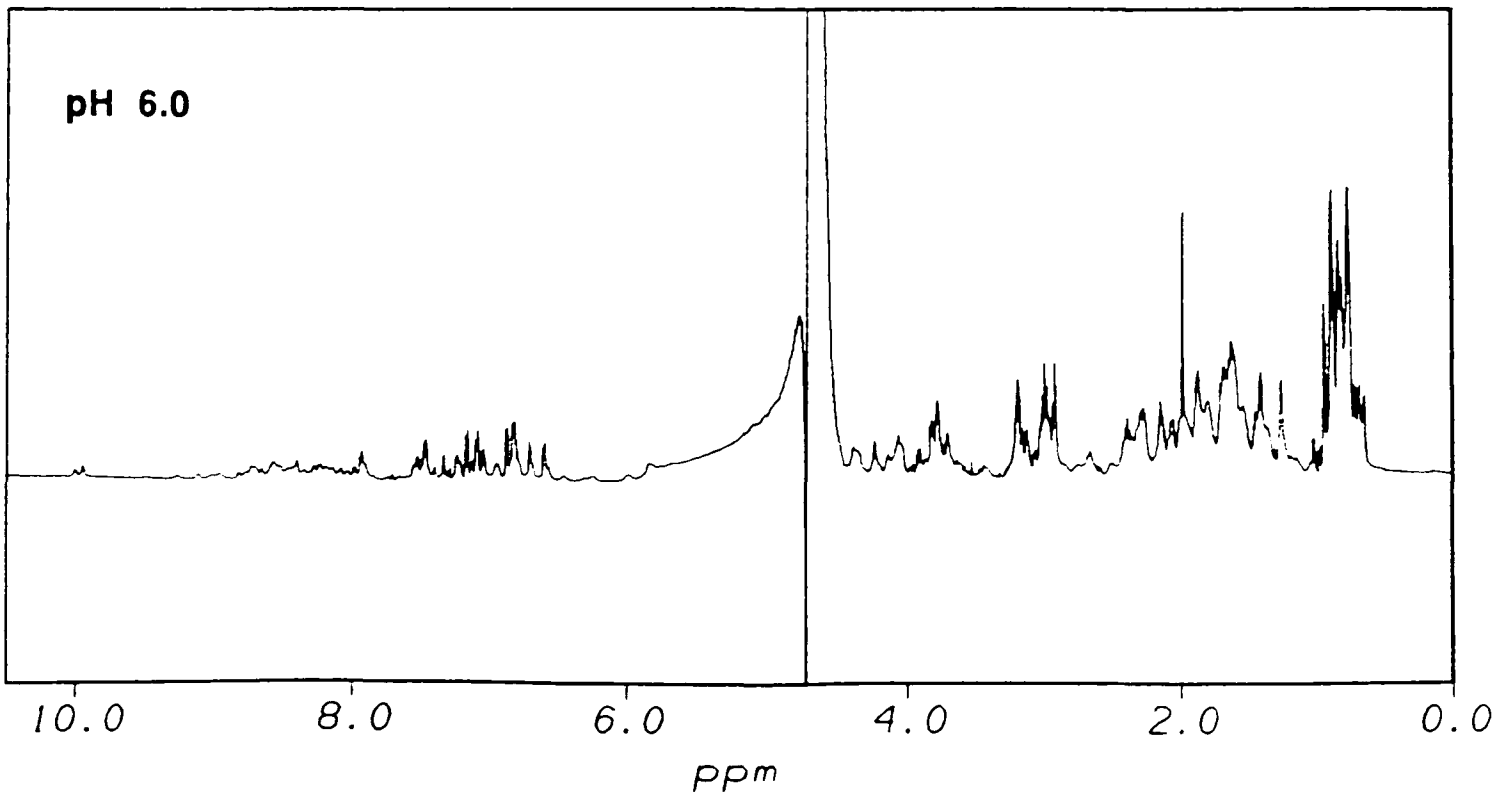

pH 4.5
10.0
8.0
6.0
4.0
2.0
0.0
ppm

Figure 2.7


pH 5.0
10.0
8.0
6.0
4.0
2.0
0.0
ppm


pH 5.4
10.0
8.0
6.0
4.0
2.0
ppm


pH 6.0
10.0
8.0
6.0
4.0
2.0
0.0
ppm

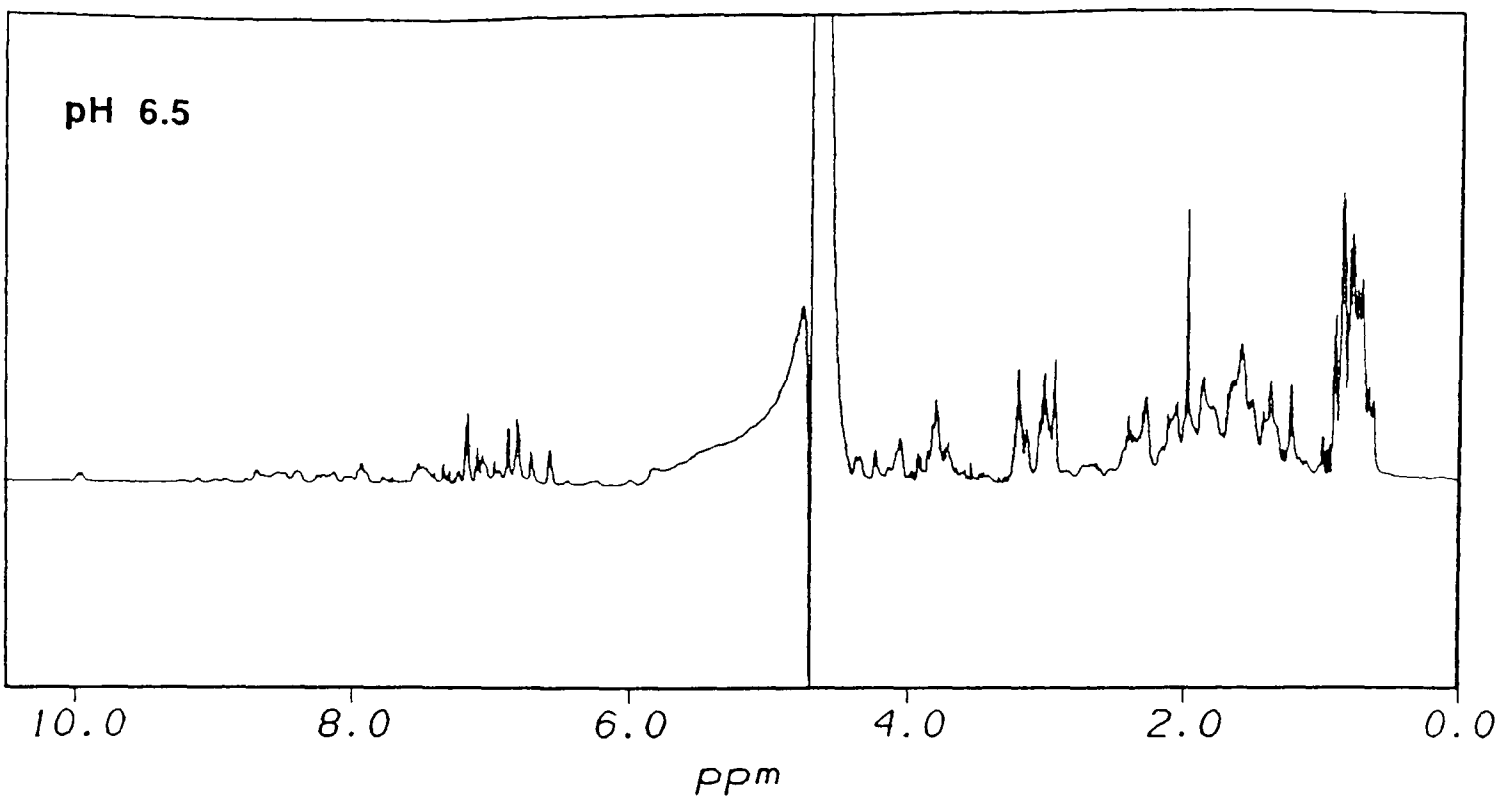


Figure 2.7

1-dimensional spectra of a 3 mM sample of t-PA F1 in 95% H_2O/5% D_2O recorded at temperature 303 K and varying pH. Peak sharpness and resolution improved with increased pH up to a value of 5.4. Consequently this pH was chosen as optimal for 2-dimensional experimentation.

dimensional spectrum recorded at pH 5.4 had the best peak sharpness and resolution, therefore this pH was chosen for 2-dimensional experiments.

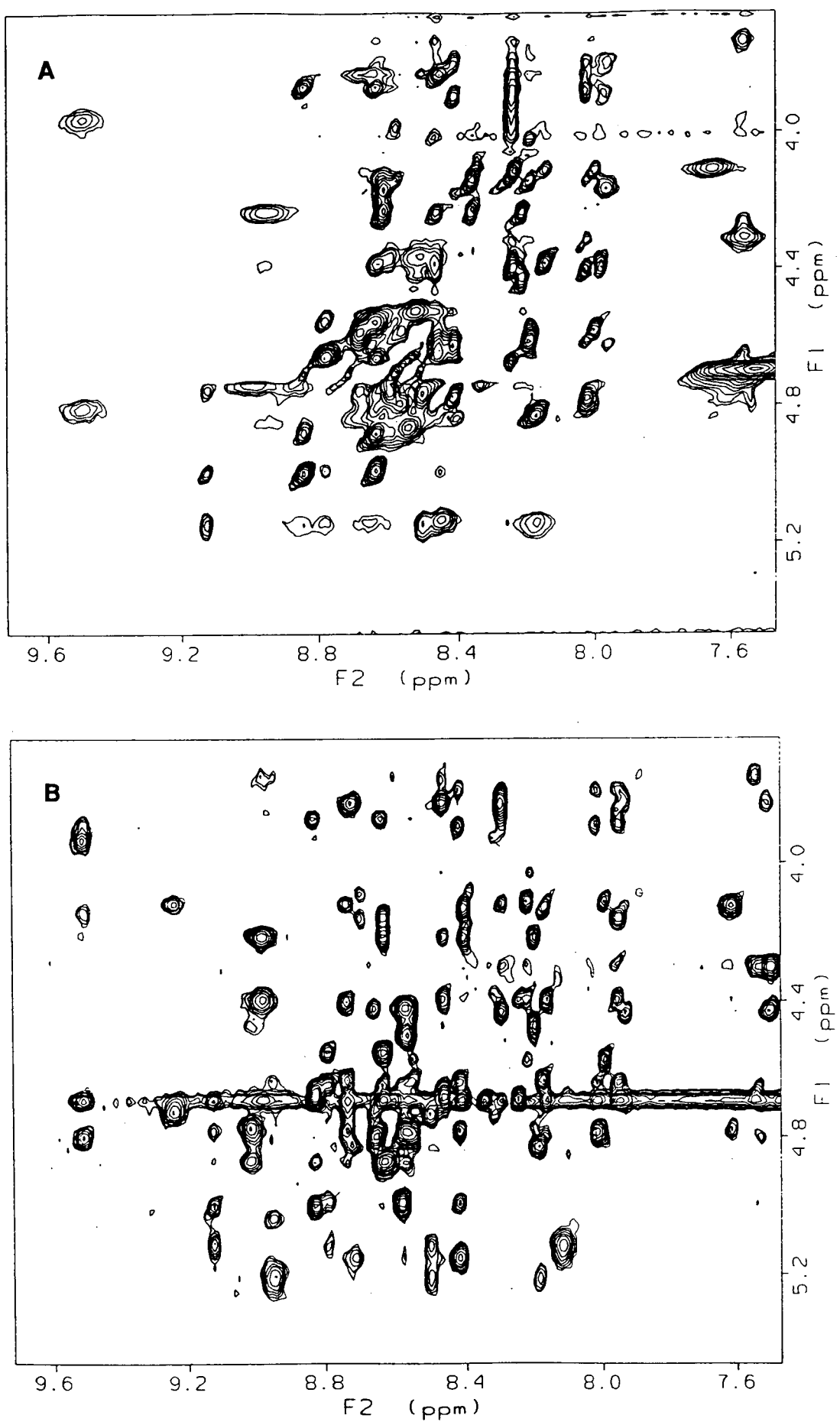
The t-PA finger module proved to be very soluble; no precipitation was observed even at a concentration of 8-10 mM. However, initial 2-dimensional n.m.r. experiments run on a sample at this concentration in 95% H_2O/5% D_2O revealed a broadening of crosspeaks most probably resulting from protein aggregation. Figure 2.8 demonstrates that when the sample was diluted the spectral quality improved significantly. Since the quality of the data collected at 5 mM and 6.5 mM concentrations was comparable, two 5 mM samples were prepared in 95% H_2O/5% D_2O and 99.996% D_2O for use in n.m.r. experiments requiring protonated and deuterated proteins, respectively.

2.5 Conclusions

The t-PA finger module was successfully expressed using the plasmid pTD4-48. The expression was directed into the cell culture supernatant using a yeast alpha secretion system, and the F1 module was the main product. Initial crude purification and concentration of the protein was achieved using batch absorption ion exchange chromatography. The crude product obtained after this process was of a higher purity and easier to dissolve than that obtained using C18 derivatised beads. Final purification was performed by reverse phase HPLC. The integrity and purity of the sample were confirmed by low-resolution mass spectrometric analysis.

Sample pH and temperature for n.m.r. experimentation were optimised based on observation of variation of crosspeak sensitivity and resolution in 1-dimensional n.m.r. spectra. A pH of 5.4 was selected for

Figure 2.8



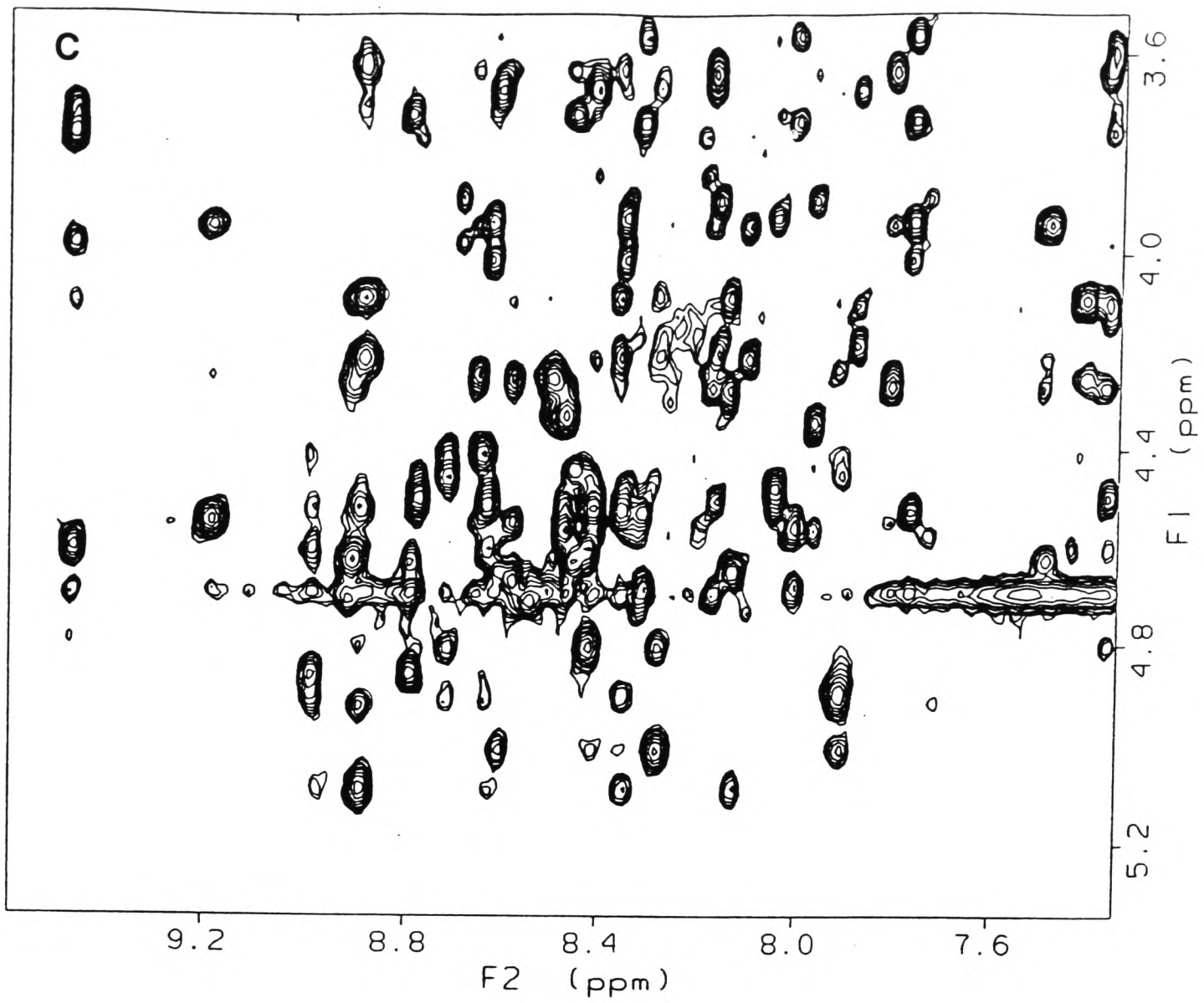


Figure 2.8

A region from three 2-dimensional NOESY spectra recorded on the t-PA finger module at various concentrations in 95% H_2O/5% D_2O and pH 5.4. At a concentration of 8-10 mM concentration and 303 K, many of the $C^{\alpha}H$/HN crosspeaks one would expect to observe in this region are broadened to such a degree that they cannot be distinguished (A). The spectrum in B, recorded at the same temperature, shows that at a concentration of 6.5 mM many more peaks are resolved. Spectra acquired on a 5 mM sample at a temperature of 295 K (C) are of similar quality to that shown in B.

2-dimensional experimentation. The t-PA finger module was highly soluble; however, inspection of preliminary 2-dimensional spectra revealed sample aggregation at high concentration. Therefore two n.m.r. samples were prepared, one in 95% H_2O/5% D_2O and one in 99.996% D_2O, each at pH 5.4 and 5 mM concentration. This enabled experiments using the protonated and deuterated samples to be run concurrently.

Chapter 3

t-PA F1 N.M.R. Data Acquisition and Assignment

3.1 Introduction

After having expressed and purified a sufficient amount of the t-PA F1 module for study, the n.m.r. data required for protein structure determination were acquired. Two-dimensional n.m.r. spectra were assigned, and a comparison was made of the n.m.r. data from the t-PA F1 module and the seventh type 1 module from human fibronectin (Baron *et al.*, 1990a) to predict their structural similarity.

The introduction to this chapter includes a concise overview of 2-dimensional ^{1}H n.m.r. with emphasis placed on the experiments performed for protein structure determination. The assignment of 2-dimensional ^{1}H protein n.m.r. and the strategy devised for assignment by homology of the t-PA F1 spectra are discussed. This introduction is followed by detailed description of n.m.r. data acquisition and assignment of the t-PA F1 spectra.

3.1.1 Introduction to 2-dimensional n.m.r.

Protein structure determination by ^1H 2-dimensional n.m.r. is most easily accomplished using three types of experiment: correlated spectroscopy (COSY) (Aue *et al.*, 1976; Bax and Freeman, 1981; Nagayama *et al.*, 1980), nuclear Overhauser enhancement spectroscopy (NOESY) (Jeener *et al.*, 1979; Macura *et al.*, 1981) and Hartman-Hahn spectroscopy (HOHAHA) (Braunschweiler & Ernst, 1983; Davis & Bax, 1985; Bax & Davis, 1985). The four basic time periods in the scheme of a 2-dimensional n.m.r. experiment are known as preparation, evolution, mixing and detection. This scheme and the COSY, NOESY and HOHAHA pulse sequences are shown in Figure 3.1. This section presents a brief overview of 2-dimensional n.m.r. and a qualitative description of the data acquired using each of these experiments with reference to their pulse sequences. Rigorous derivation of the relationship between the pulse sequence and acquired data for each experiment requires use of product operators (discussed in Ernst *et al.*, 1987) and lies beyond the scope of this thesis.

In all 2-dimensional experiments a signal, modulated as a function of t_1, is later detected as a function of t_2. The signal recorded during t_2 is called the free induction decay (FID). The duration of the evolution period, t_1, is varied incrementally in a series of experiments in order to resolve the data into two independent time dimensions. The experimental matrix $S(t_1, t_2)$ is Fourier transformed in both dimensions to obtain a 2-dimensional frequency domain matrix $S(F_1, F_2)$. Often the data in each dimension are multiplied by a window function and zero-filled prior to transformation to improve spectral sensitivity and resolution. The effects of these data manipulations are illustrated in Figure 3.2.

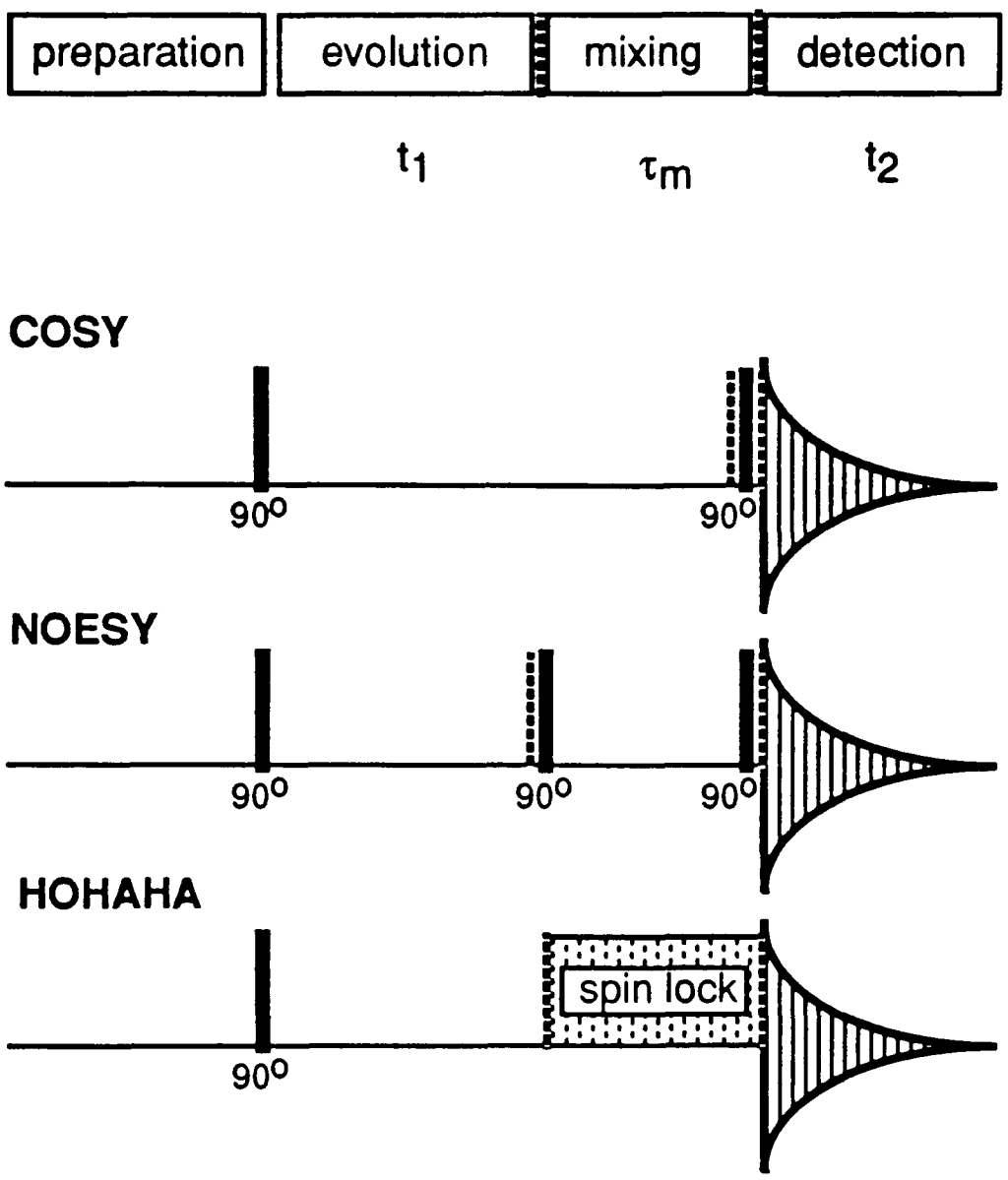


Figure 3.1

The four basic time periods in the scheme of a 2-dimensional n.m.r. experiment and the basic pulse sequences for the three most commonly used experiments in protein structure determination by ^1H n.m.r. are outlined schematically. In each diagram the mixing period for the experiment is bracketed by dashed lines.

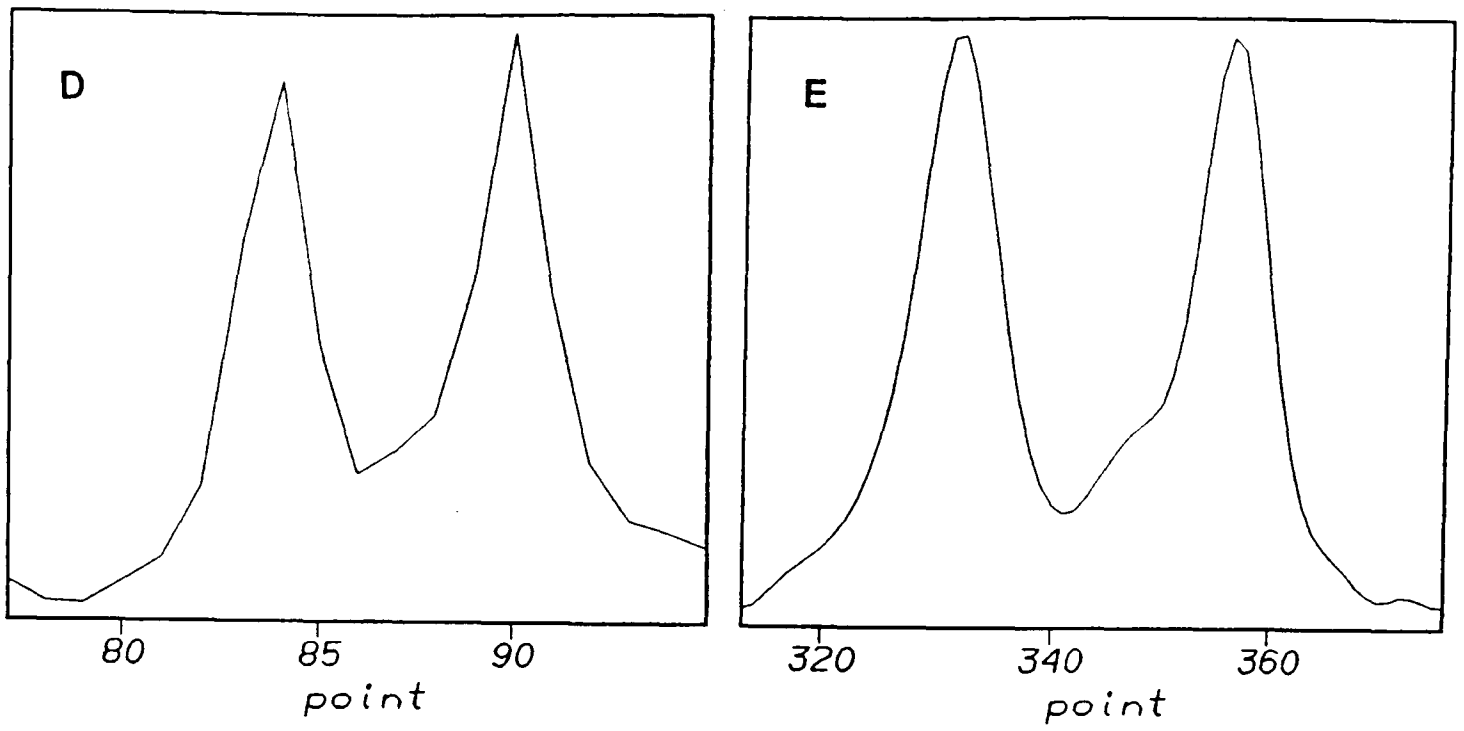


Figure 3.2

Effect of use of window functions and zero-filling in processing of n.m.r. data. Multiplication of the FID (A) by a window function that enhances the early part of the signal results in a transformed spectrum with increased signal to noise ratio but attenuated spectral resolution (B). Conversely, use of a window function that emphasises the later part of the signal improves resolution with a concomitant reduction in sensitivity (C). The signal shown in B is approximately 3-fold more intense than in C. Multiplication by a window function ensures that the acquired signal decays completely to zero which minimises data convolution due to truncation effects. Often data are zero-filled after the window function has been applied to increase digital resolution. Prior to transformation the unenhanced data in D were zero-filled to 4-times the number of points, resulting in the more well-defined peaks shown in E.

In general, 2-dimensional n.m.r. experiments are set up such that magnetisation which evolved with some frequency during t_1 evolves differently during t_2 as the consequence of magnetisation transfer between coupled spins. This gives rise to crosspeaks in the Fourier transformed matrix at the intersection of the chemical shifts of the coupled spins.

The COSY experiment is used to delineate networks of scalar coupled spins. As an empirical rule, connectivities between spin-1/2 nuclei separated by 3 bonds or less are observed. The pulse sequence for the COSY experiment consists of two 90° pulses separated by the t_1 evolution period. In a coupled network of spins the first 90° pulse creates phase coherence of the magnetisation which is redistributed amongst the associated transitions by the second pulse. Consider, for example, the energy level diagram for a two spin system which is shown in Figure 3.3.

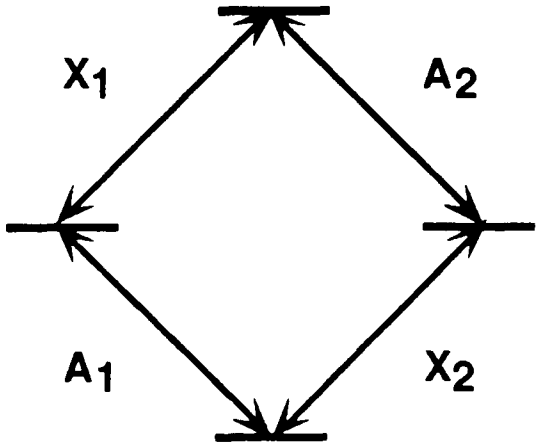


Figure 3.3

Energy levels of an AX spin system.

Coherent magnetisation across transition A_1, created by the first 90° pulse, evolves during t_1 and is redistributed amongst all four transitions A_1, A_2, X_1 and X_2 by the second 90° pulse. How much coherence is transferred to each place depends on the values of t_1 and the spin-spin

coupling constant (J), and on the duration of the second pulse (discussed in Bax, 1982, section 2.3.3 and Appendix III).

If phase coherence is transferred from spin A to spin X by the second pulse, off-diagonal peaks appear in the spectrum with coordinates ($\delta_A \pm 1/2\ J_{AX}$, $\delta_X \pm J_{AX}$) and ($\delta_X \pm 1/2\ J_{AX}$, $\delta_A \pm J_{AX}$). The phase shifts of the crosspeaks alternate in sign around a square pattern as shown in Figure 3.4. The coupling which gives rise to the coherence transfer, J_{AX}, is designated as the active coupling of the crosspeak. If another proton, M, couples to this two-spin system, additional couplings are passive and introduce new splittings. The alternating phase pattern of the COSY crosspeak is described as its antiphase multiplet fine structure. Active and passive couplings are further discussed with reference to the PE-COSY experiment which is described in Chapter 4.

Diagonal peaks in the COSY spectrum appear when the signals do not change frequency between the evolution and detection periods, i.e., when no magnetisation is transferred from spin A to spin X. In the conventional COSY experiment the diagonal peaks are in pure dispersion when the phase is adjusted for pure absorption lines of the crosspeaks. Consequently crosspeaks close to the diagonal may be unresolvable. Double quantum filtration (DQF) is commonly used in COSY experiments so that both diagonal and crosspeaks can be adjusted into pure absorption phase (Rance *et al.*, 1983).

The 2-dimensional NOESY experiment (Figure 3.1) is used to map all protons which are near (typically $r_{ij} \leq 5$ Å) to each other in space. In the NOESY pulse sequence, the 90^o mixing pulse of the COSY scheme is replaced by two 90^o pulses separated by the mixing time τ_m. A knowledge of the origin of the nuclear Overhauser effect (NOE) is necessary to explain response to this pulse sequence.

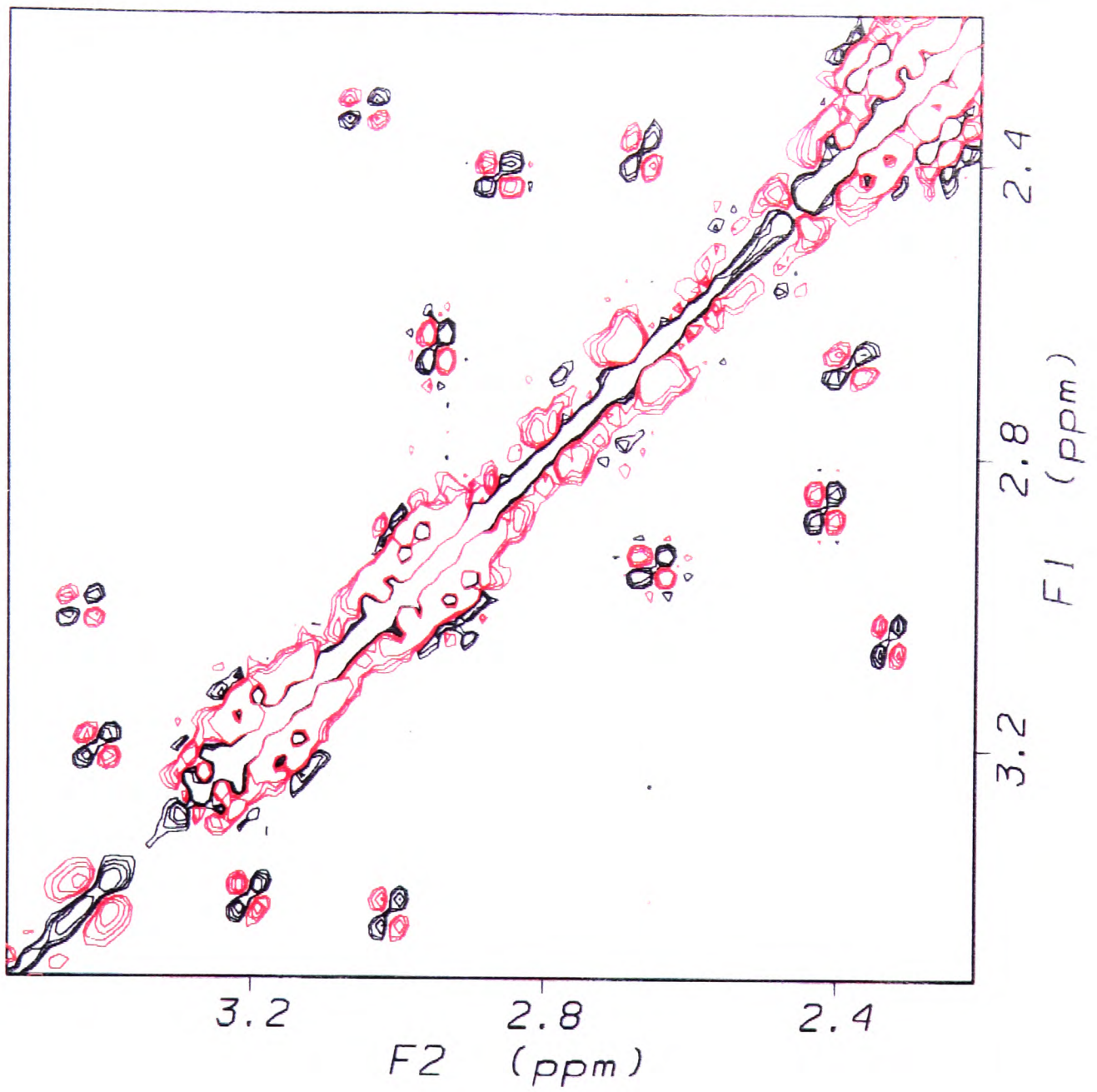


Figure 3.4

Crosspeaks in a COSY spectrum are symmetrically arranged with respect to the diagonal are split by spin-spin couplings. The antiphase spliting pattern provides structural information about the spatial proximity of the protons giving rise to the crosspeak.

The rate of dipolar relaxation for two spin-1/2 nuclei is a function of the molecular tumbling rate, which is parameterised by the molecular correlation time (τ_c), and the distance separating the nuclei (r). Specifically, the transition probabilities for single, zero and double quantum coherence (W_1, W_0 and W_2), defined with respect to the energy levels of a two spin system as shown in Figure 3.5, are all a function of τ_c and $1/r^6$. If a nonequilibrium population difference is established across the transitions of one spin, then a flux of spins proportional to the appropriate population differences and relaxation transition probabilities acts to restore equilibrium. The NOE is defined as the fractional change in the intensity of the n.m.r. resonance of a dipolar coupled spin when cross relaxation occurs in a effort to restore equilibrium energy populations. The maximum observable NOE is defined relative to the transition probabilities by the nuclear Overhauser enhancement factor (η) where (for homonuclear spins),

$$\eta = \frac{W_2 - W_o}{2W_1 + W_2 + W_0}$$

For molecules with long correlation times, such as proteins, W_o is much greater than W_1 and W_2. Hence η = -1, corresponding to a maximum observable proton-proton NOE in a protein of -100%.

As noted previously, when cross relaxation between nuclei 1 and 2 occurs directly, the intensity of the observed NOE will be proportional to $1/r^6$. However, in proteins where there are a large number of like spins in close spatial proximity, the intensity of the NOE can be severely affected by spin diffusion, i.e., the transfer of magnetisation via two or several relaxation steps (Figure 3.6). The initial build-up rate of the NOE in proteins is nonetheless directly related to the distance separating the

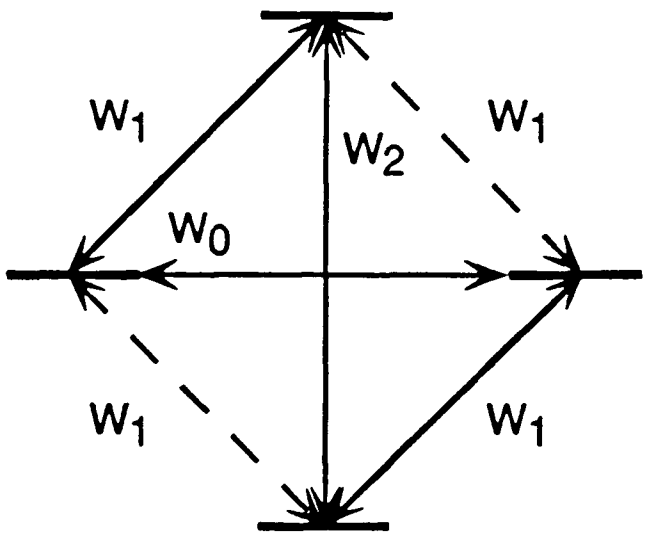


Figure 3.5

Energy levels of an AX homonuclear spin system showing pathways associated with single (W_1), zero (W_0) and double (W_2) quantum transition probabilities. Single quantum transitions associated with spin A and spin X are shown with dashed and solid lines respectively.

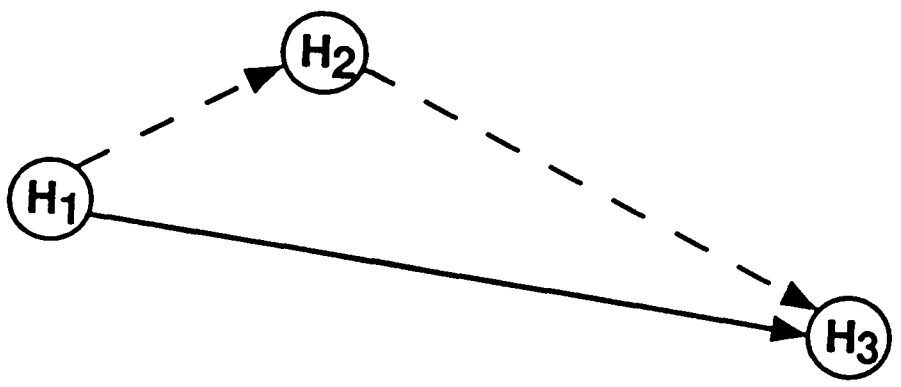


Figure 3.6

Cross relaxation between protons 1 and 3 can take place directly (solid arrow) or indirectly via a spin diffusion pathway (dashed arrow). Relaxation via spin diffusion limits the accuracy of calibration of NOE intensity and interproton distance.

coupled protons (Noggle & Schirmer, 1971; Solomon, 1955; Wagner & Wüthrich, 1979).

Relating this discussion to the NOESY pulse sequence, the first two 90° pulses establish nonequilibrium populations across energy transitions of dipolar coupled spins which give rise to the NOE, and the mixing time τ_m corresponds to the amount of time during which the NOE is allowed to build up. The shorter τ_m is chosen to be, the more accurately the NOE intensity will correlate with interproton distance. At the same time, longer choice of τ_m is associated with greater NOE intensity and higher signal to noise.

In HOHAHA spectra crosspeaks appear connecting protons which are spin coupled, either directly or through a chain of spin-spin couplings. Hence the HOHAHA experiment (Figure 3.1) is an alternative to the COSY experiment for obtaining the scalar coupling network of protons. The extent of magnetisation transfer along an amino acid side chain is dependent on the duration of the HOHAHA mixing period, and experiments are routinely conducted in which scalar connectivities between protons separated by up to 5 bonds (and sometimes more) are resolvable. Thus scalar coupling networks are presented in two different formats by HOHAHA and COSY spectra. Unlike the COSY experiment, the HOHAHA experiment generates phase-sensitive spectra without intensity alternation. The antiphase multiplet fine structure of the COSY crosspeak can lead to partial cancellation and loss of intensity. Therefore, the HOHAHA experiment is expected to be more sensitive than the COSY experiment.

The HOHAHA experiment is based on the fact that in a low effective applied field, the scalar coupling between protons will be greater than their chemical shift. Such a condition can be achieved by application of a long spin-locking pulse which serves to eliminate the

chemical shift difference between two coupled protons and allow magnetisation transfer. It is for this reason that the duration of the mixing period in a HOHAHA experiment determines the extent of transfer of magnetisation along the side chains of the amino acids.

3.1.2 Assignment of ^1H 2-dimensional protein n.m.r. spectra

N.m.r. assignment of proteins relies on comparison of spectra acquired using protein samples dissolved in H_2O and D_2O at various temperatures. The reason for the use of two solvents is because many protons, including the majority of backbone amide protons, are labile. Since D_2O resonates at a different frequency from protons, deuteration of a protein sample leads to loss of ^1H n.m.r. data for labile protons. However, the large magnitude of the solvent signal in H_2O spectra often obscures signals from protons at or near the water resonance. Additionally, signals from protons exchanging with the water are diminished by selective saturation of the H_2O signal. For these reasons n.m.r. experiments must be recorded both in H_2O and D_2O. Assignment can be further complicated by crosspeak overlap. Chemical shift is a function of pH and temperature, thus these assignment ambiguities are usually resolved by collecting additional data sets under different conditions for comparison.

Since the chemical shift of a proton is a function of its chemical environment, different types of protons within an amino acid residue have characteristic shifts. The spin systems of the 20 amino acids give rise to 10 distinct patterns of n.m.r. signals which are shown in Figure 3.7 as well as those for aromatic ring protons. Specific values of the chemical shifts of these protons in a short denatured peptide (GGXA) have been determined for use as a reference and are described as "random coil"

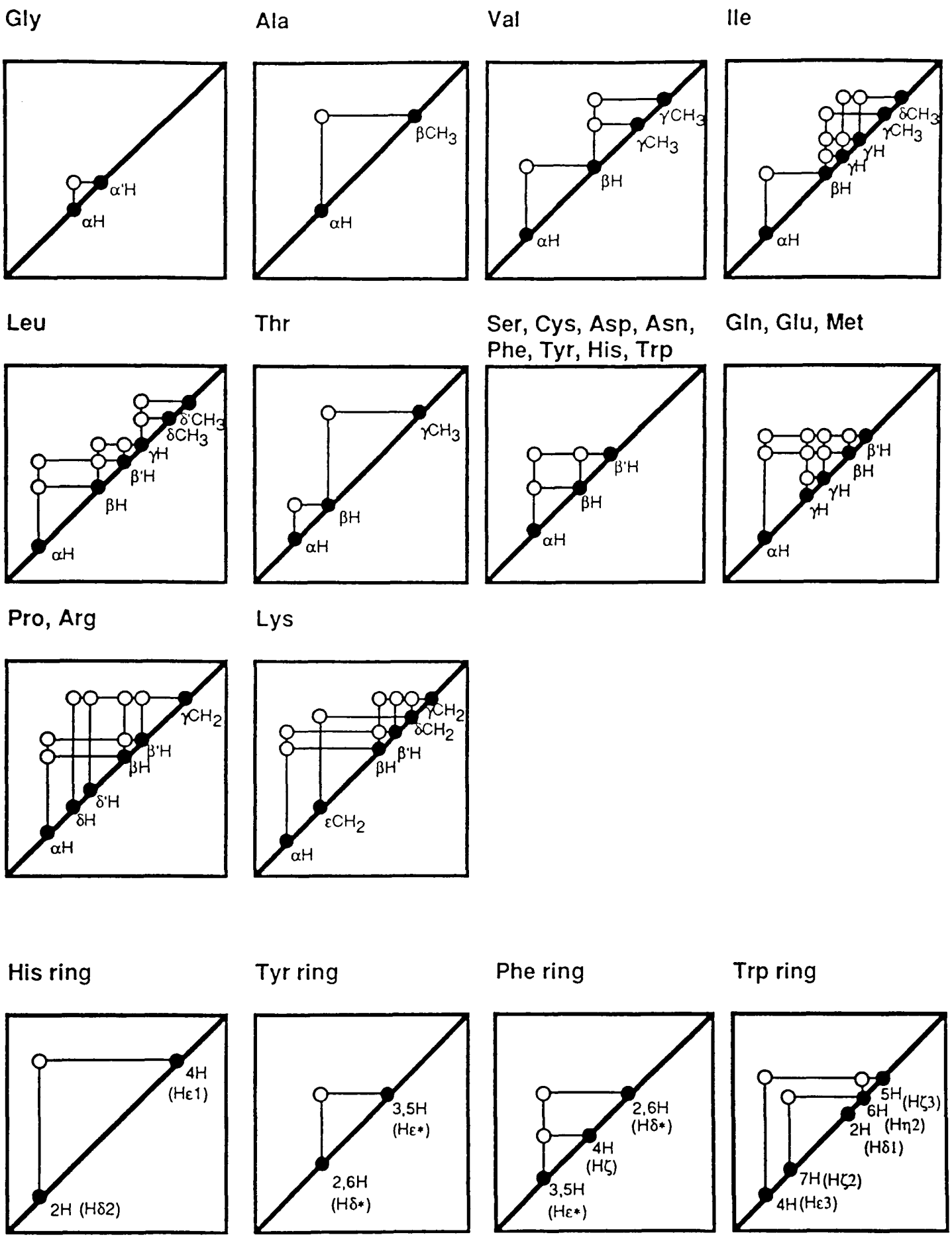


Figure 3.7

The spin systems of the 20 amino acids and the aromatic side chains and the pattern of crosspeaks generated. Open circles indicate positions of intraresidue COSY crosspeaks. The number and intensity of NOESY and HOHAHA crosspeaks for each spin system is dependent the geometry of the side chain and on choice of experimental mixing time.

(Wüthrich, 1986). Perturbations from these values are caused primarily by secondary and tertiary structural interactions. The qualitative comparison of chemical shift data with random coil positions often allows the identification of commonly occurring secondary structural motifs (Wishart *et al.*, 1991).

3.1.3 Strategy for assignment of the t-PA F1 spectra

In the assignment of the n.m.r. spectra of the t-PA F1 module it was anticipated that this process would be simplified by the knowledge of the structure of the seventh type 1 module from human fibronectin determined previously by Baron *et al.* (1990a). Of the 15 residues that are highly conserved in the secondary structure of human fibronectin finger type 1 modules (Baron *et al.*, 1990a), the 8 that are conserved in the t-PA finger module are highlighted in Figure 3.8.

```
consensus residues:  *        *  ** * * *          * * ** * *  * *
fibronectin-7:       ICTTNEG -VMYR IG DQWDKQHDMG -HMMRCTC VGNGRGE WTCI
t-PA:                ICRDEKT QMIYQ QH QSWLRPVLRS NRVEYCWC -NSGR-- AQCH
conserved in t-PA:    *        *       * *          * *    *      *
```

Figure 3.8

Alignment of the sequences of the seventh type 1 module from human fibronectin and the t-PA F1 module. The 15 residues highly conserved in fibronectin type 1 modules and the 8 which are conserved in the t-PA F1 sequence are indicated by asterisks.

The alignment of the two peptides was used as the starting point for modelling the structure of the t-PA finger. Figure 3.9 shows the secondary structure of the fibronectin finger which is composed of a

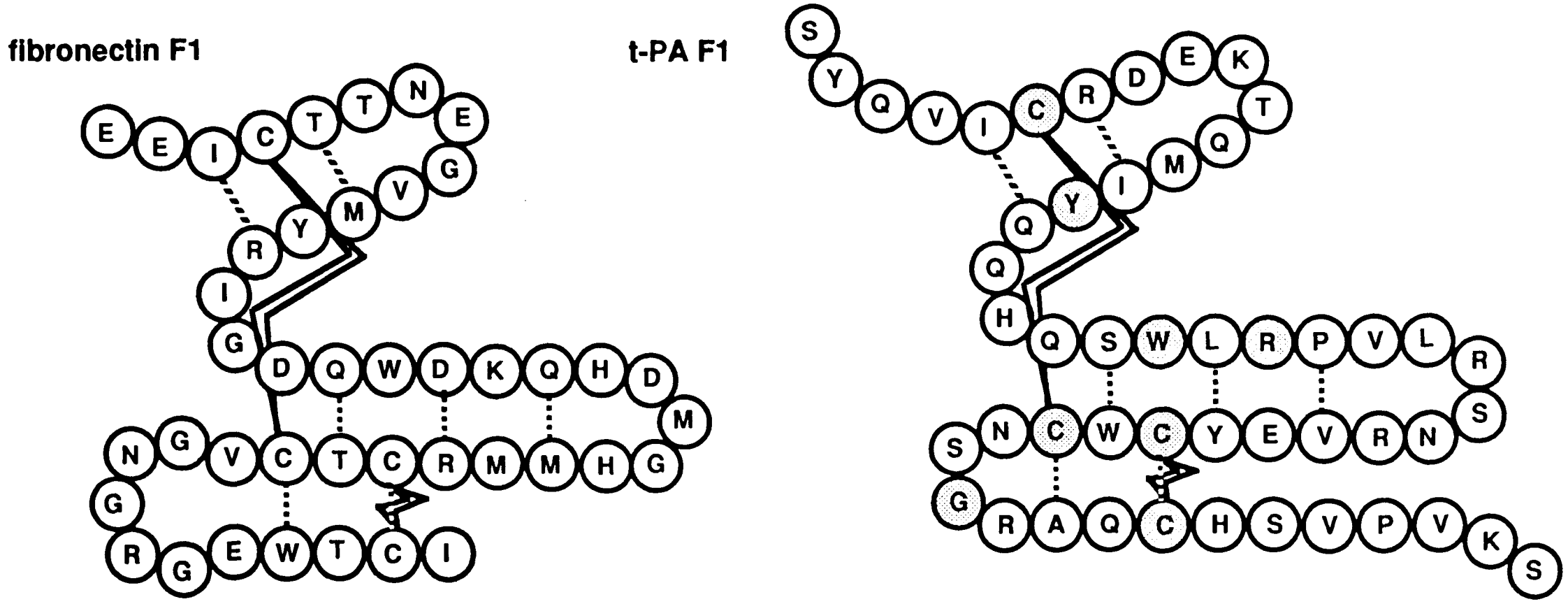


Figure 3.9

Secondary structure of the seventh type 1 module from human fibronectin and predicted secondary structure of the t-PA F1 module. Conserved residues in the t-PA finger module are shaded. Disulphide bridges linking cysteine residues are shown with jagged lines. Hydrogen bonds in the fibronectin F1 module and predicted for the t-PA F1 module are indicated by dashed lines.

double-stranded and a triple-stranded β-sheet. Also shown is the predicted secondary structure for t-PA F1. Upon successful completion of sequential assignment, this model was used as an aid in searching for NOEs and backbone hydrogen bonds defining β-sheets. Then a search was made for NOEs defining tertiary structure based on the fibronectin F1 fold and the disulphide bonding pattern.

3.2 N.M.R. Data Acquisition for the t-PA F1 Module

Samples for n.m.r. spectroscopy contained approximately 5 mM t-PA F1 in either 95% H_2O/5% D_2O or 99.996% D_2O, pH 5.4. All 2-dimensional experiments were recorded in a phase-sensitive manner on a Bruker AM 600 spectrometer with a sweep width of 7.6 kHz unless otherwise noted. Time proportional phase incrementation (TPPI) was used for quadrature detection in the F_1 dimension. The pulse sequences for all of the n.m.r. experiments performed are included in Appendix I.

In H_2O solution, NOESY experiments (Jeener *et al.*, 1979; Macura *et al.*, 1981) with mixing times of 50, 200 and 300 ms were performed in which the water resonance was suppressed by a "jump and return" pulse (Plateau & Guéron, 1982) in place of the last 90° pulse of the NOESY sequence. The jump and return pulse is a composite $1\bar{1}$ pulse used as an alternative to selective saturation of the water signal to avoid HN crosspeak attenuation. When the magnetisation of a large signal such as the unsaturated water is excited, the transverse magnetisation decays faster than expected from the relaxation processes alone due to an effect known as radiation dampling (explained in Freeman, 1988, pp177-179). In H_2O jump and return NOESY experiments radiation damping was minimised by shifting the phase cycling of the mixing pulse by 45° with respect to the evolution and detection pulses (Driscoll *et al.*, 1989). All of

these experiments were recorded at 303 K, and an additional data set with a 200 ms mixing time was recorded at 295 K. HOHAHA spectra (Braunschweiler & Ernst, 1983; Davis & Bax, 1985; Bax & Davis, 1985) were recorded with a WALTZ-17y spin lock sequence (Bax *et al.*, 1987) at 303 K and 295 K. The mixing time was of the order of 45 ms, and 1.5 ms "trim" pulses bracketed the WALTZ-17y sequence. Trim pulses are relatively long pulses applied along one axis which select magnetisation along that axis and dephase perpendicular magnetisation by taking advantage of radiofrequency (B_1) field inhomogeneity. Trim pulses are used to eliminate the dispersive component of the HOHAHA peak shape. To attenuate t_2 ridges arising from the strong solvent resonance in H_2O experiments, the free induction decays were deconvoluted in the time domain with a Gaussian function (Marion *et al.*, 1989) prior to Fourier transformation in the F_2 dimension. Typically 800-1024 increments of 2K data points were collected per experiment. Data processing involved a Lorentzian-to-Gaussian convolution in F_2 and a $\pi/3$ sine-squared bell window function in F_1. For all experiments, the receiver phase was optimised to reduce base-line distortions (Marion & Bax, 1988a).

A COSY spectrum was recorded in H_2O at 303 K with use of the SCUBA technique to recover crosspeaks coincident with the H_2O resonance (Brown *et al.*, 1987). The data from this experiment were processed using an unshifted sinebell window function in both dimensions. Zero filling yielded a spectrum with digital resolution of 1.1 Hz/pt in the F_2 dimension.

NOESY experiments were recorded in D_2O with mixing times of 50, 200 and 300 ms. At the 50 ms mixing time zero-quantum coherence effects which distort peak shape were attenuated with a 5% pseudo-random variation of the mixing time. HOHAHA experiments in D_2O were recorded with 20 ms and 37 ms mixing times. For identification of slowly

exchanging hydrogens, a fast HOHAHA experiment (approximately 6 hours long) was recorded on a protonated sample immediately after dissolution in cold D_2O. DQF-COSY spectra (Rance *et al.*, 1983) were recorded using selective irradiation during the relaxation delay period to suppress the H_2O resonance. These D_2O experiments were recorded using a Bruker AM 500 spectrometer at 303 K with a sweep width of 6.5 kHz at 303 K.

For the measurement of $^3J_{\alpha\beta}$ spin-spin coupling constants (described in Chapter 4), a 35° mixing pulse PE-COSY spectrum (Mueller, 1987; Marion & Bax, 1988b) was recorded in D_2O with 1024 increments and 4K real data points in the F_2 dimension. Prior to analysis, zero-filling was used to increase the digital resolution to 1.1 Hz/pt in the F_2 dimension.

3.3 Assignment of Sequential and Short Range NOEs

The first data set to be considered in the assignment of the t-PA F1 module was the fingerprint region of the SCUBA COSY experiment which is shown in Figure 3.10. In principle this region of the spectrum contains a single crosspeak $C^{\alpha}H/HN$ for each non-proline residue. However, crosspeaks for residues near the termini of the molecule or in highly flexible regions may not show up due to rapid HN exchange. The dispersion of HN chemical shifts indicated that the module was folded. Of the 48 non-proline residues in the t-PA F1 sequence, 45 $C^{\alpha}H/HN$ crosspeaks were unambiguously observed and numbered randomly from 1-45. Four other crosspeaks possibly corresponding to $C^{\alpha}H/HN$ connectivities were also identified as shown.

The corresponding 45 numbered peaks were assigned in a H_2O HOHAHA spectrum recorded at the same temperature. These peaks as

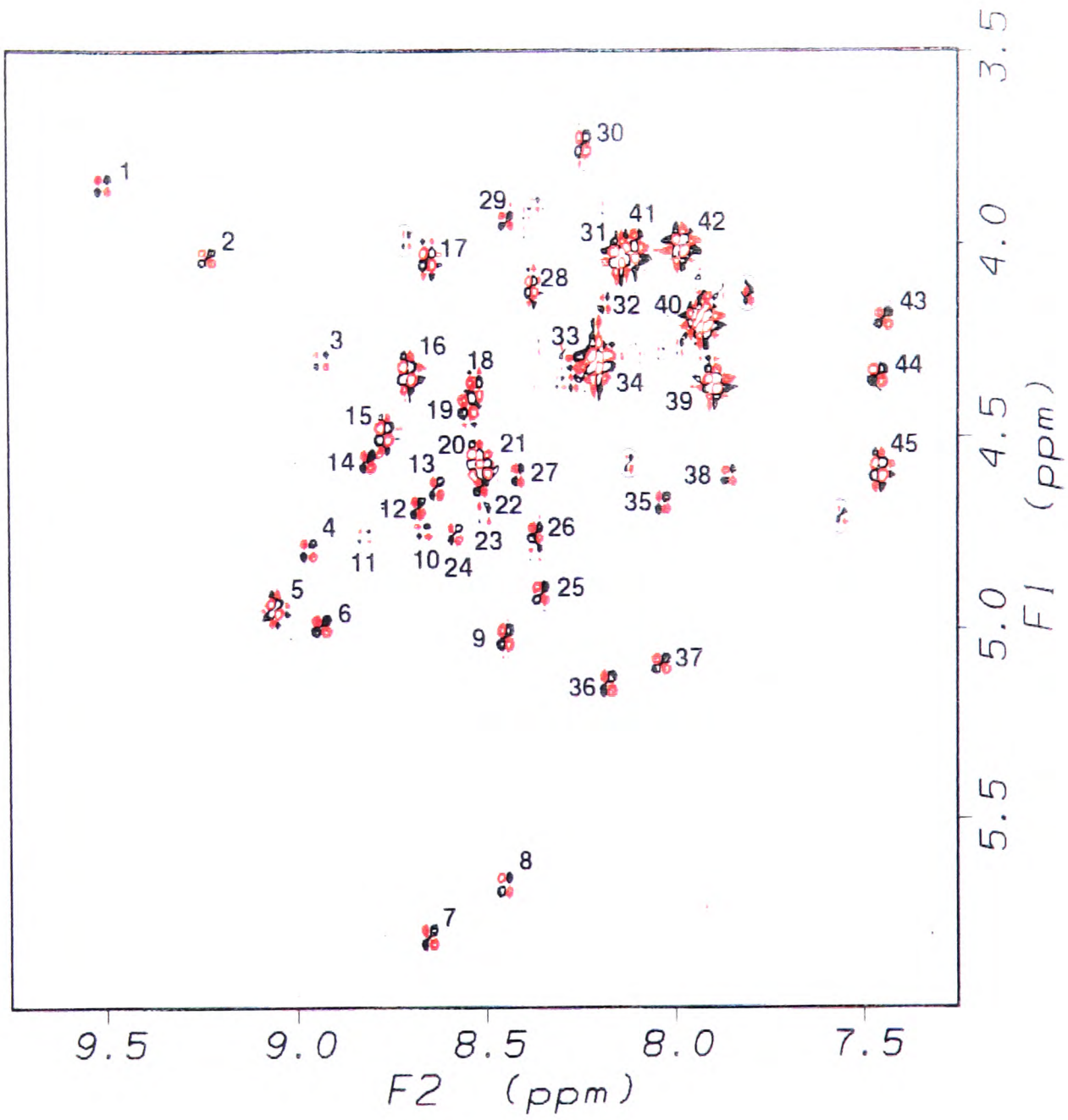


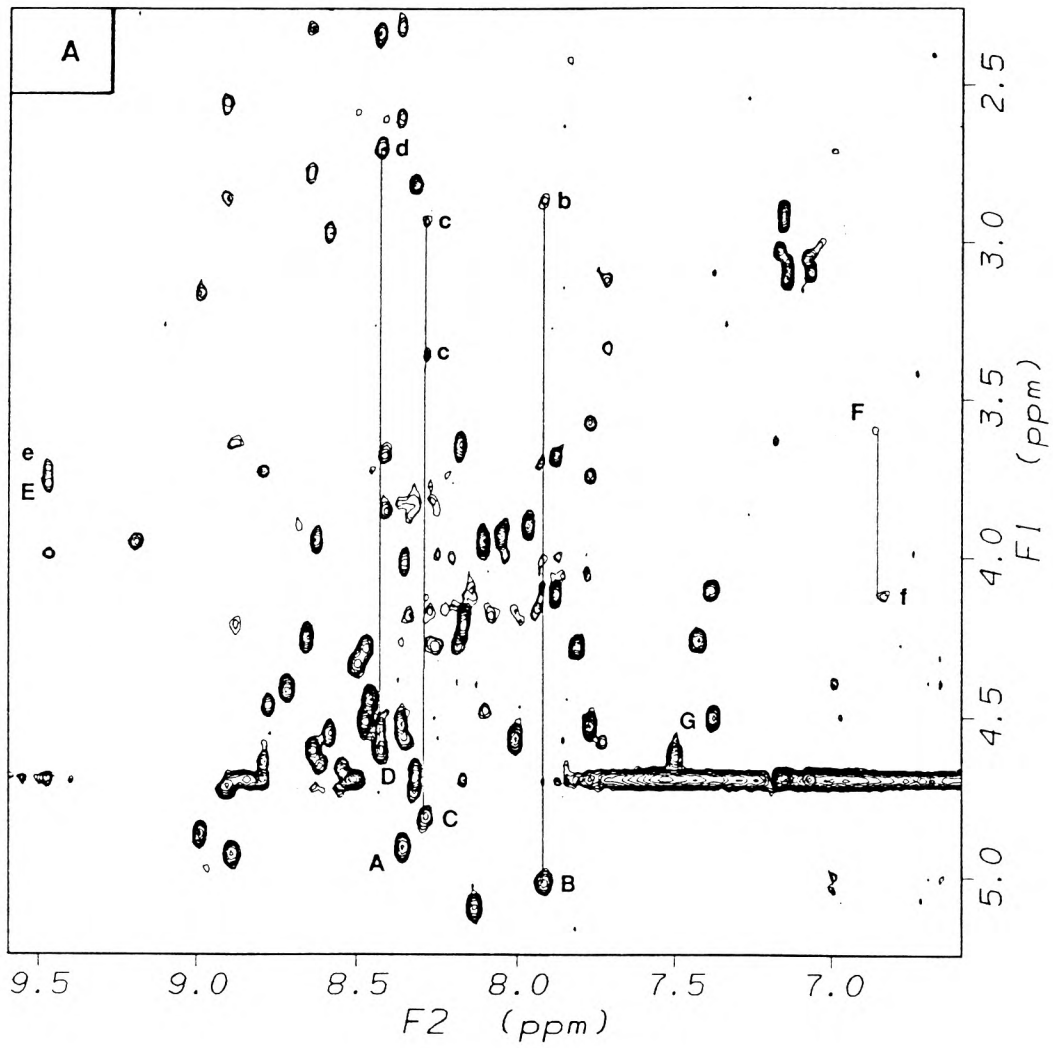
Figure 3.10

Fingerprint region of the SCUBA COSY spectrum of the t-PA F1 module recorded at 303 K, pH 5.4. Unambiguous $C^{\alpha}H$/HN crosspeaks are numbered consecutively from 1-45, and 4 peaks which may also correspond to $C^{\alpha}H$/HN connectivities are circled.

well as all other observable HOHAHA crosspeaks in the amide region were superimposed on a H_2O NOESY spectrum also conducted at 303 K. The HOHAHA and NOESY spectra recorded at 295 K had sharper peaks than the spectra recorded at 303 K presumably due to a decrease in chemical exchange line broadening at the lower temperature. Consequently, the intraresidue crosspeaks were identified in these spectra and the 295 K data set was used primarily. A region of this data set is shown in Figure 3.11. Sequential assignments were obvious when corresponding $C^{\alpha}H/HN$ and $C^{\alpha}H/HN_{(i,\ i+1)}$ crosspeaks were not degenerate in chemical shift with any other protons. These assignments were confirmed by identification of sequential side chain to amide proton connectivities. When sequential assignment was ambiguous, comparison was made with the data set recorded at 303 K. In this manner the majority of the residues were sequentially assigned, but these assignments remained to be confirmed by assignment of the amino acid side chains.

Of the 20 amino acids, 7 generate unique patterns of crosspeaks and are thus more readily identified. These seven amino acids are gly, ala, val, ile, leu, thr and lys. The side chain spin systems for several of these types of residues were resolvable in the D_2O COSY recorded on the t-PA F1 module as is shown in Figure 3.12. Typical complications encountered in the assignment process are evident with reference to this spectrum. Comparison of the patterns of COSY crosspeaks that one would expect to see for a given spin system with the actual data shows that in practice not all of the connectivities appear. The discrepancy between the theoretically derived and experimentally observed patterns can usually be explained either by (i) chemical shift degeneracy, particularly for chemically indistinguishable protons such as the two valine $CH_{3\gamma}$ groups, or (ii) crosspeak cancellation due to the finite proton

Figure 3.11



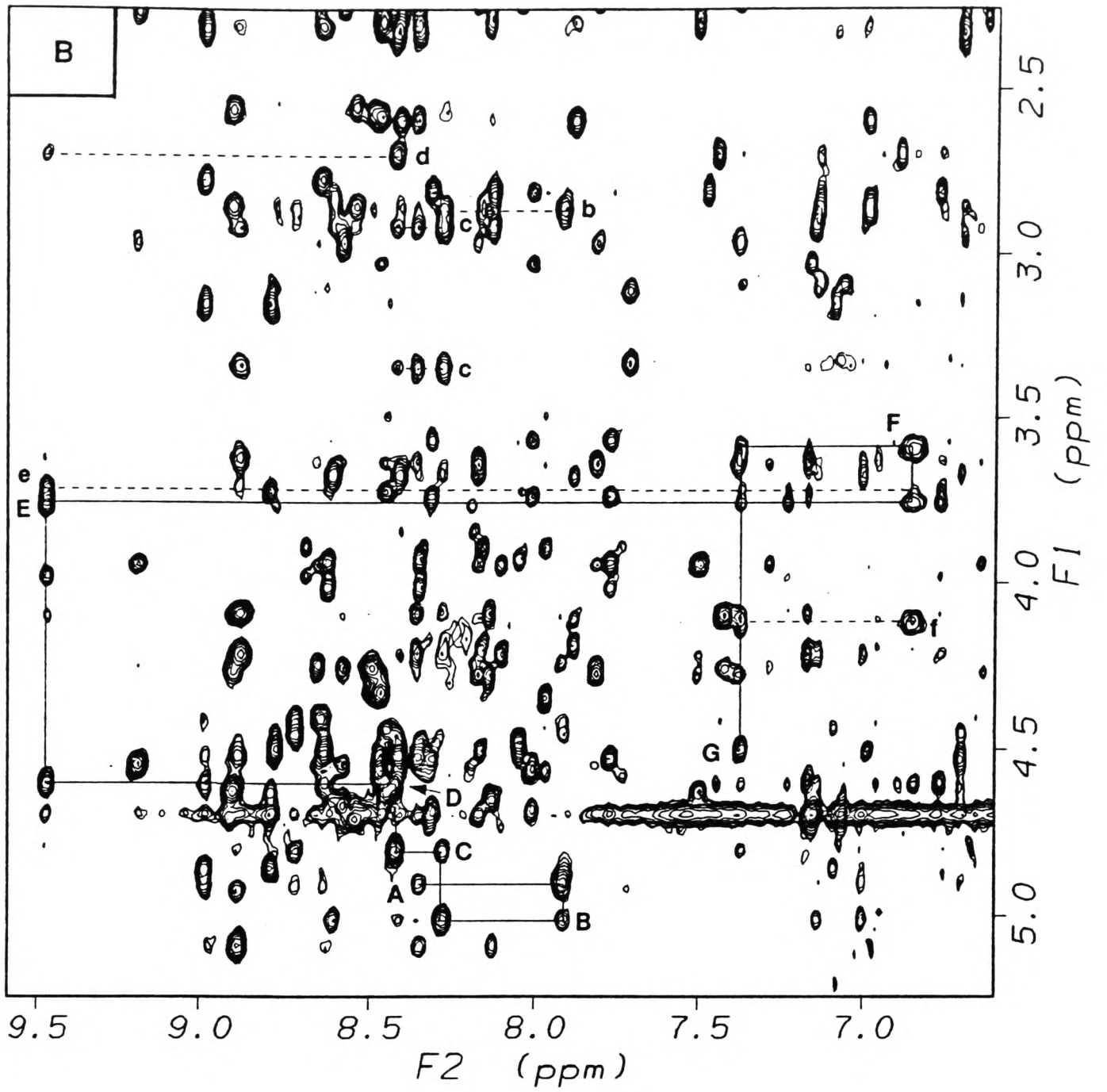


Figure 3.11

Two-dimensional n.m.r. spectra of t-PA F1. (A) Portion of a 37 ms HOHAHA spectrum in H_2O recorded at 295 K, pH 5.4, showing selected $C^{\alpha}H/HN$ crosspeaks (labelled A-G) and intraresidue side chain connectivities (labelled b-f). (B) The corresponding region of a 200 ms NOESY spectrum in H_2O recorded under the same conditions. Sequential $C^{\alpha}H/HN$ and $C^{\alpha}H/HN_{(i,\ i+1)}$ crosspeaks are joined by solid lines. Sequential side chain to amide proton connectivites are highlighted by dashed lines.

B

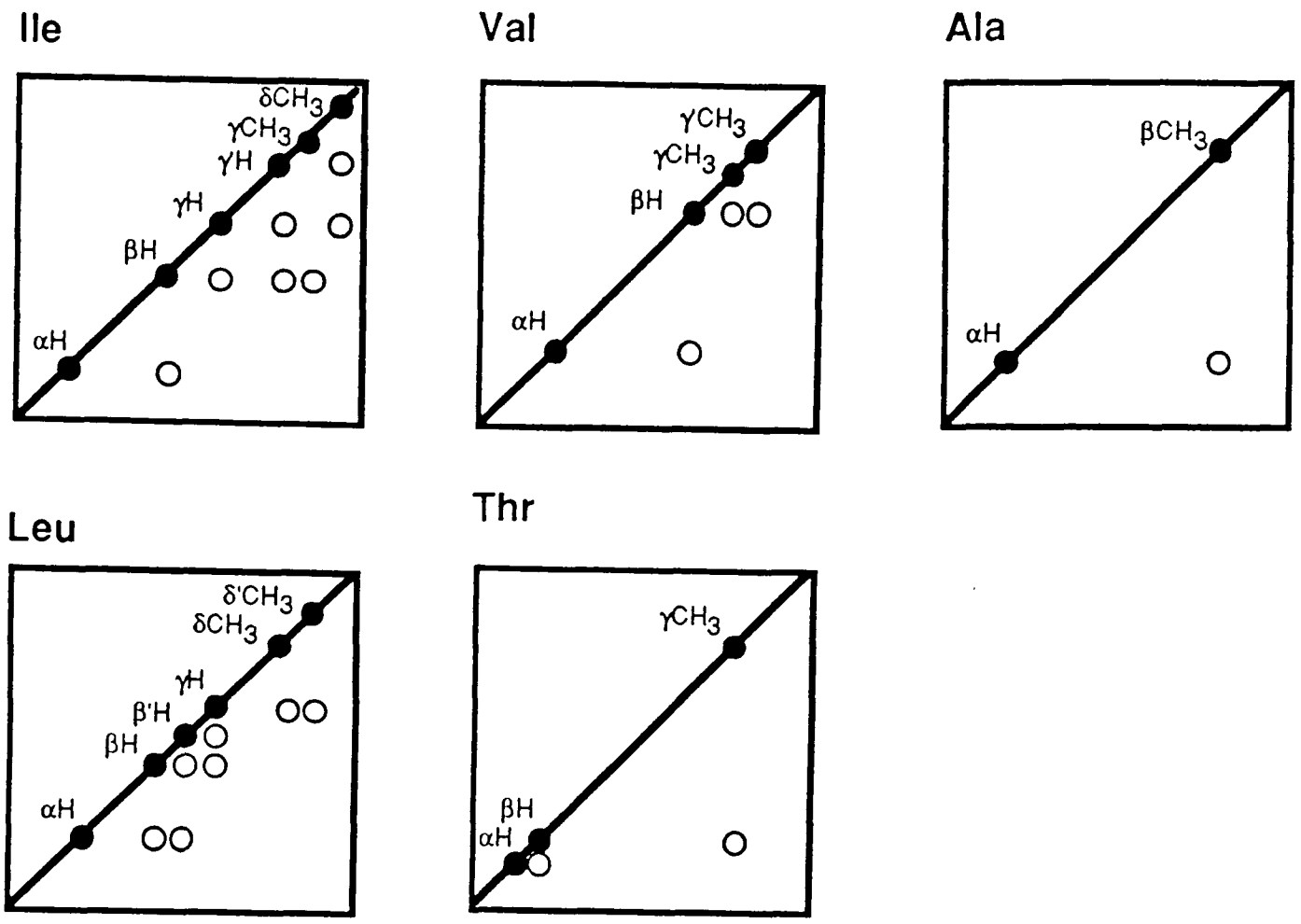


Figure 3.12

A region of the D_2O COSY spectrum of the t-PA F1 module recorded at 303 K and pH 5.4 is shown (A) with specific side chain spin systems delineated. Each amino acid is identified by its single letter code. The predicted pattern of resonances generated by these spin systems is also presented (B) in order to highlight the discrepancies between experimental and idealised data.

line width. As explained previously, those crosspeaks which cancel in the phase-sensitive D_2O COSY spectrum should appear in the corresponding D_2O HOHAHA spectrum. Another noteworthy observation is that occasionally the relative chemical shifts of two protons can invert. For example, the isoleucine $CH_3\delta$ is sometimes shifted downfield of the $CH_3\gamma$. Chemical shift assignments for the side chains identified in Figure 3.12 were confirmed by inspection of a D_2O HOHAHA spectrum recorded at the same temperature (Figure 3.13). The assignments for these residues in the H_2O spectra were then completed. Using these assignments as reference points, short stretches of amino acids were assigned and linked until the sequential assignment process was finished.

Next all of the NOEs between residues separated by 3 or fewer amino acids were tabulated. A summary of the short range NOEs found for the t-PA F1 module is shown in Figure 3.14. This graphical presentation of the data immediately allowed prediction of regions of secondary structure based on their characteristic patterns of short range NOEs (Figure 3.14). It was concluded from this summary that the structure of the t-PA finger module, like the fibronectin finger, is primarily composed of β-strands and contains no α-helical regions.

The chemical shifts for the t-PA F1 module are tabulated in Table 3.1. Secondary $C^{\alpha}H$ chemical shifts used to predict secondary structure, were calculated by subtracting random coil $C^{\alpha}H$ chemical shifts from the measured ones for each residue. The secondary $C^{\alpha}H$ chemical shifts for the fibronectin finger were also calculated (Baron, 1990) and compared in order to improve sequence alignment (Figure 3.15). The correlation of secondary $C^{\alpha}H$ chemical shifts in the β-sheet regions of the fibronectin finger module promised very similar structures for the two molecules.

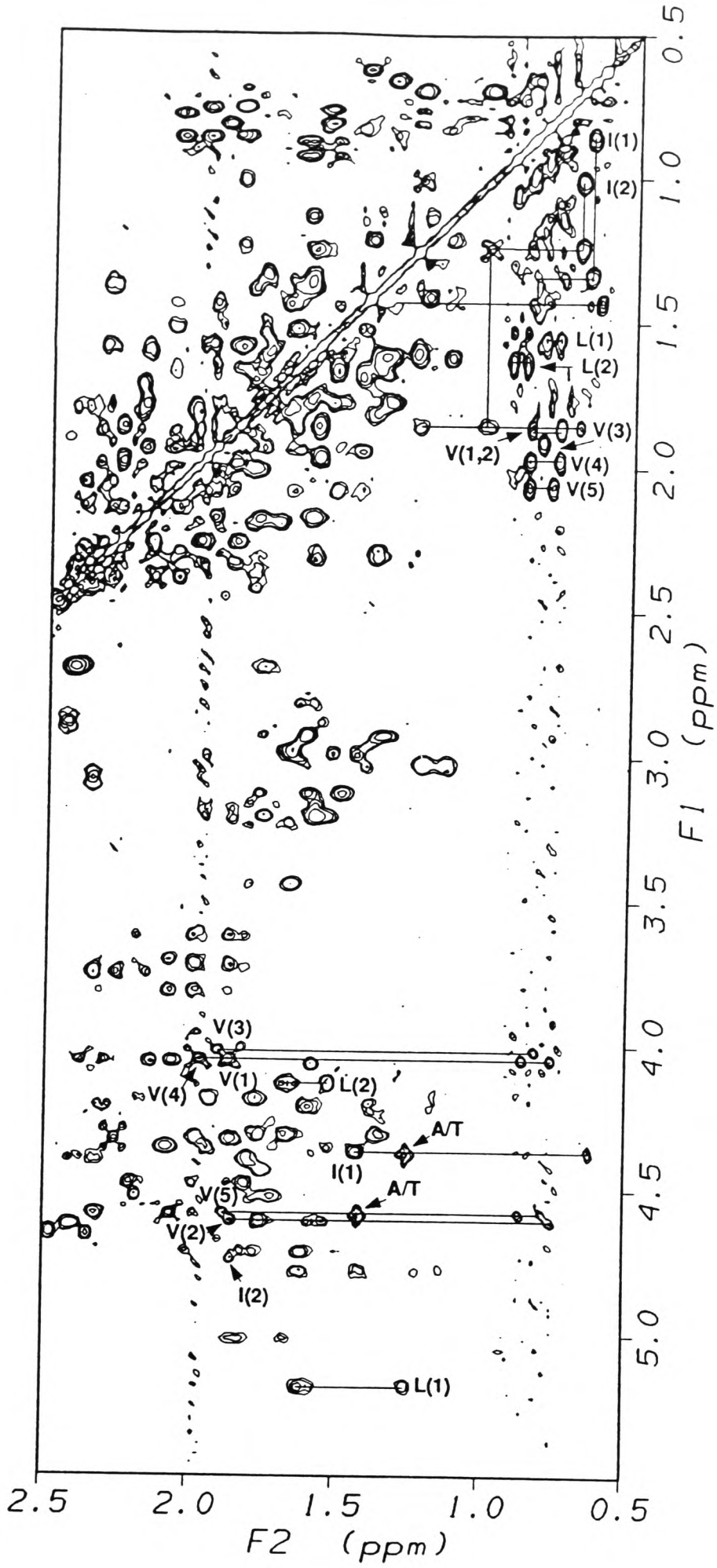


Figure 3.13

A region of a D_2O HOHAHA spectrum corresponding to the COSY in Figure 3.12 recorded under the same conditions. Comparison of this data demonstrates the complimentary nature of the two experiments. Specifically, the COSY data are more easily interpreted because crosspeaks only appear between protons linked by three or fewer covalent bonds. The HOHAHA spectrum presents more complete data at the expense of greater spectral crowding and complexity.

Figure 3.14

Summary of the short range NOEs tabulated for the t-PA F1 module (A), and the expected pattern of NOEs for commonly occuring secondary structural motifs (B). Short ^1H-^1H distances are indicated by lines linking the residues that contain the connected hydrogen atoms. The thickness of the lines is proportional to the NOESY crosspeak intensities. In an α-helix $d_{\beta N(i,\ i+1)}$ varies from 2.5 to 4.1 Å, while in a β-sheet this distance varies from 3.2 to 4.7 Å. Hence strong $d_{\beta N(i,\ i+1)}$ NOEs are not normally observed in β-sheet regions. The extended stretches of strong $d\alpha N_{(i,i+1)}$ NOEs and the lack of $d\alpha N_{(i,i+3)}$ and $d\alpha N_{(i,i+4)}$ NOEs demonstrate that the secondary structure of the t-PA F1 module is predominantly β-sheet with little or no α-helix.

Table 3.1

Proton Resonance Assignments of t-PA F1 at 295 K and pH 5.4[a]

residue	NH	$C^{\alpha}H$	$C^{\beta}H$	others
Ser 1	-	-	-	
Tyr 2	-	4.52	2.86*	$C^{\delta}H$ 6.99*; $C^{\varepsilon}H$ 6.69*
Gln 3	8.16	4.21	1.87, 1.76	$C^{\gamma}H$ 2.16*; $N^{\delta}H_2$ 7.35, 6.71
Val 4	8.10	3.94	1.86	$C^{\gamma}H_3$ 0.76, 0.65
Ile 5	7.49	4.62	1.75	$C^{\gamma}H$1.14, 0.91; $C^{\gamma}H_3$ 0.65*; $C^{\delta}H_3$ 0.59*
Cys 6	8.91	4.71	2.86, 2.56	
Arg 7	8.54	4.66	1.51, 1.31	$C^{\gamma}H$1.02, 1.11; $C^{\delta}H$ 2.92*; NH 7.15
Asp 8	8.56	4.55	2.97, 2.25	
Glu 9	9.18	3.95	2.02, 1.95	$C^{\gamma}H$ 2.28, 2.20
Lys 10	8.64	4.26	1.98, 1.84	$C^{\gamma}H$1.34,1.41; $C^{\delta}H$1.56*; $C^{\varepsilon}H$ 2.87*
Thr 11	7.81	4.28	4.28	$C^{\gamma}H_3$ 1.15*
Gln 12	8.17	3.65	2.23, 2.06 [b]	$C^{\gamma}H$ 2.16*; $N^{\delta}H_2$ 7.28, 6.64
Met 13	7.37	4.11	1.50, 1.28	$C^{\gamma}H$ 2.18*
Ile 14	7.41	4.26	1.33	$C^{\gamma}H$1.25, 0.75; $C^{\gamma}H_3$ 0.50*; $C^{\delta}H_3$ 0.55*
Tyr 15	8.50	4.34	2.56, 1.66	$C^{\delta}H$ 5.85*; $C^{\varepsilon}H$ 5.71*
Gln 16	8.46	4.28	1.69* [b]	$C^{\gamma}H$ 2.29, 2.24; $N^{\delta}H_2$ 7.50, 6.62
Gln 17	8.14	4.10	1.83, 1.66	$C^{\gamma}H$ 2.06*; $N^{\delta}H_2$ 7.22, 6.76
His 18	8.88	4.21	3.62*	$C^{\delta 2}H$ - ; $C^{\varepsilon 1}H$ 7.16
Gln 19	8.35	4.52	2.31*	$C^{\gamma}H$ 2.60*; $N^{\delta}H_2$ 7.88, 6.98
Ser 20	8.41	5.61	3.67*	

Trp 21	8.61	4.65	2.93, 2.82	$C^{\delta 1}H$ 6.45; $C^{\varepsilon 3}H$ 6.18; $C^{\zeta 3}H$ 6.33; $C^{\eta}H$ 6.12; $C^{\zeta 2}H$ 6.69 [c] ; $N^{\varepsilon 1}H$ 9.91
Leu 22	8.13	5.10	1.51, 1.13	$C^{\gamma}H$1.46; $C^{\delta}H_3$ 0.72*, 0.67*
Arg 23	8.89	4.92	1.77, 1.72	$C^{\gamma}H$1.58*; $C^{\delta}H$ 3.34, 3.12; $N^{\varepsilon}H$ 7.71
Pro 24		4.46	2.23, 1.78	$C^{\gamma}H$1.96, 1.89; $C^{\delta}H$ 3.70, 3.60
Val 25	8.05	3.92	1.75	$C^{\gamma}H_3$ 0.75, 0.65
Leu 26	8.36	4.01	1.55, 1.42 [d]	$C^{\gamma}H$1.52; $C^{\delta}H_3$ 0.82*, 0.77*
Arg 27	8.61	3.94	1.88, 1.77	$C^{\gamma}H$1.47*; $C^{\delta}H$ 3.11*; $N^{\varepsilon}H$ 7.12
Ser 28	7.76	4.52	3.75, 3.56	
Asn 29	8.30	4.70	2.81*	NH_2 7.47, 6.76
Arg 30	8.00	4.56	1.69, 1.40	$C^{\gamma}H$1.50*; $C^{\delta}H$ 3.02*; $N^{\varepsilon}H$ 7.16
Val 31	8.47	4.52	1.76	$C^{\gamma}H_3$ 0.66*
Glu 32	8.62	4.60	1.64, 1.46 [b]	$C^{\gamma}H$1.88, 1.72
Tyr 33	8.42	4.53	2.36*	$C^{\delta}H$ 6.70*; $C^{\varepsilon}H$ 6.49*
Cys 34	8.35	4.90	-	
Trp 35	7.90	5.01	2.86*	$C^{\delta 1}H$ 6.99; $C^{\varepsilon 3}H$ 7.14; $C^{\zeta 3}H$ 6.77 $C^{\eta}H$ 6.84; $C^{\zeta 2}H$ 6.95; $N^{\varepsilon 1}H$ 9.83
Cys 36	8.29	4.81	3.35, 2.93	
Asn 37	8.41	4.60	2.72*	NH_2 7.44, 6.89
Ser 38	9.46	3.76	3.98, 3.72	
Gly 39	6.85	4.12, 3.58		
Arg 40	7.37	4.51	1.65*	$C^{\gamma}H$1.48, 1.42; $C^{\delta}H$ 3.10*; $N^{\varepsilon}H$ 7.06
Ala 41	8.77	4.46	1.32	
Gln 42	8.72	4.41	1.65, 1.60	$C^{\gamma}H$ 2.07*; $N^{\delta}H_2$ 7.42, 7.02
Cys 43	8.63	5.76	2.78, 2.26	

His 44	8.98	4.86	3.16, 3.14	$C^{\delta 2}H$ - ; $C^{\varepsilon 1}H$ 7.09
Ser 45	8.78	4.64	3.73*	
Val 46	8.45	4.45	1.94	$C^{\gamma}H_3$ 0.75, 0.66
Pro 47		4.35	2.09, 1.69	$C^{\gamma}H$1.86, 1.76; $C^{\delta}H$ 3.64, 3.49
Val 48	7.96	3.90	1.80	$C^{\gamma}H_3$ 0.72*
Lys 49	8.16	4.21	1.66,1.54	$C^{\gamma}H$1.23*; $C^{\delta}H$1.47*; $C^{\varepsilon}H$ 2.67*
Ser 50	7.88	4.12	3.68*	

Footnotes to Table 3.1:

[a] Chemical shifts are with respect to dioxane; (-) indicates that the resonance was not observed or could not be assigned; (*) indicates protons with degenerate chemical shifts.

[b] $C^{\beta}H$ and $C^{\gamma}H$ assignments ambiguous.

[c] $C^{\zeta 2}H$ degenerate with Tyr 33 $C^{\delta}H$.

[d] $C^{\beta}H$ and $C^{\gamma}H$ protons overlap.

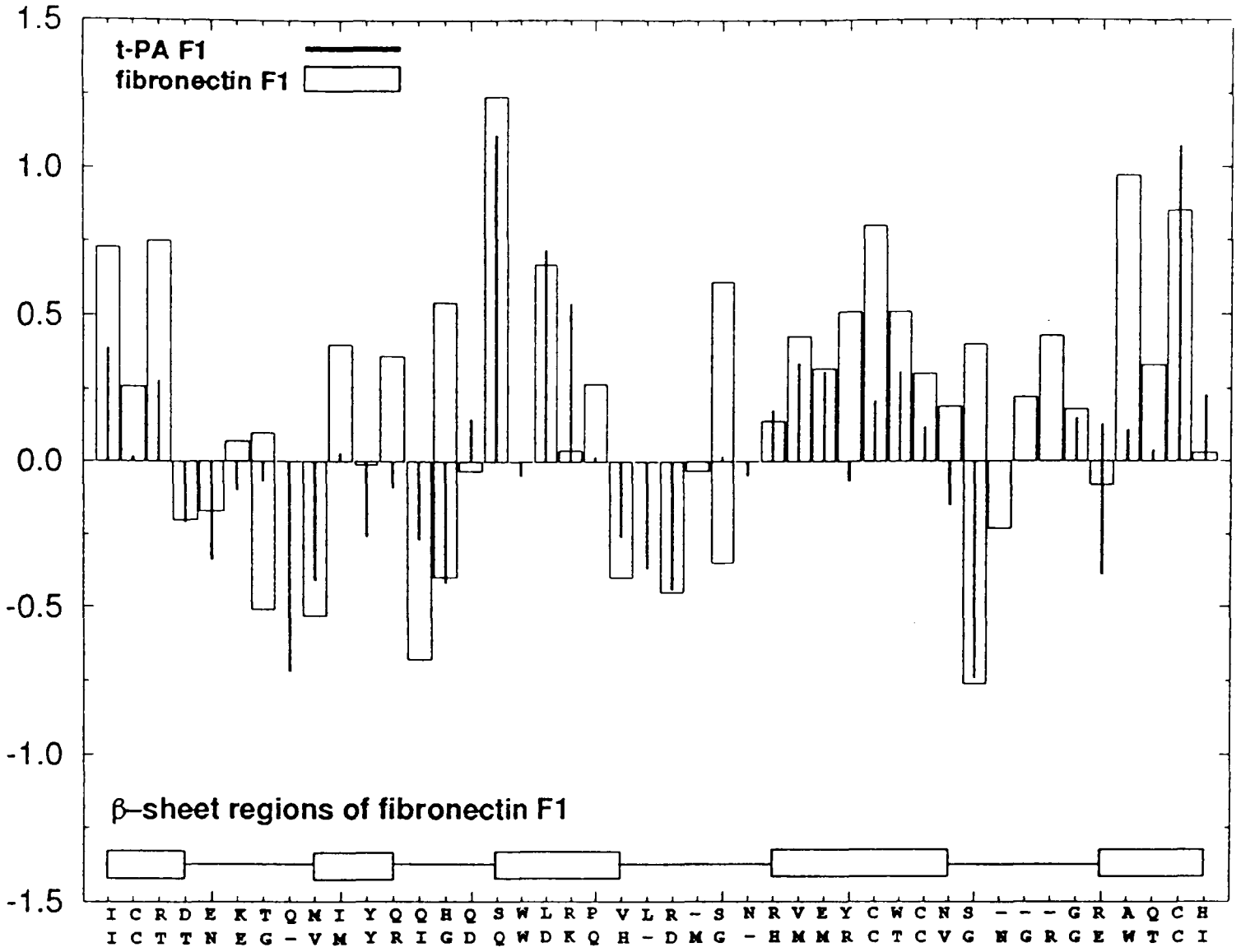


Figure 3.15

Graph of the secondary $C^{\alpha}H$ chemical shifts for the t-PA F1 module and the fibronectin F1 module. Chemical shift displacement from random coil positions is well conserved, particularly in the regions of fibronectin F1 secondary structure. The sequence alignment has been optimised. The only changes to the alignment in Figure 3.8 occur in turns connecting the β-strands. For this reason it was not necessary to revise the predicted secondary structure of the t-PA F1 module presented in Figure 3.9.

3.4 Long Range NOE Assignments Based on Homology

The next step in the n.m.r. assignment of the t-PA F1 module was to search for data defining the double-stranded and a triple-stranded β-sheets composing the fibronectin F1 structure based on the aligned sequences. Specifically, alternating close $C^{\alpha}H/C^{\alpha}H$ contacts and hydrogen bonds between the strands were sought. Short $C^{\alpha}H/C^{\alpha}H$ distances are manifested by medium or strong NOE connectivities between the protons involved. Backbone amide protons involved in hydrogen bonds are less labile than those which are not, therefore these slowly exchanging protons appear in a quickly acquired HOHAHA spectrum recorded on a protonated sample freshly dissolved in D_2O. However, because the spectrum for this experiment is collected rapidly, it has reduced signal to noise ratio and inferior peak resolution. Consequently hydrogen bonds are only assigned where NOE connectivities defining secondary structure have already been established.

Two $C^{\alpha}H/C^{\alpha}H$ connectivities were found to define the double-stranded β-sheet in fibronectin. Thus the corresponding contacts were sought in the H_2O NOESY of the t-PA F1 module linking residues cys 6/tyr 15 and asp 8/met 13. No such NOEs were found, but $C^{\alpha}H/C^{\alpha}H$ connectivities were observed between ile 5/gln 16 and arg 7/ile 14. In addition slow HN exchange was observed for residues cys 6/tyr 15 and asp 8/met 13. It was concluded that the double-stranded β-sheet was forming but with the alternating pattern of close $C^{\alpha}H/C^{\alpha}H$ contacts and hydrogen bonds shifted by one residue relative to the fibronectin F1. Similarly, $C^{\alpha}H/C^{\alpha}H$ connectivities were found between residues ser 20/trp 35, leu 22/tyr 33, cys 36/ala 41, cys 34/cys 43 and arg 30/pro 47, and hydrogen bonds were unambiguously identified linking residues arg

23/glu 32 and trp 35/gln 42. These assignments were used to define the triple-stranded β-sheet in the t-PA F1 module. Figure 3.16 summarises the NOE and hydrogen bond information for the two β-sheet regions.

$^3J_{HN\alpha}$ spin-spin coupling constants, which are measured in a COSY spectrum recorded in H_2O, vary according to the ϕ peptide backbone torsion angle as shown in Figure 3.17. In antiparallel β-sheets ϕ = -139° which corresponds to a $^3J_{HN\alpha}$ coupling constant of ~ 9 Hz. In order to correct partially for distortions arising from the finite amide proton line widths which limit the accuracy with which coupling constants can be measured, cross sections parallel to F_2, through $C^{\alpha}H$/HN crosspeaks were extracted and fitted to simulated lineshapes (Redfield & Dobson, 1990). A cut-off of 9.0 Hz was chosen for assignment of $-90° \geq \phi \geq -150°$ for residues cys 6, met 13, ile 14, tyr 15, val 31, glu 32, tyr 33, and cys 34 of the t-PA F1 module.

Once the regions of secondary structure had been defined, all that remained was to search for NOEs which would define the packing of the sheets. This task was simplified by the two known disulphide bonds linking cys 6/cys 36 and cys 34/cys 43. Initially NOEs in the vicinity of these covalent bonds were sought. Then close contacts between the two conserved residues in the hydrophobic core of the molecule, tyr 15 and trp 21 were found. Figure 3.18 shows a qualitative comparison of the NOEs used to calculate the fibronectin F1 structure and those tabulated at this stage for the t-PA F1 module. The similarity of the two plots confirmed the structural homology of the two finger modules. Furthermore, the number of long range contacts found for the t-PA module was of the order of those used to calculate the fibronectin structure. Since assignment of long range contacts is facilitated by consultation of initial structures, at this point data analysis was temporarily abandoned and structure calculations were begun.

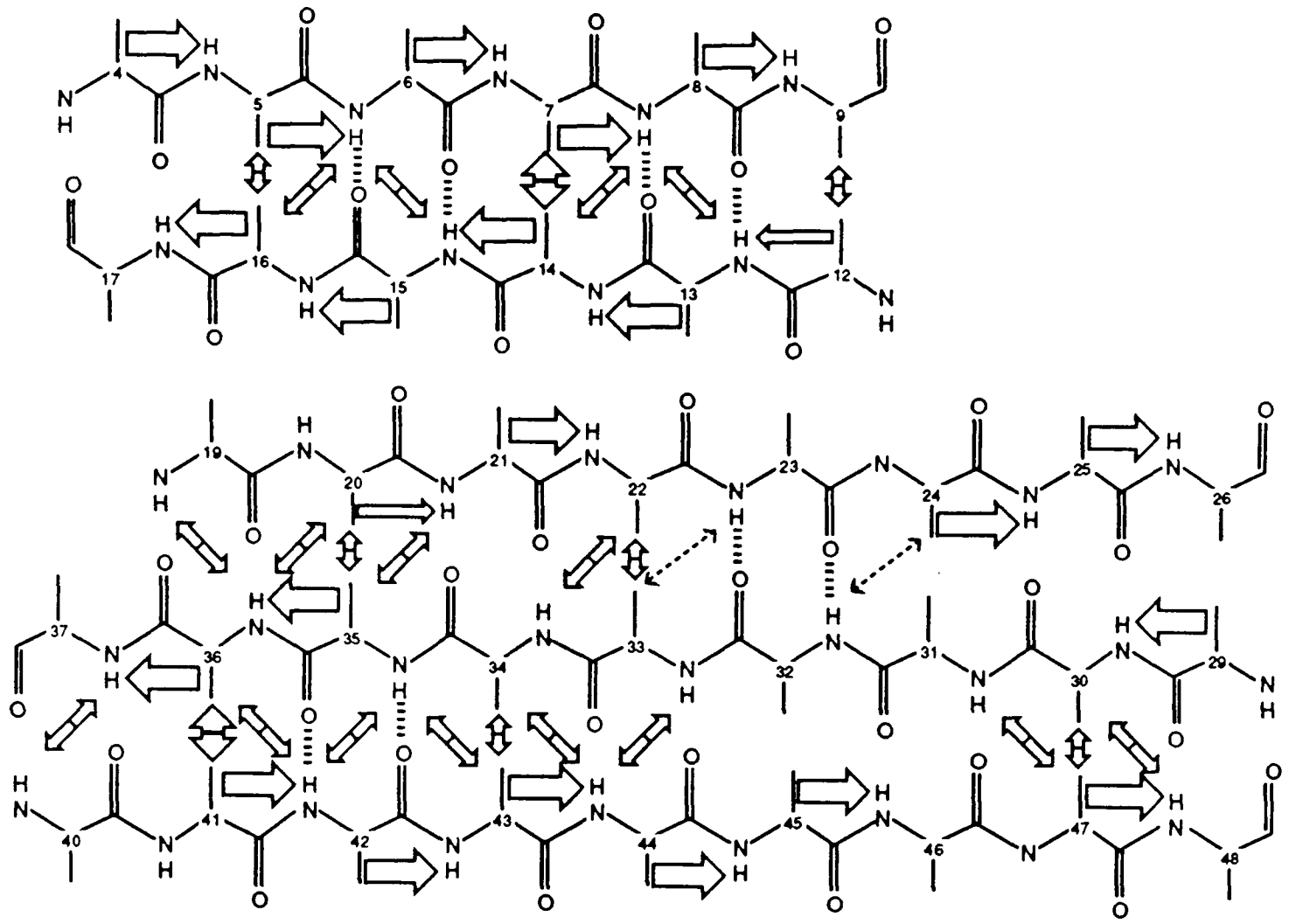


Figure 3.16

Summary of the NOE and hydrogen bond information defining the double-stranded and triple-stranded β-sheets forming the secondary structure of the t-PA F1 module. Strong, medium and weak NOEs are indicated by thick, thin and dashed arrows respectively. Horizontally dashed lines join hydrogen bonded atoms across the sheets.

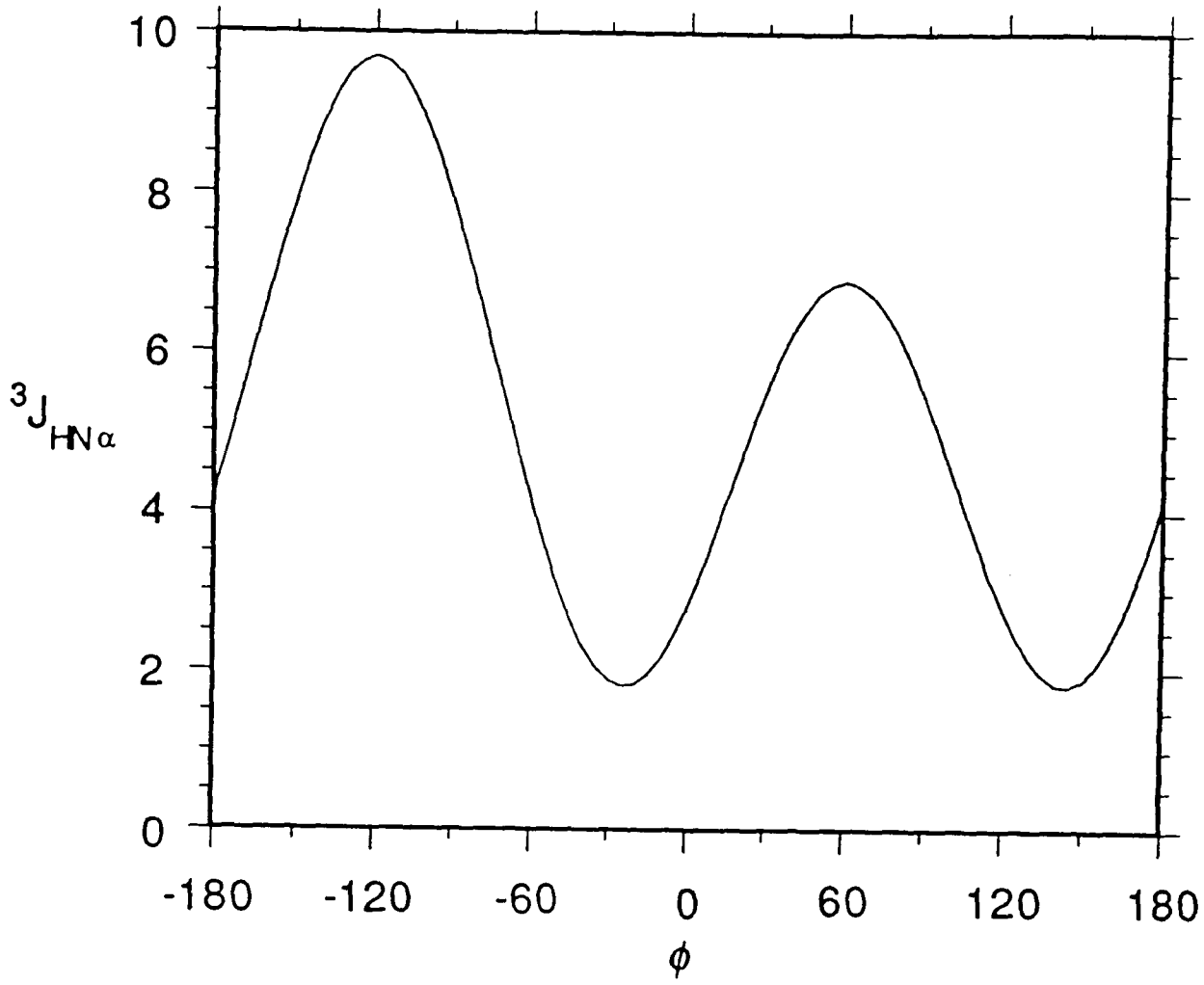


Figure 3.17

Plot of $^3J_{HN\alpha}$ versus the torsion angle ϕ based on the general relation between the size of the spin-spin coupling constant and the intervening torsion angle derived by Karplus (1959).

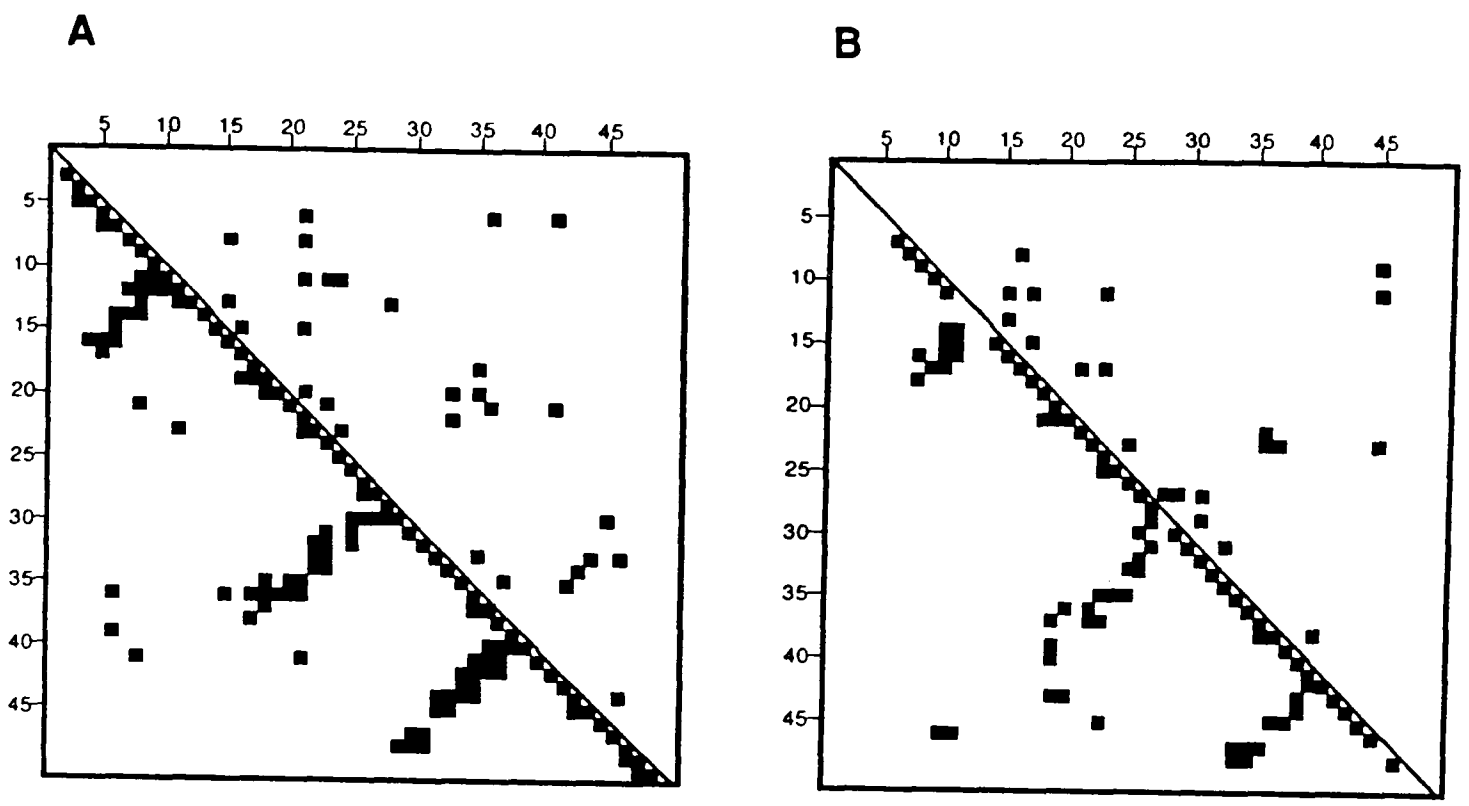


Figure 3.18

NOE block diagrams illustrating interproton connectivities in the t-PA (A) and the fibronectin (B) F1 modules. The sequence is numbered along the axes, and each square denotes an observed NOE between two residues. Filled squares below the diagonal indicate connectivities involving at least one backbone atom, while squares above the diagonal correspond to those involving only side chain atoms. The reverse diagonals below the main diagonal are characteristic of NOEs between β-sheets. The similarity of the two plots suggested homologous structures for the two F1 modules.

3.5 Conclusions

N.m.r. spectra of the t-PA F1 module were recorded in H_2O and D_2O using NOESY, HOHAHA and COSY pulse sequences. Using these data, sequential assignment was performed followed by identification of all short range NOEs. A qualitative inspection of this information and comparison of secondary $C^{\alpha}H$ chemical shifts with those of the fibronectin F1 module indicated that the two structures were likely to be similar. Alternating close $C^{\alpha}H/C^{\alpha}H$ contacts and hydrogen bonds were sought based on the β-strands comprising the fibronectin finger secondary structure. It was found that the pattern was shifted by one residue relative to the fibronectin sequence. Nonetheless, the secondary structural motif of a double-stranded β-sheet followed by a triple-stranded one appeared to be well conserved. NOEs defining the tertiary structure of the t-PA finger were assigned with reference to the disulphide bonding pattern and the fibronectin finger fold.

Chapter 4

Structure Calculation and Refinement of the t-PA F1 Module

4.1 Introduction

A flow chart illustrating the steps involved in solving the solution structure of a protein by 2-dimensional n.m.r., which highlights the focus of this chapter, is presented in Figure 4.1. Prior to structure calculations based on n.m.r. assignments NOE, hydrogen bond and torsion angle data were converted into a set of experimental restraints. The structures were calculated iteratively and refined by inclusion of stereospecific restraints for β-methylene protons.

The first introductory section of this chapter describes the techniques used in conversion of n.m.r. data to experimental restraints which take into account inaccuracies in the data and assignment ambiguities. An overview of the commonly used methods for protein structure calculation follows which includes discussion of the principal advantages and disadvantages associated with each. Finally,

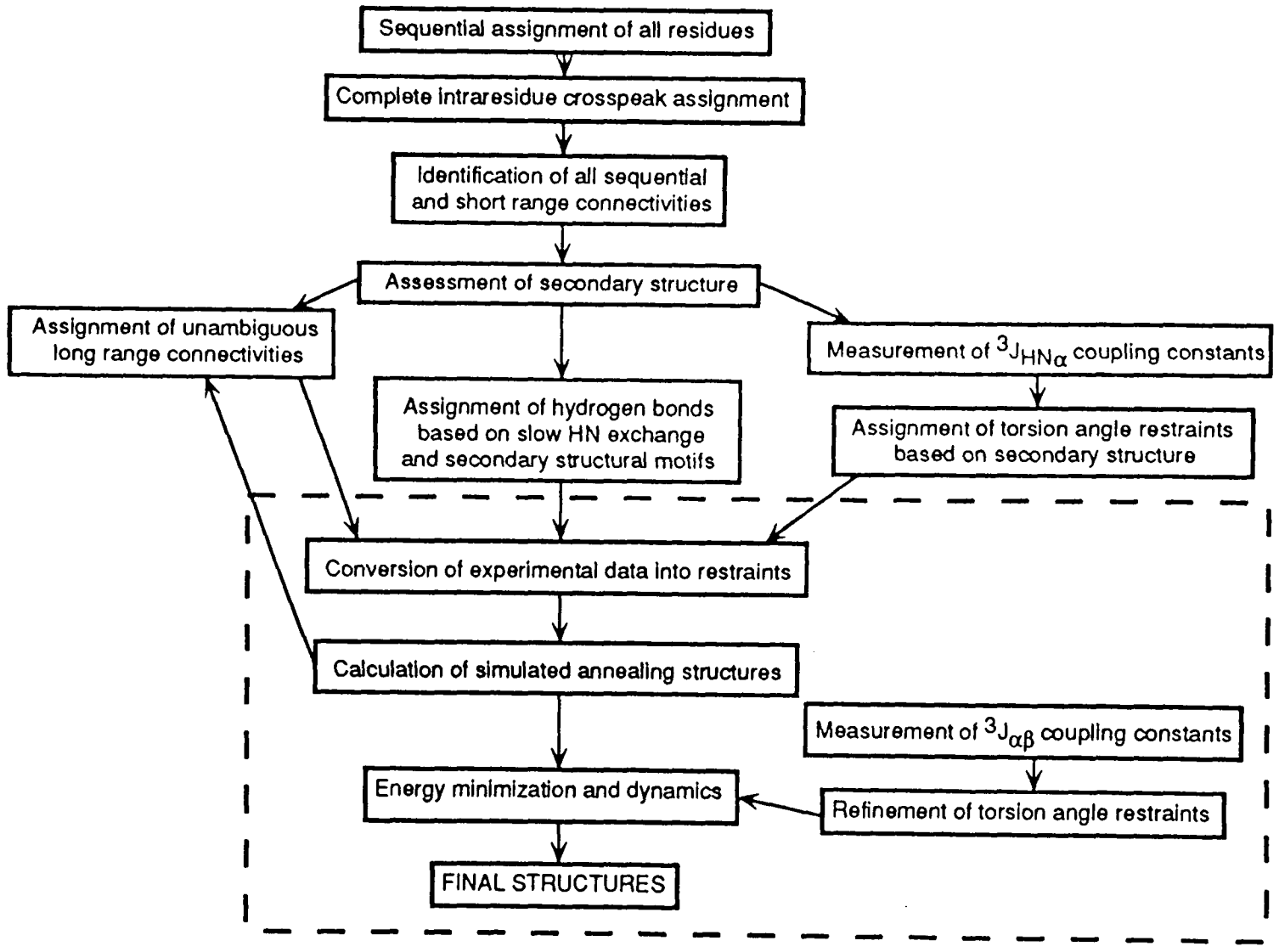


Figure 4.1

Flow chart illustrating the steps involved in ^1H n.m.r. derived protein structure determination. This chapter addresses the second stage of the process which is outlined by dashed lines.

techniques for refinement of ^1H protein n.m.r. structures are presented with emphasis on stereospecific assignment of β-methylene protons. Introductory discussion is followed by details of the t-PA F1 structure calculations and refinement and a description of the final structures.

4.1.1 Conversion of n.m.r. data to experimental distance restraints

Due to the inherent inaccuracy in the calibration of NOE intensity with interproton distance caused by spin diffusion effects, the n.m.r. data do not define exact distances between hydrogen atoms. Hence NOE intensities are usually approximately quantified and then grouped into three classes: strong, medium and weak corresponding to short, medium and long distances respectively. This loose correlation of NOE intensity with ^1H-^1H distance normally compensates for inaccuracies in the data. Furthermore when an NOE dominated by spin diffusion effects is assigned to the incorrect class of restraint, this error is usually detected and corrected during the structure calculation process. The probability of identifying such improperly assigned restraints increases with the number of NOEs used in the calculations.

Distances associated with NOEs attributed to chemically indistinguishable protons such as β-methylene protons must be adjusted as shown in Figure 4.2. If the protons have degenerate chemical shift or if one or more of the NOEs to chemically indistinguishable protons is unresolvable, a pseudoatom correction is applied, i.e., a fixed distance is added to the distance constraint, and the restraint vector is defined from the geometric centre of the identical protons. If their chemical shifts are nondegenerate and NOEs to each proton are resolvable, no pseudoatom correction need be applied. However, since the protons cannot be

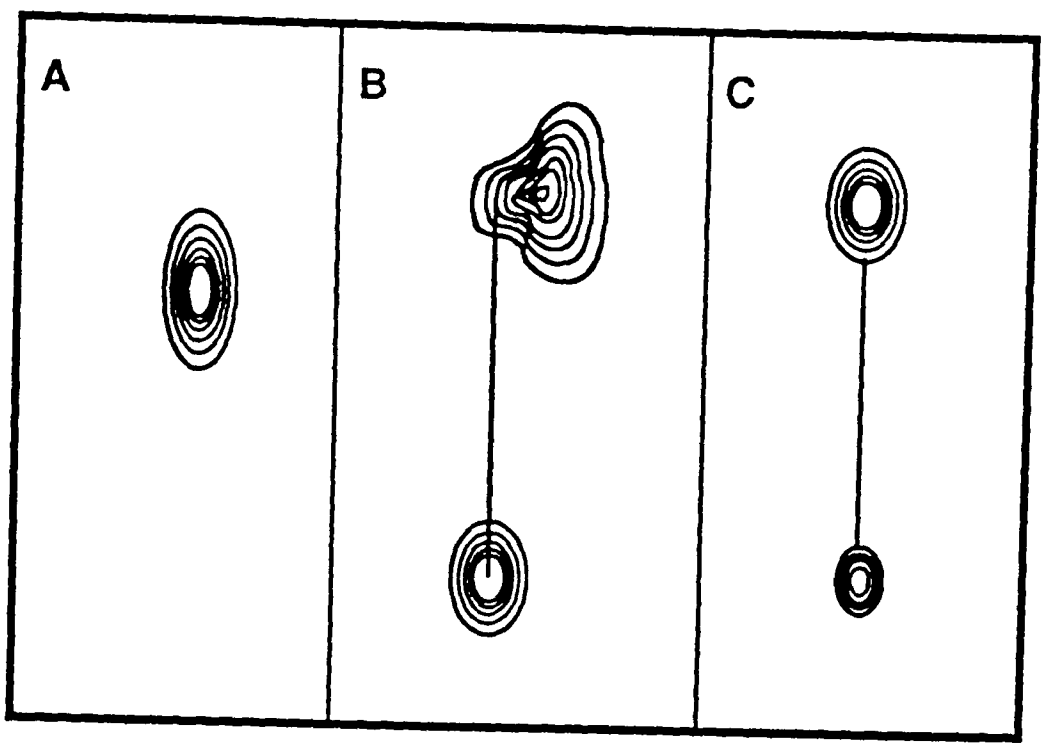


Figure 4.2

Three patterns of NOEs commonly observed for chemically indistinguishable protons such as for HN/Hβ2 and HN/Hβ3 connectivities. In A the chemical shift of the two protons is degenerate and thus only one crosspeak is observed, while in B the upper HN/Hβ crosspeak is overlapped. In these two cases a pseudoatom correction must be applied. When both HN/Hβ crosspeaks are resolvable (C), no such correction need be applied. However, if the two Hβ protons are not stereospecifically assigned, the two distance restraints $d_{\beta 2N}$ and $d_{\beta 3N}$ must both be set to the class defined by the weaker of the two NOEs.

distinguished based on the experimental data, the distance constraint to each such proton must be identical and set to the class defined by the weakest of the NOEs observed.

Hydrogen bonds are defined by two distance restraints, between the backbone carbonyl oxygen and the backbone amide proton and between the backbone carbonyl oxygen and the backbone nitrogen in order to ensure linearity of the CO-HN bond during structure calculations. Torsion angles are energetically restrained during structure calculations to lie within discrete upper and lower limits which are chosen based on the accuracy of the coupling constant data.

4.1.2 Methods for protein structure calculation

The techniques used to calculate protein structures can be broadly grouped into two classes: those which use metric matrix distance geometry (Havel *et al.*, 1983; Crippen & Havel, 1988) to calculate the structure from a matrix of interatomic distances, and those which employ a restraint satisfaction algorithm to fold an initial structure into a conformation consistent with experimentally derived distances. This section reviews the distance geometry algorithm and two restraint satisfaction algorithms in wide-spread use, minimisation in torsion angle space (Güntert *et al.*, 1991) and dynamical simulated annealing (Nilges *et al.*, 1988a), and discusses their relative accuracy and efficiency in the calculation of protein structures from n.m.r. data.

The first step in 3-dimensional structure calculation using metric matrix distance geometry is construction of a matrix of upper and lower bounds on the distances between all pairs of atoms. These distances are computed with the equations of Crippen & Havel (1988) using known bond lengths, dihedral angles, planarity restraints and van der Waals

radii, together with distance ranges derived from NOE measurements and dihedral angle ranges derived from coupling constant measurements. These bounds are "smoothed" by determining the bounds implied by triangle inequalities. Then Cartesian coordinates are produced from the matrix of interatomic distances. This procedure, known as "embedding", is done by randomly assigning distances between the upper and lower bounds. The embedded 3-dimensional structures typically have poor geometry requiring regularisation and subsequent refinement.

The minimisation of a protein structure in 3-dimensional torsion angle space involves location of the global minimum of a target function where the only degrees of freedom are the dihedral angles of the rotatable single bonds of the structure. The form of the target function used is

$$T = T_{distance} + T_{dihedral}$$

where $T_{distance}$ describes the contribution of the distance restraint violations (including those corresponding to steric overlap) and $T_{dihedral}$ describes the contribution of the dihedral angle restraint violations. Initially only restraints between residues close together in sequence are included in the calculations, and as the minimisation proceeds longer range restraints are included. Consequently local structure forms first, and the global fold of the protein is adopted towards the end of the procedure.

The protocol of dynamical simulated annealing (SA) involves solving Newton's equations of motion using molecular dynamics (Brooks *et al.*, 1988; Brünger, 1990) to find the global minimum of a target function representing the potential energy of the system. The target function is composed of the following terms:

$$F_{tot} = F_{covalent} + F_{repel} + F_{NOE} + F_{tor}$$

$F_{covalent}$ maintains the bond lengths, bond angles and improper torsions of the structure. Poor nonbonded contacts are prevented by F_{repel} which is a function of the van der Waals radii. The experimental distance and torsion angle restraints are incorporated by square-well potentials F_{NOE} and F_{tor}. The SA method efficiently overcomes potential energy barriers along the path to the lowest energy structure by initially raising the temperature of the system then slowly cooling it down again. Simulated annealing is described in more detail with reference to the t-PA F1 structure calculations in section 4.2.

When protein structures are calculated from n.m.r. data the number of experimental restraints is usually less than the molecule's degrees of freedom. Therefore the conformational sampling of the structure calculation method used is of great practical importance. Also, the spread of a family of independently-determined structures is often used to estimate the precision of n.m.r. structures (for example, Havel & Wüthrich, 1984). The fact that the sampling properties of the metric matrix distance geometry algorithm are not always optimal has been of concern for some time (Brünger *et al.*, 1987; Nilges *et al.*, 1988b; Thomason & Kuntz, 1989). Kuszewski *et al.* (1992) compared structures calculated with a variety of distance geometry protocols with those calculated using simulated annealing. The Ramachandran (ϕ,ψ) plot for the SA structures exhibited the most uniform sampling of allowed regions. The sampling properties of the simulated annealing algorithm were also shown to be superior to those of torsion angle space minimisation by Hartshorn (1992). High temperature dynamics performed during SA calculations accounts for the good sampling properties of this method.

The presence of false local minima can complicate location of the global minimum of a target function and lead to incorrectly folded

structures. Nilges *et al.* (1988a) developed a reliable method for circumventing this problem during SA calculations. Random starting structures (which may be regarded as very high temperature conformations of the protein) are simulated at high temperature with very low values of all of the force constants for the covalent, interproton distance and repulsive van der Waals terms in the target function. Consequently energy barriers between different folds of the protein are easily overcome, and the global minimum of the target function is reliably located. Additionally, the atoms are initially only weakly coupled and can move essentially independently to satisfy the restraints. Over the course of the calculations the force constants are gradually increased such that the final structures satisfy the experimental restraints within the error bounds and have nearly perfect stereochemistry and no close non-bonded contacts. To avoid folding problems when using minimisation in torsion angle space the target function varies during the calculations.

In order to test the relative accuracy of structures calculated using simulated annealing and minimisation in torsion angle space, an n.m.r. data set was generated from a high-resolution crystal structure and used to calculate structures using the two methods (Hartshorn, 1992). Comparison of the resulting structures revealed significantly smaller root mean square (r.m.s.) deviations in atomic coordinates for the structures calculated using simulated annealing.

The greatest disadvantage of structure calculation by simulated annealing is that this method is at least a factor of 2 times more computationally expensive than the alternative methods discussed (Kuszewski *et al.*, 1992; Hartshorn, 1992). However, once the initial fold of a protein has been reliably determined, an efficient variation of the simulated annealing protocol (Nilges *et al.*, 1988b; Downing *et al.*, 1992) can be used for subsequent calculations over the course of refinement.

This refinement protocol is reviewed in the context of the t-PA F1 structure calculations in section 4.2.

4.1.3 Techniques for refinement of ^{1}H n.m.r. protein structures

Chemical shift degeneracy normally gives rise to a number of NOEs which can be attributed to alternative pairwise interactions. Such ambiguities can in most cases be resolved by inspection of low-resolution structures. Hence initial structures are refined using an iterative approach to structure calculation in which more and more distance restraints are incorporated as the definition of the structure improves (Kraulis *et al.*, 1989; Forman-Kay *et al.*, 1991). When the NOE assignment process is complete, the precision of an n.m.r. structure can be significantly improved by incorporation of stereospecific restraints for β-methylene protons (Driscoll *et al.*, 1989; Folkers *et al.*, 1989).

$^3J_{\alpha\beta}$ coupling constant data is required for stereospecific assignment of β-methylene protons. The PE-COSY variation of the COSY experiment, in which the 90° mixing pulse is replaced by a 35° pulse, is commonly acquired and used for accurate measurement of these coupling constants. COSY and PE-COSY crosspeak patterns are compared in Figure 4.3. Typically the distribution of antiphase components in the crosspeak multiplets from a COSY experiment is dependent on the number and relative magnitudes of active and passive spin-spin couplings involved in the crosspeak (Boyd & Redfield, 1986). This distribution has important consequences for the amount of cancellation which occurs in the multiplets as linewidth is varied. Consequently, the multiplet splittings measured from the COSY experiment cannot be accurately correlated with the true active and passive couplings. As is shown in Figure 4.3, the 35° mixing pulse of the

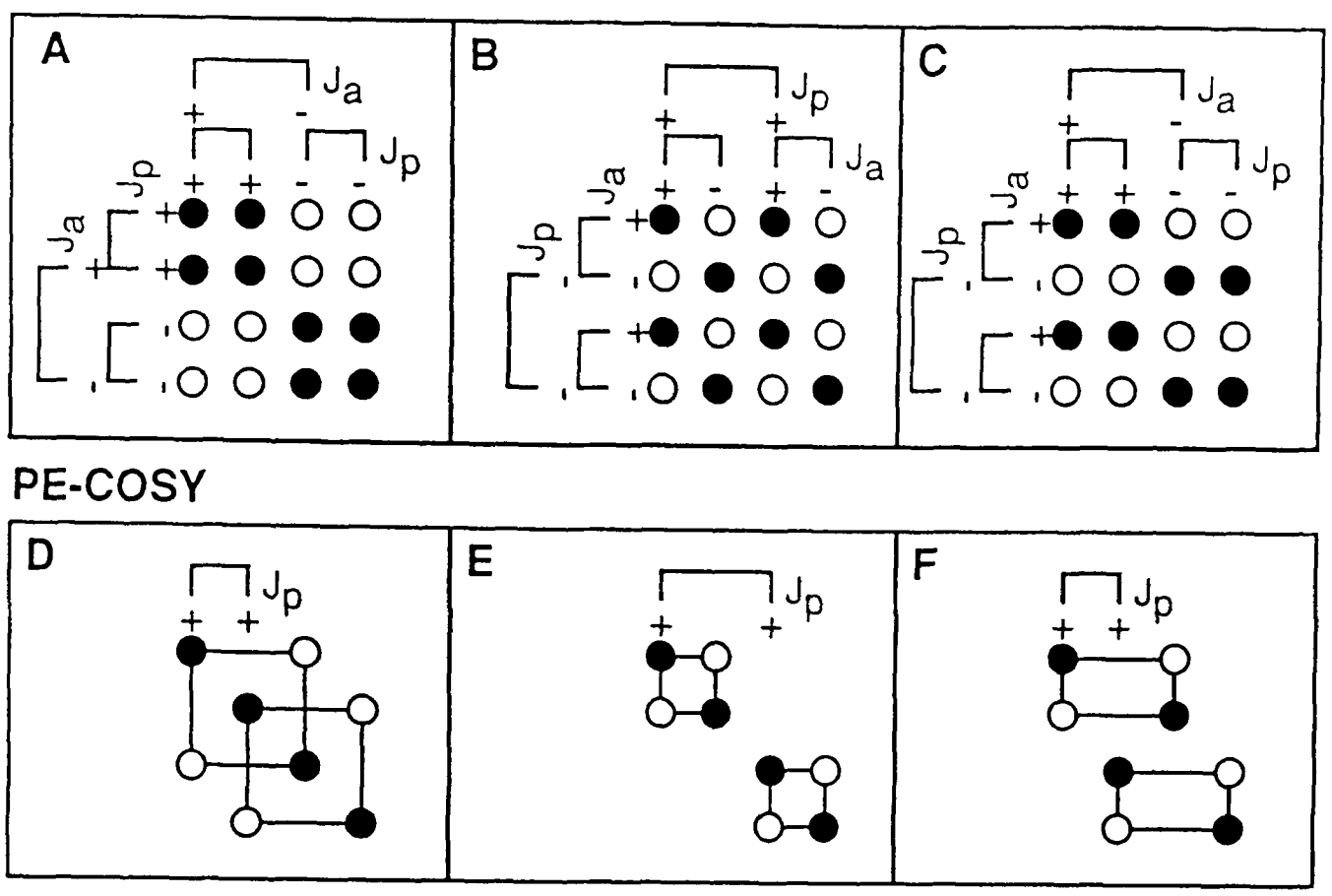


Figure 4.3

The fine structure patterns of β-methylene Hα/Hβ crosspeak multiplets are represented schematically for COSY and PE-COSY spectra. In A and D the passive couplings in both dimensions are smaller than the active coupling. In B and E both passive couplings are larger than the active coupling, and in C and F one passive coupling is greater than the active coupling while the other passive coupling is smaller than the active coupling. Of the 16 COSY crosspeak components only 8 appear in the PE-COSY spectrum, and the two square antiphase patterns are shifted relative to each other by the passive coupling of the crosspeak.

PE-COSY experiment reduces the number of components in a single Hα/Hβ crosspeak from 16 to 8. Furthermore, the two square patterns in the PE-COSY experiment are shifted by the passive coupling of the crosspeak, which can thus be measured with precision. For each pair of β-methylene crosspeaks, the passive coupling of one is equivalent in magnitude to the active coupling for the other. Thus active coupling constants can be measured accurately from a PE-COSY spectrum without distortion of the splitting by the finite proton line width or crosspeak cancellation. COSY and PE-COSY spectra of the t-PA F1 module which highlight such effects are presented in Figure 4.4.

Stereospecific assignment of β-methylene protons is facilitated by use of the program STEREOSEARCH (Nilges, *et al.*, 1990). This program attempts to match experimental parameters with those calculated for conformations in a data base. Specifically, the input experimental data consists of:

(i) the coupling constants $^3J_{HN\alpha}$, $^3J_{\alpha\beta2}$ and $^3J_{\alpha\beta3}$, which are related to the dihedral angles via the Karplus equations (Karplus, M., 1963):

$$^3J_{HN\alpha} = [6.4\cos^2(\phi - 60^o) - 1.4\cos(\phi - 60^o) + 1.9]$$

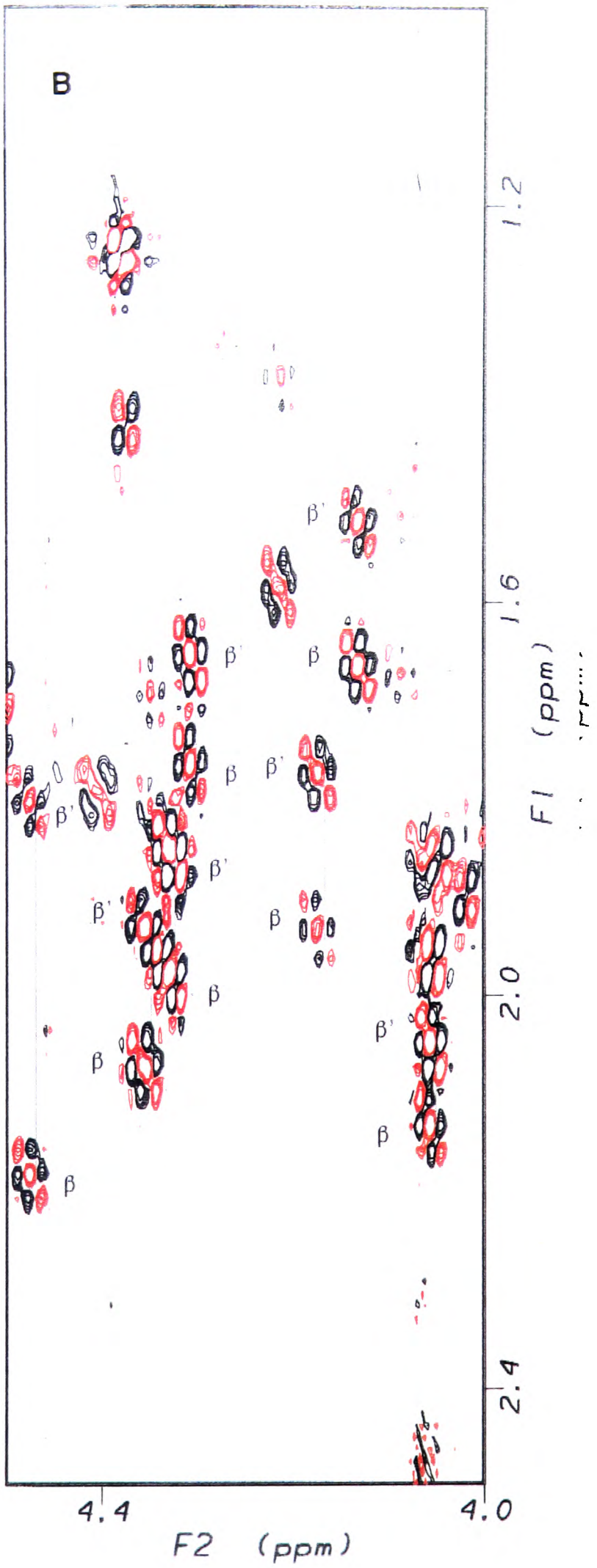
$$^3J_{\alpha\beta} = [9.5\cos^2(\theta) - 1.6\cos(\theta) + 1.9]$$

where θ is χ^1 for the β3 proton, $\chi^1 - 120^o$ for the β2,

(ii) the intraresidue NOE connectivities $d_{\alpha N}(i,i)$, $d_{\alpha\beta2}(i,i)$, $d_{\alpha\beta3}(i,i)$, $d_{\beta2N}(i,i)$ and $d_{\beta3N}(i,i)$, and

(iii) the sequential NOE connectivities $d_{\beta2N}(i,i+1)$, $d_{\beta3N}(i,i+1)$, $d_{\alpha N}(i,i+1)$ and $d_{NN}(i,i+1)$.

Uncertainty estimates may be included corresponding to each data entry. The values for ϕ, ψ and χ^1 that are consistent with the input data are calculated and then compared with entries in a database. Two databases from which to choose are provided with STEREOSEARCH, and an understanding of the derivation of each is critical to intelligent use



Each crosspeak in the PE-COSY spectrum consists of two antiphase squares which are split by the passive coupling in F_2.

of the program. The first of these is a *crystallographic* database with entries derived from 34 high resolution crystal structures found in the Brookhaven databank. While conformations representing small deviations from ideality are included in this database, the number of entries it includes is very limited, and its use may lead to artificially precise results. The other database is a *systematic* one that was generated by varying the torsion angles of the central residue of a model tripeptide with idealised geometry in 10° increments. It should be noted that this database has the advantage that all the possible conformations in ϕ, ψ and χ^1 torsion angle space are searched. One might expect this database, which contains many more entries than the crystallographic one, would be the more likely to yield accurate, unbiased results. However, this database is only as accurate as the model used to generate it. The model tripeptide is truncated insofar as the first residue comprises only a carbonyl group and the third only an amide group. The central residue is a serine which was chosen because it has the smallest non-glycine side chain. One can appreciate that this model provides only a crude approximation of the conformational environment of residues in a compact protein structure. Thus the output of the program STEREOSEARCH must be examined carefully before it is incorporated into structure calculations in the form of experimental restraints.

In many instances stereospecific assignments of β-methylene protons can be obtained from a qualitative analysis of NOE and coupling constant data alone (Hyberts, *et al.*, 1987; Arseniev, *et al.*, 1988). Therefore it is advisable to confirm that STEREOSEARCH results are consistent with this interpretation. The variation of $^3J_{\alpha\beta 2}$ and $^3J_{\alpha\beta 3}$ with χ^1 as defined by the Karplus equations is shown graphically in Figure 4.5. Of the three classical staggered rotamer positions, $\chi^1 = 60°$, $\chi^1 = 180°$ and $\chi^1 = -60°$, it is clear from this graph that only $\chi^1 = 60°$ can be

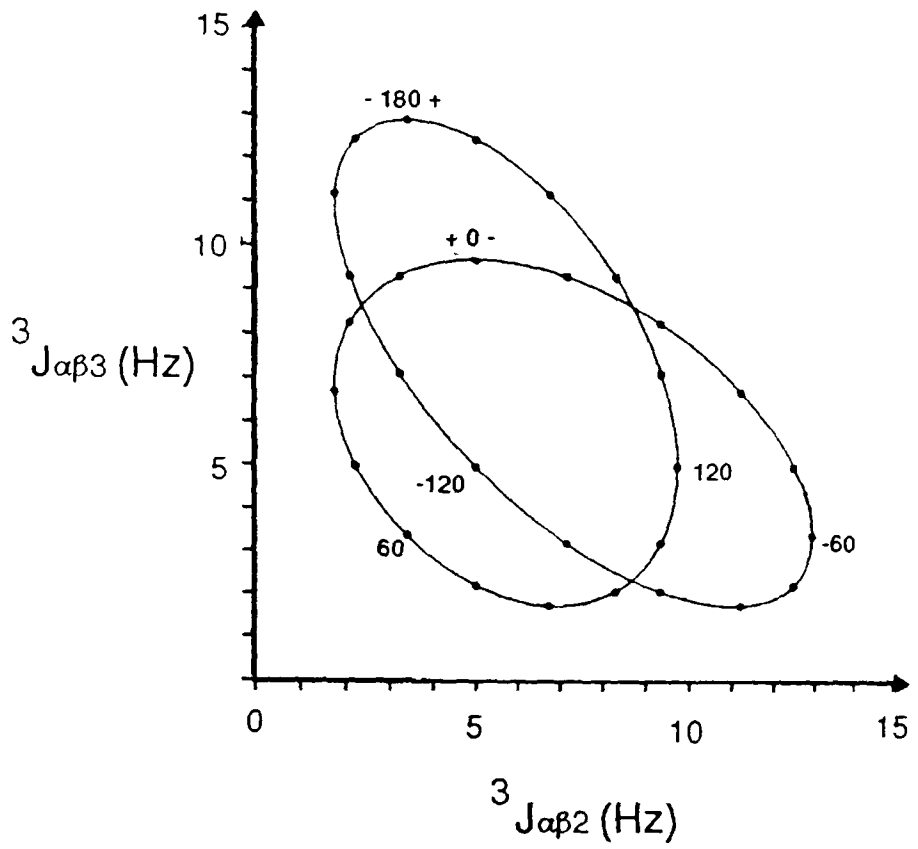
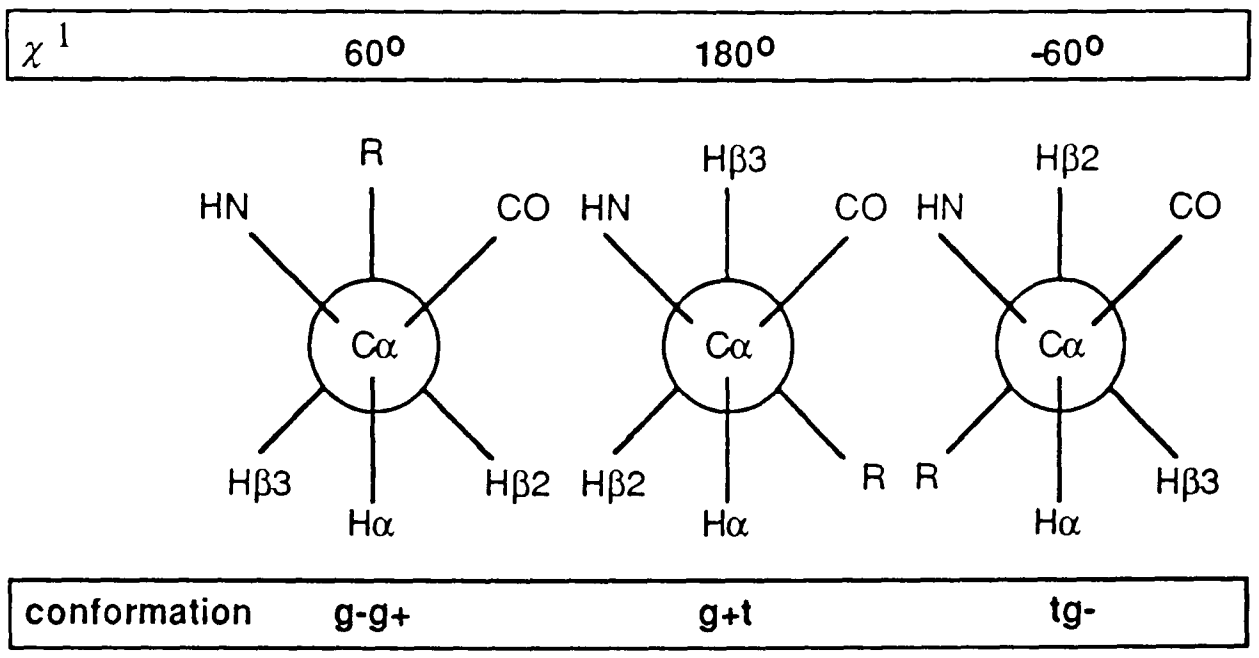


Figure 4.5

Variation of the coupling constants $^3J_{\alpha\beta2}$ and $^3J_{\alpha\beta3}$ with dihedral angle χ^1 as defined by the Karplus equations (Karplus, M., 1963).

distinguished unambiguously based on coupling constant data alone. In this case both $^3J_{\alpha\beta 2}$ and $^3J_{\alpha\beta 3}$ will be less than 5 Hz. If one of these coupling constants is greater than 10 Hz and the other is less than 5 Hz χ^1 is either 180° or -60°, and NOE distance information is required to distinguish between these two conformations. If one or both of the $^3J_{\alpha\beta}$ coupling constants in the range 5 Hz < $^3J_{\alpha\beta}$ < 10 Hz it is likely that the side chain in question is not locked in a unique conformation, i.e., it is in a dynamic equilibrium involving 2 or 3 of the staggered rotamers. This is particularly likely to be the case for residues whose side chains are highly solvent accessible. Note that while a statistical interpretation of STEREOSEARCH results has been advocated by Nilges *et al.* (1990), this approach should only be used to assign χ^1 for those side chains with $^3J_{\alpha\beta}$ coupling constants that are <u>not</u> in the range 5 Hz < $^3J_{\alpha\beta}$ < 10 Hz.

The variation in interproton HN_i-$H\beta_i$ distances for the three staggered rotamer conformations with χ^1 is shown schematically in Figure 4.6. In the case of χ^1 = 180° both of the β-protons are equidistant from the amino proton, however if χ^1 = -60°, one will be closer than the other. Consequently, the χ^1 = 180° conformation is characterised by 2 strong $HN_i/H\beta_i$ crosspeaks, and the χ^1 = -60° by 1 strong and 1 weak crosspeak. If the intraresidue $HN_i/H\beta_i$ crosspeak information is unresolvable, stereospecific assignment can sometimes be made based on sequential $H\beta_i/HN_{i+1}$ NOESY data. The variation in sequential interproton distance $d_{\beta N}(i, i+1)$ with backbone dihedral angle ψ is presented graphically in Figure 4.7. If ψ is in the allowed region from -165° to 60° one will observe 2 weak $H\beta_i/HN_{i+1}$ crosspeaks for the χ^1 = 180° conformation and 1 strong and 1 weak peak for χ^1 = -60°. The data upon which qualitative assignment of rotamer conformation is based is summarised in Table 4.1.



Hβ2	Hβ3	χ^1
g-	g+	60°
g+	t	180°
t	g-	-60°

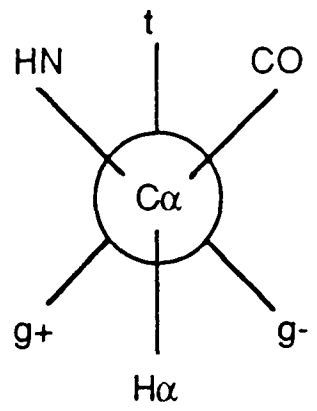


Figure 4.6

Newman projections corresponding to the three staggered χ^1 rotamer conformations. g and t refer to *gauche* or *trans* orientaion of the β-protons with respect to the α–proton, where β2 and β3 are defined according to the IUB-IUPAC convention. The relationship between the g+, g- and t positions and χ^1 angle is also shown.

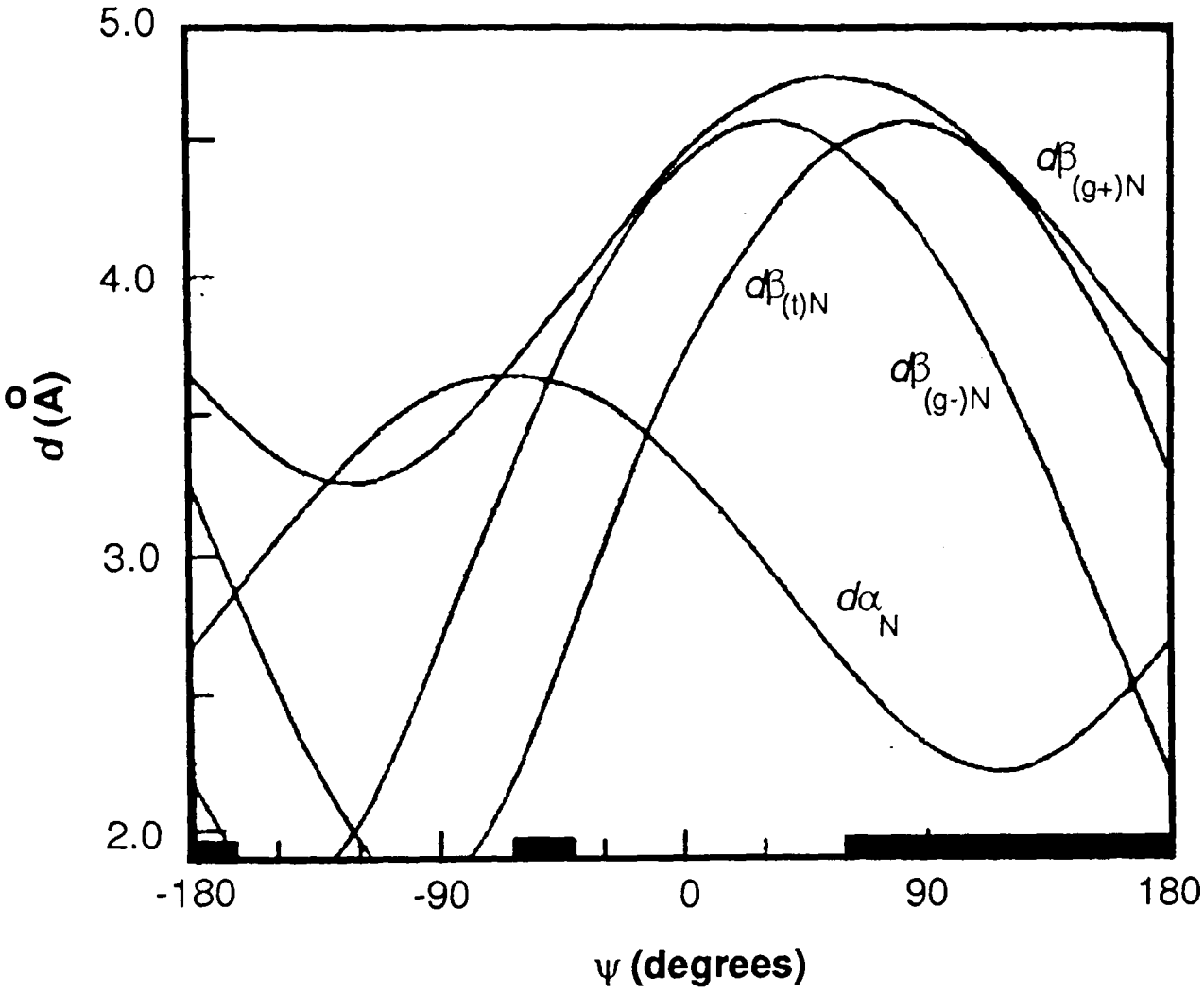


Figure 4.7

Dependence of the sequential distances from $H\alpha_i$, $H\beta_i(g+)$, $H\beta_i(g-)$ and $H\beta_i(t)$ to HN_{i+1} on the torsion angle ψ. The sterically allowed ranges for ψ are indicated by shaded bars at the bottom (reproduced from Arseniev *et al.*, 1988).

Table 4.1

Coupling Constant and NOE Data Used in Derivation of Stereospecific Hβ Assignments and χ^1 Torsion Angle Restraints

χ^1	60°	180°	-60°	
$^3J\alpha\beta_2$	< 5 Hz	< 5 Hz	> 10 Hz	
$^3J\alpha\beta_3$	< 5 Hz	> 10 Hz	< 5 Hz	
$H\alpha_i/H\beta2_i$	strong	strong	weak	
$H\alpha_i/H\beta3_i$	strong	weak	strong	
$HN_i/H\beta2_i$	weak	strong	strong	
$HN_i/H\beta3_i$	strong	strong	weak	
$H\beta2_i/HN_{i+1}$	strong	weak	weak	$(-165^o < \psi < 60^o)$
$H\beta3_i/HN_{i+1}$	weak	weak	strong	$(-165^o < \psi < 60^o)$

Over the course of structure refinement the selective weighting of torsion angle restraints is an effective means of pinpointing NOEs which have been assigned to the incorrect class of distance restraint as a consequence of spin diffusion effects. For example, if the dihedral angle force constant is weighted heavily with respect to the NOE force constant for ϕ restraints in well-defined regions secondary structure (based on all available n.m.r. data), misclassified interproton backbone restraints will usually be consistently violated in the resulting structures. Similarly overly constrained distances to stereospecifically assigned β-methylene protons are highlighted by the appropriate scaling of χ^1 torsion angle restraints.

4.2 t-PA F1 Structure Calculations

NOE connectivities assigned from 200 ms and 300 ms mixing time experiments were quantified by the counting of contours on 2-D plots of 50 ms and 200 ms experiments. These intensities were subsequently converted to three categories of distance restraints. Protons connected by strong, medium and weak NOEs were assigned upper distance limits of 2.5 Å, 3.5 Å, and 5.0 Å, respectively. The lower limit for all distance constraints was the sum of the van der Waals radii. The appropriate pseudoatom corrections were made to distances involving methyl protons or non-stereospecifically assigned methylene protons (Wüthrich *et al.*, 1983).

Hydrogen bonds in the β-sheet regions of the molecule that could be unambiguously assigned based on the qualitative analysis of amide exchange rates were included in the structure calculations. Each hydrogen bond was defined by two distance restraints, between the backbone carbonyl oxygen and the backbone amide proton (2.0 ± 0.3 Å)

and between the backbone carbonyl oxygen and the backbone nitrogen (3.0 ± 0.3 Å).

For eight residues with $^3J_{HN\alpha}$ coupling constants larger than 9.0 Hz, ϕ torsion angle restraints were defined as $-150^o < \phi < -90^o$. $^3J_{\alpha\beta}$ spin-spin coupling constants were measured from a 35° mixing pulse PE-COSY spectrum. Intraresidue and sequential NOE intensities were quantified from 50 ms mixing time NOESY data. These data were analysed to make qualitative stereospecific assignments. Then the program STEREOSEARCH (Nilges *et al.*, 1990) employing a systematic conformational grid search was used to confirm these assignments and to derive further ϕ, ψ and χ_1 torsion angle restraints for twenty-two residues. The minimum ranges employed for ϕ, ψ and χ_1 restraints were ±30°, ±50° and ±20° respectively (Kraulis *et al.*, 1989).

The final structure calculations were based on 723 interproton distance restraints including 197 sequential ($|i - j| = 1$), 66 short range ($1 < |i - j| \leq 4$), and 210 long-range ($|i - j| > 4$) restraints; 250 intraresidue restraints; 16 distance restraints for 4 backbone hydrogen bonds; and 43 dihedral angle restraints made up of 24 ϕ , 13 ψ, and 6 χ_1 restraints. The assignments of side chain protons were nearly complete, with the exception of the β-methylene protons of cys 34. Under no experimental conditions encountered in this study were intraresidue $C^{\alpha}H/H\beta$ or HN/Hβ NOEs observed for this residue. In the $^4F1.^5F1$ pair of type 1 modules from human fibronectin the chemical shifts for the Hβ protons corresponding to this cysteine were observed at 0.48, 1.59 ppm and 0.43, 1.43 ppm (Williams, 1992). The large displacement of these chemical shifts from random coil (~ 2 ppm) suggests that the cys 34 $C^{\alpha}H/H\beta$ and HN/Hβ resonances were broadened in the t-PA F1 spectra by chemical exchange effects. Few NOEs were observed involving protons near the C-terminus or the N-terminus, indicating a lack of

structural definition at the ends of the molecule. A complete list of the experimental restraints is included in Appendix II.

Three-dimensional structures were computed from the experimental restraints with the program XPLOR (Brünger *et al.*, 1987; Brünger, 1990). Sun-4 computers were used to perform all calculations, and the resulting structures were visualised using a Silicon Graphics 4D/20 graphics system with the program Insight (Biosym Technologies).

Initial structures were generated using a dynamical simulated annealing protocol (Nilges *et al.*, 1988a; Brünger, 1990) starting from randomly generated backbone configurations with extended side chains as outlined in Figure 4.8. The first phase consists of 9 ps of restrained molecular dynamics at 1000 K which is performed for initial minimisation. A very low weight is set on the van der Waals repulsive term such that atoms are allowed to pass each other easily. NOE and other distance restraints are incorporated using a "soft-square" potential with a harmonic plus linear switching function set to 0.5 Å above the upper bound. Before cooling the system to 300 K, the linear component of the distance restraint potential and the van der Waals repulsive term are increased over 9 ps. The temperature is reduced in steps of 25 K, then 200 steps of conjugate gradient minimisation complete the initial structure calculation. These structures were refined using successive rounds of an efficient simulated annealing protocol developed by T. S. Harvey based on that used in the hybrid method of Nilges *et al.* (1988b). Compared with the hybrid method of Nilges *et al.* (1988b), this protocol relies on the use of the SHAKE algorithm to maintain bond lengths (Ryckaert *et al.*, 1977), thus reducing high frequency vibrations in the system. This allows the use of a long integration timestep (here 4 fs). A further feature is the rapid cooling to 300 K from 1000 K using the method of Berendsen (Berendsen *et al.*, 1984). This was found to be faster than

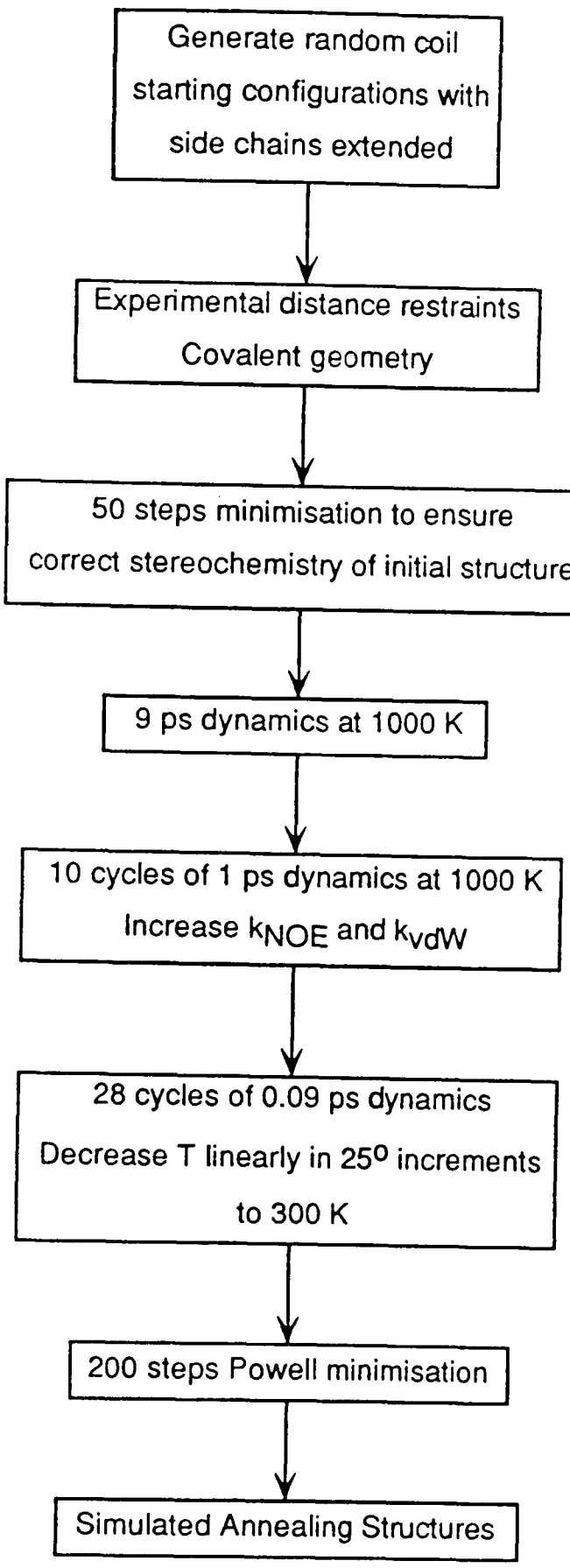


Figure 4.8

Flow chart illustrating the steps involved in calculation of the t-PA F1 structures by simulated annealing from random coil starting configurations.

the original slow cooling routine, whilst producing comparable results. The XPLOR protocols used in structure calculations are included in Appendix III.

A total of 85 refined simulated annealing structures converged from 100 random coil configurations. All 85 SA structures showed good covalent geometry and nonbonded contacts and satisfactory agreement with experimental distance restraints, as indicated by a low value of the NOE energy term, $F_{NOE} < 400$ kJ·mol^{-1}. Of these, 28 SA structures were selected based on agreement with the experimental data, with the cut-off taken at $F_{NOE} < 110$ kJ·mol^{-1}. These are shown in Figure 4.9, best-fitted on the backbone atoms of residues 3-47.

The selection of the residue range(s) that are used in structure superimposition and that are included in calculations of r.m.s deviation of atomic coordinates is of significant importance because the spread of the family of n.m.r. structures is routinely used to estimate the precision of the structure determination. Fewer NOEs are observed for more flexible regions of a protein due to faster exchange of amide protons and chemical exchange effects. Also, residues with defined secondary structure are, on average, within the distance observable in a NOESY experiment (~ 5 Å) from a greater number of residues than those residues with less well-defined structure. Figure 4.10 shows the number of short range ($1 < |i - j| \leq 4$) and long range ($|i - j| > 4$) NOEs used in the t-PA F1 structure calculations plotted as a function of residue number. Also shown is the r.m.s. deviation in atomic coordinates by residue number. While intraresidue, sequential and short range NOEs are relatively evenly distributed over the molecule, certain regions (notably at the N- and C-termini, and also residues 26-28, which are included in the 8:8 turn at the end of the major β-sheet) lack long range distance restraints. A good correlation between the number of experimental

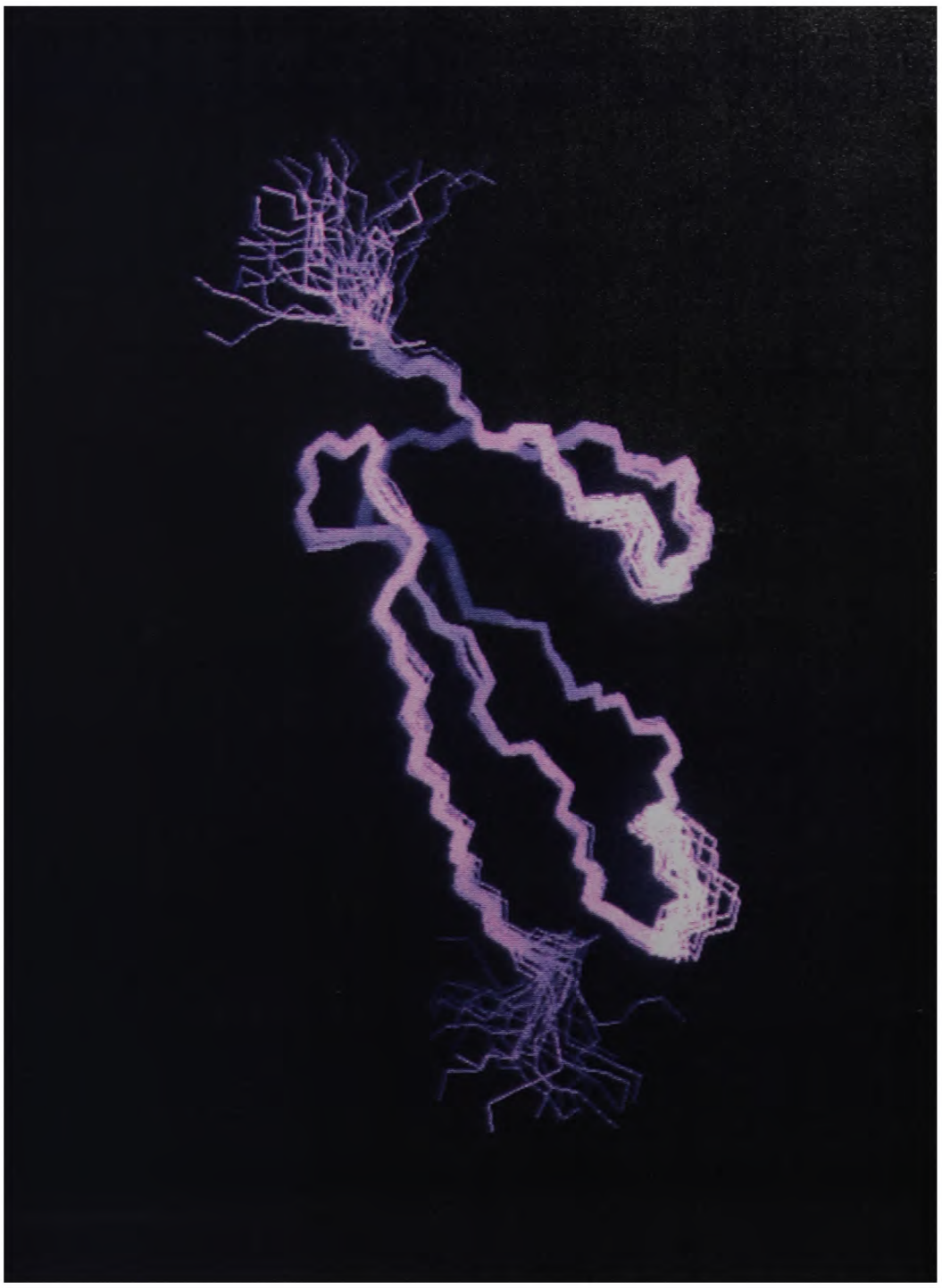
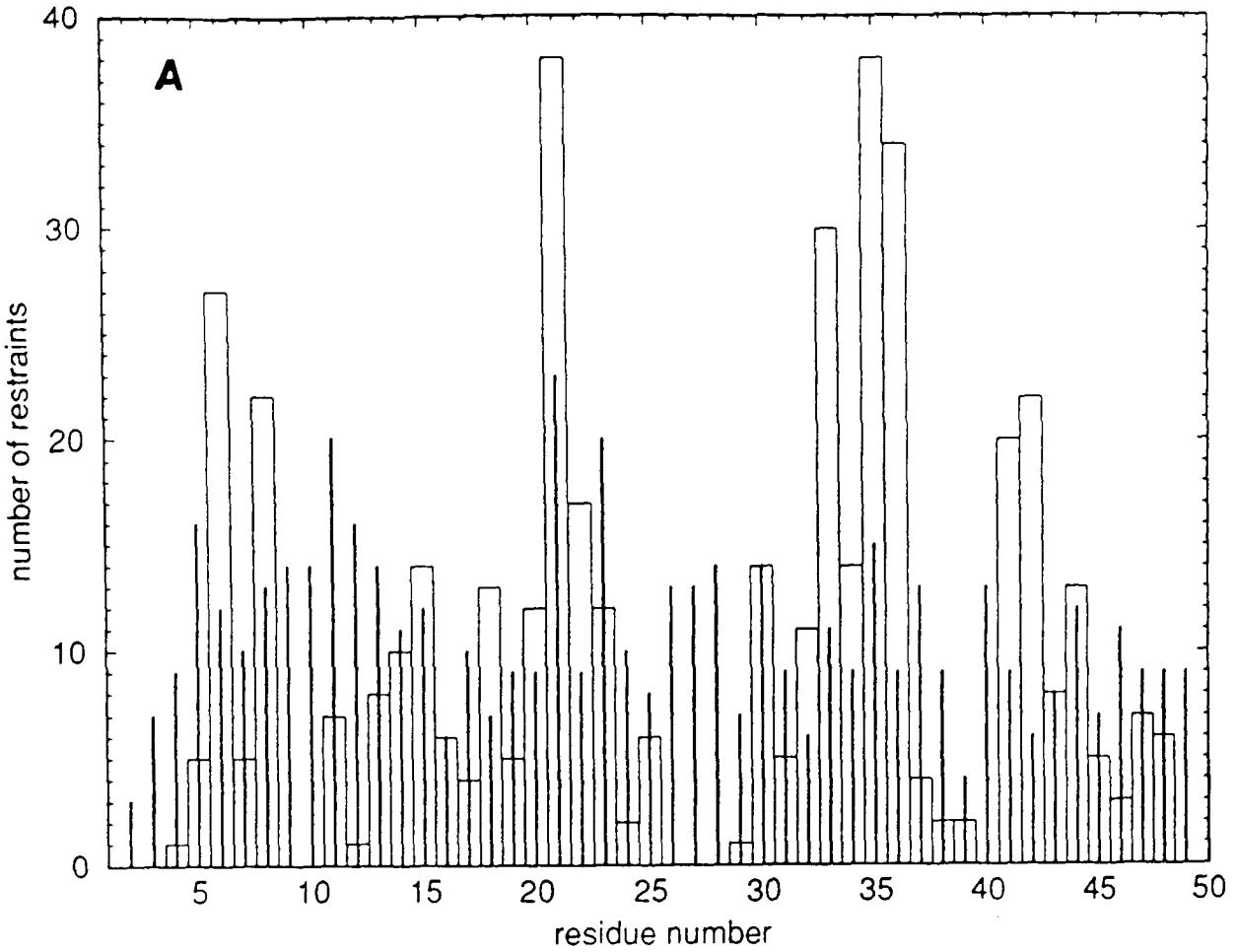


Figure 4.9

Best fit superposition of the backbone (N, C^{α} and C) atoms of the 28 final refined simulated annealing structures of t-PA F1.



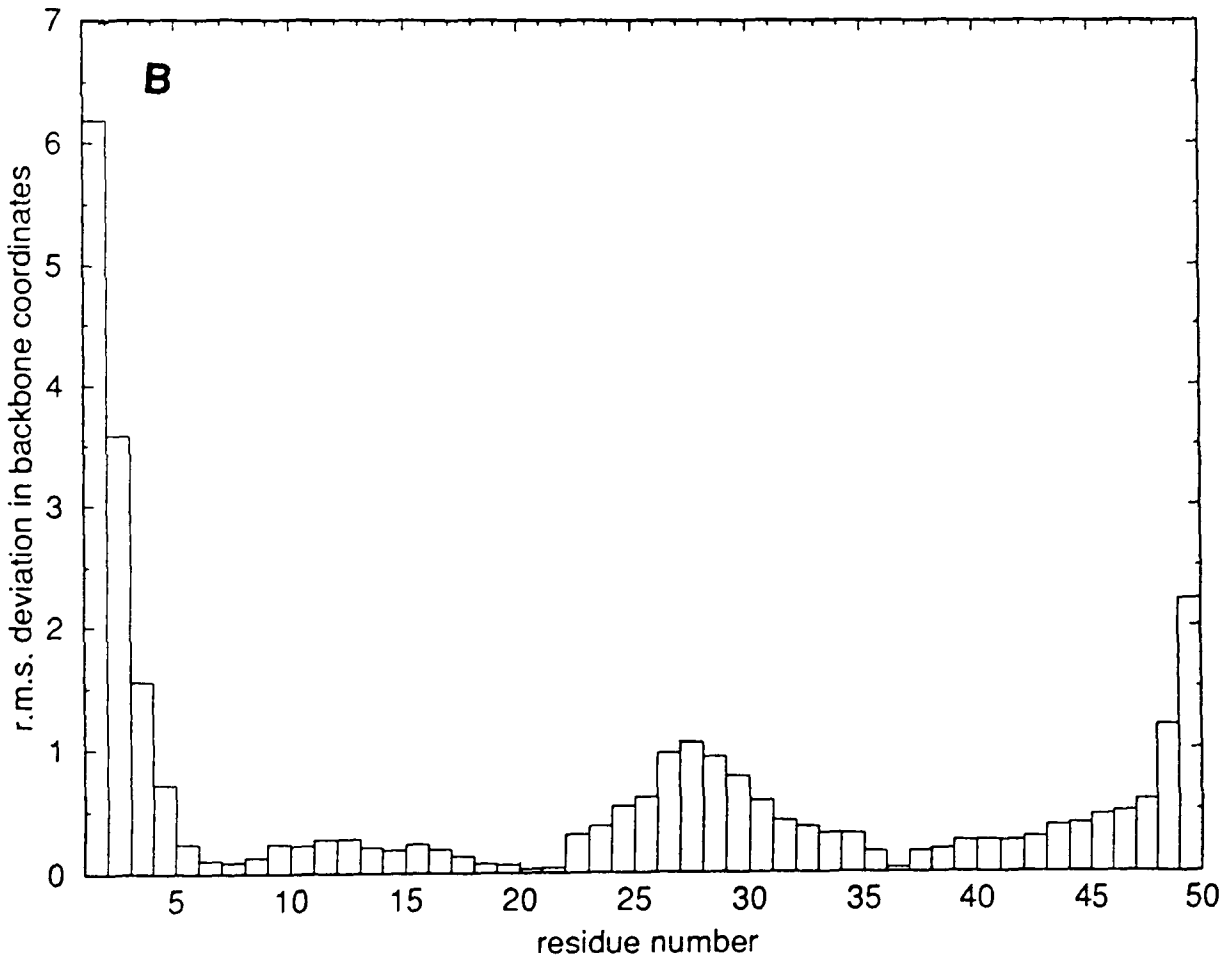


Figure 4.10

All short range ($1 < |i - j| \leq 4$) and long range ($|i - j| > 4$) NOEs (A), and the r.m.s. deviation in atomic coordinates (B) plotted as a function of residue number. In A the numbers of short and long range NOEs are indicated by a line and a bar, respectively.

restraints per residue and the precision of atomic coordinates is apparent. Hence contiguous residues manifesting the highest r.m.s. deviations in atomic coordinates were selectively excluded in structure superposition and from subsequent calculations.

A mean structure was calculated from the 28 individual best-fitted structures by coordinate averaging. The covalent geometry of the mean structure was regularised by restrained minimisation as in the last stage of the refinement. This restrained minimised average structure $(SA)_r$ satisfies the experimental restraint data as well as any of the individual SA structures.

A summary of the structural statistics for the individual SA structures and for the $(SA)_r$ structure is presented in Table 4.2. The data demonstrate that the structure of the t-PA F1 module is well defined. The overall atomic rms difference between the individual structures and the mean coordinate positions is 0.41 ± 0.13 Å for the backbone atoms (residues 4 - 47) and 0.98 ± 0.14 Å for all heavy atoms (residues 3 - 48). All of the chosen structures satisfy the n.m.r. restraints within the experimental error, assumed to be at least ±0.5 Å, with no restraint violations over 0.3 Å and no torsion angle violations greater than 2°. Deviations from idealised geometry are small, and the negative value of -570 $kJ \cdot mol^{-1}$ for the Lennard-Jones energy (which is not used in the target function for the simulated annealing calculations) indicates that nonbonded contacts are good.

Analysis of the coordinates of protein structures deposited in the Brookhaven databank has highlighted a correlation between stereochemical quality and resolution. Specifically, as the resolution improves, the distribution of ϕ,ψ and χ^1 torsion angles becomes more clustered (Morris *et al.*, 1992). Figure 4.11 illustrates the distribution of backbone ϕ and ψ angles for the 28 SA t-PA F1 structures. All backbone

Table 4.2

Structural Statistics for the t-PA F1 Module[a]

	<SA>	$(SA)_r$
RMS deviations from exptl distance restraints (Å)[b]		
all (723)	0.031±0.002	0.025
interproton distances		
intraresidue (250)	0.030±0.003	0.025
sequential (197)	0.028±0.004	0.021
interresidue short range ($1 < \|i-j\| \leq 4$) (66)	0.032±0.009	0.024
interresidue long range ($\|i-j\| > 4$) (210)	0.037±0.003	0.030
H-bonds (16)[c]	0.012±0.007	0.012
RMS deviations from exptl dihedral restraints (deg) (43)[b,d]	0.25±0.07	0.17
Deviations from idealized covalent geometry		
bonds (Å)	0.0096±0.0005	0.009
angles (deg)	2.229±0.006	2.228
impropers (deg)[e]	0.941±0.012	0.935
F_{bond} (kJ.mol^{-1})	160.1±6.1	149.6
F_{angle} (kJ.mol^{-1})	4719.3±27.5	4712.2
$F_{improper}$ (kJ.mol^{-1})[e]	214.2±5.4	210.4
F_{NOE} (kJ.mol^{-1})[f]	95.6±8.5	60.6
F_{repel} (kJ.mol^{-1})[g]	71.6±13.7	55.3
F_{VDW} (kJ.mol^{-1})[h]	-570.3±31.9	-576.4

[a] The notation of the structures is as follows: <SA> are the 28 refined simulated annealing structures; $(SA)_r$ is the restrained minimised mean structure, where the mean structure was obtained by averaging the coordinates of the individual <SA > structures best fitted to each other. The number of terms for the various restraints is given in parentheses.

[b] None of the structures exhibit distance violations greater than 0.3 Å or dihedral violations greater than 2 °.

[c] Each hydrogen bond is characterized by two distance restraints: r_{NH-O}, 2.1-2.3 Å and r_{N-O}, 2.1-3.3 Å. All hydrogen bonding restraints involve slowly exchanging backbone amide protons and were only included after they could be unambiguously assigned following initial structure calculations.

[d] The torsion angle restraints comprises 24 ϕ, 13 ψ and 6 χ^1 angles.

[e] The improper torsion restraints serve to maintain planarity and chirality.

[f] The value of the square-well NOE (F_{NOE}) is calculated with a force constant of 210 kJ.mol^{-1}.Å^{-2}.

[g] The value of the quartic van der Waals repulsion term F_{rep} is calculated with a force constant of 17 kJ.mol^{-1}.Å^{-4} with the hard sphere van der Waals radius set to 0.8 times their standard values.

[h] The van der Waals energy F_{VDW} is calculated with the CHARMM (Brooks *et al.*, 1983) empirical energy function and is *not* included in the target function for simulated annealing or restrained minimisation.

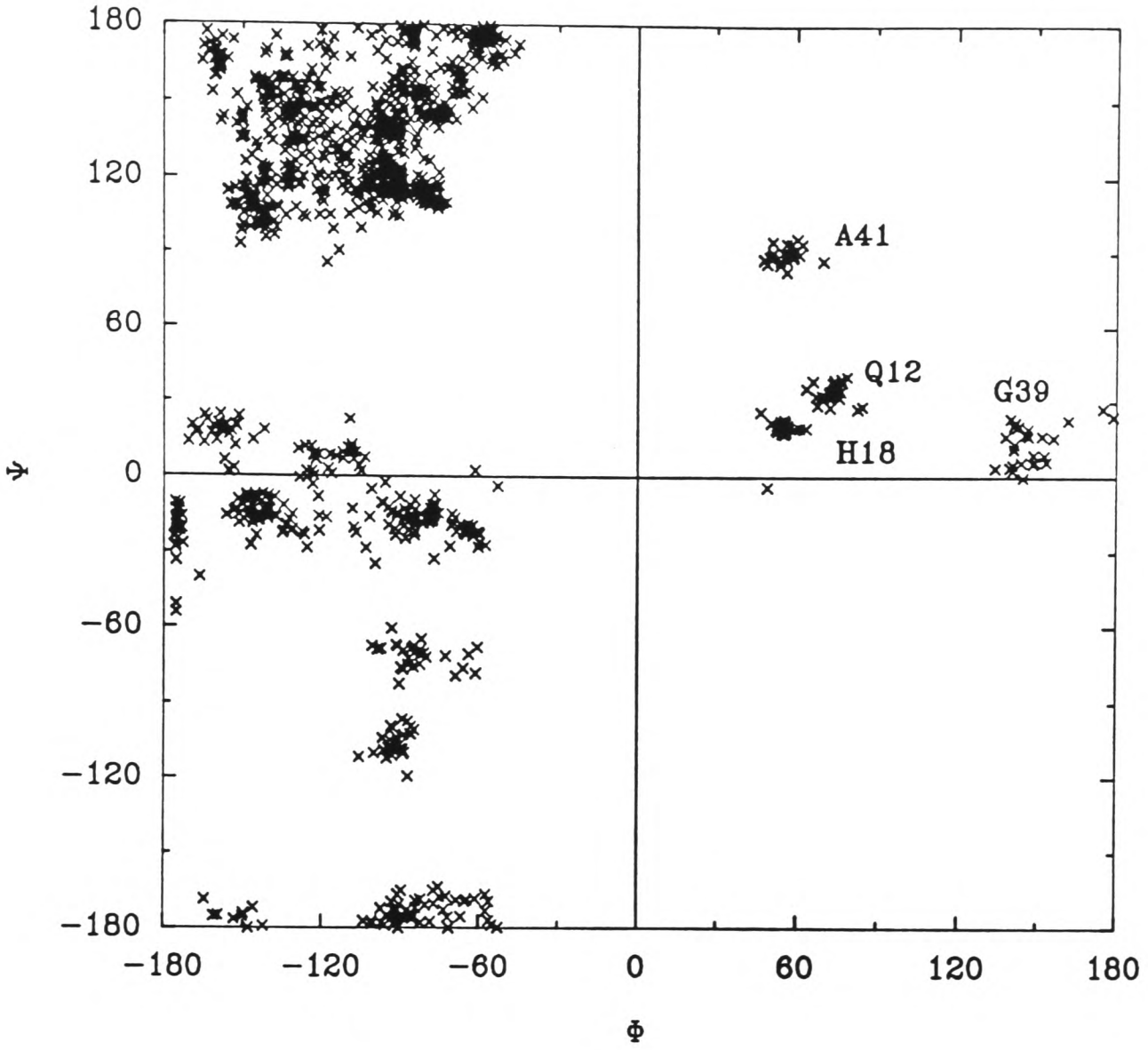


Figure 4.11

Ramachandran ϕ, ψ plot for the 28 structures (residues 4 to 47). The region of the plot corresponding to positive ϕ, ψ angles is labelled by residue. All residues with positive ϕ angles lie in turns.

ϕ and ψ torsion angles fall into allowed regions of the plot as defined by Morris *et al.* (1992). Furthermore, the torsion angles of each residue are well-defined and reflect the high resolution of the structure. It is interesting to note that the non-glycine residues with positive ϕ, gln 12, his 18, and ala 41, all lie in turns and all correspond to conserved glycines in the sequence of the seventh type 1 module found in human fibronectin for which positive ϕ and ψ torsion angles are allowed.

4.3 Description of the Structure of the t-PA F1 Module

The structure of the t-PA F1 module is presented schematically in Figure 4.12. As predicted, the topology of the module is dominated by two regions of antiparallel β-sheet which are held together by two disulphide bridges, cys 6-cys 36 and cys 34-cys 43. Residues cys 6 to arg 8 form a short, well defined double-stranded β-sheet with residues met 13 to tyr 15. The hydrogen bond between residues 8 and 13 precedes a 4:4 turn (Sibanda *et al.*, 1989) connecting these two strands, with gln 12 in place of the glycine normally found at position 4. The double stranded β-sheet is linked to a triple-stranded β-sheet by a second tight turn formed by residues gln 16 to gln 19. This turn closely resembles a 2:2 turn with a hydrogen bond connecting the carbonyl oxygen of gln 16 to the amide proton of gln 19. This hydrogen bond was not restrained in structure calculations because the $C^{\alpha}H$/HN resonance for gln 19 is not resolved in the experiment performed to identify slowly exchanging amide protons. The triple-stranded β-sheet, formed by residues ser 20 to pro 24, val 31 to trp 35, and gln 42 to val 46, is well defined by the NOE restraints, and forms with only two pairs of residues restrained by hydrogen bonds. The three strands are connected by two loops formed by residues pro 24 to val 31 and trp 35 to gln 42. Few NOE

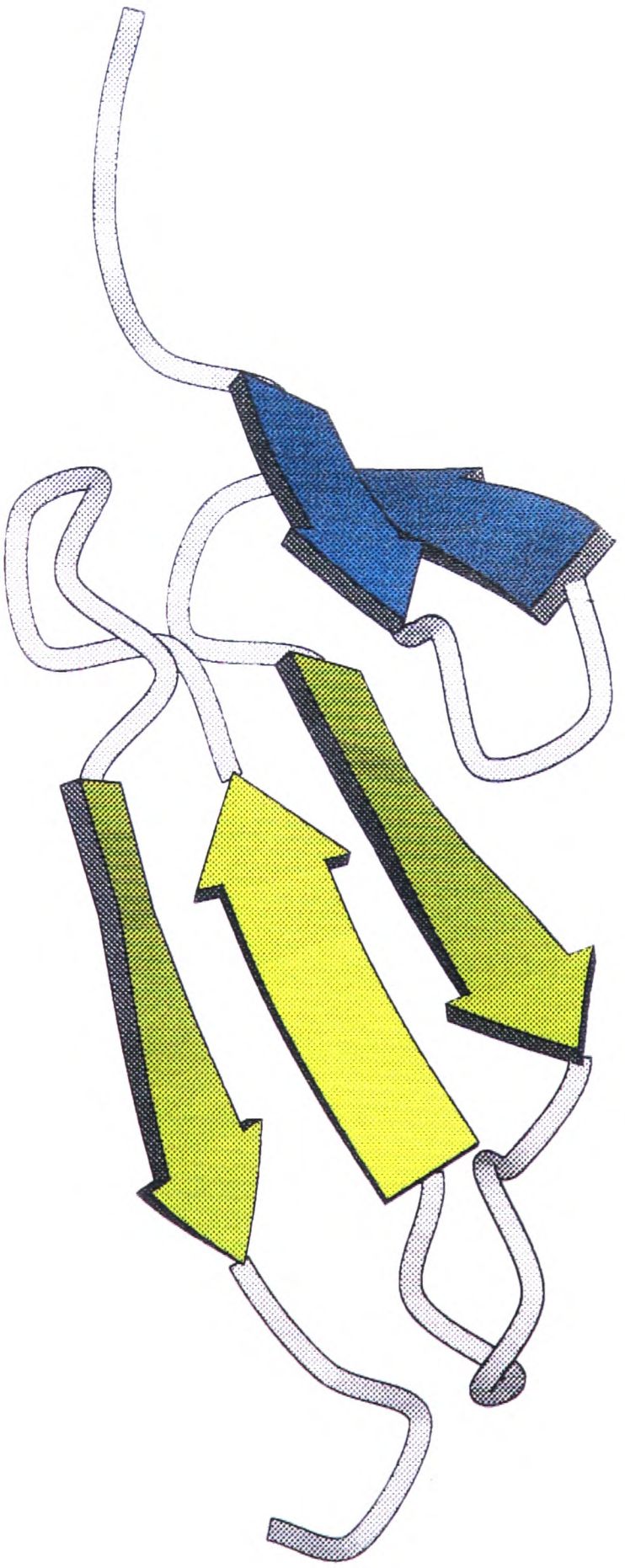


Figure 4.12

Schematic representation of the 3-dimensional structure of t-PA F1. Arrows indicate strands of β-sheet connected by lines that pass through the exact C^{α} positions of the minimised average structure (Kraulis, P. J., 1991).

restraints were found in the region of the first wide 8:8 residue loop, most probably due to conformational flexibility in this region.

4.4 Conclusions

The intensities of NOE crosspeaks in 50 ms and 200 ms mixing time experiments were approximately quantified and classified into three categories of distance restraint: short ($r_{ij} \leq 2.5$ Å), medium ($r_{ij} \leq 3.3$ Å) and long ($r_{ij} \leq 5.0$ Å). The appropriate pseudoatom corrections were applied to these distances. Qualitatively assigned ϕ torsion angle restraints based on $^3J_{HN\alpha}$ coupling constant data as well as hydrogen bond restraints were also included in structure calculations. Simulated annealing structures were calculated iteratively with more experimental restraints included in each successive round of calculations. These structures were energy minimised and further refined by the incorporation of additional torsion angle restraints. These restraints resulted from a qualitative analysis of coupling constant and NOESY data which was corroborated quantitatively by the program STEREOEOSEARCH (Nilges *et al.*, 1990).

The final 28 structures were calculated using 723 interproton restraints and 43 dihedral angle restraints. Considerable attention has been given to the correlation between precision and accuracy of protein structures and the number of experimental restraints per residue (Gronenborn & Clore, 1990; Clore and Gronenborn, 1991a). In order to standardise the assessment of protein structures, a categorisation into four classes of increasing quality has been introduced by Clore & Gronenborn (1991b). In the calculation of the structure of the t-PA F1 module, 766 restraints were used to define the positions of residues 3 - 48, an average of over 16 restraints per residue. Therefore, on the basis

of their criteria, the structure of the t-PA F1 module falls into the fourth-generation category, with errors in atomic coordinates comparable to those of a 2 to 2.5 Å resolution x-ray structure. The n.m.r. data on which the restraints were based were of high quality. A number of broadened and overlapped peaks gave evidence of motional averaging on a millisecond time scale, but the cause is not clearly evident in the structure.

The 3-dimensional topology of the t-PA F1 module is comprised of a double-stranded β-sheet overlaying a triple-stranded β-sheet. These two regions are held together by the disulphide bond linking residues cys-6/cys-36 and hydrophobic interactions. The structure is in close agreement with its prediction based on the structure of the seventh type 1 module from human fibronectin.

Chapter 5

Structural Significance of the t-PA F1 Module

5.1 Introduction

Once the structure of the t-PA F1 module was completed, it was possible to compare it to the previously calculated seventh type 1 module from human fibronectin (Baron *et al.*, 1990a). Of the 14 residues that are highly conserved in human fibronectin finger type modules (including the seventh type 1 module), only eight are conserved in t-PA F1. Given this limited degree of sequence homology between the two finger modules, the extent of their structural similarity is an indication of the general value of consensus structures in the study of protein modules (Campbell & Baron, 1991). Comparison of the two structures was also useful because it provided insight into possible differences in the intact structures of human fibronectin and tissue-type plasminogen activator.

A well recognised advantage of n.m.r. over x-ray crystallography in the study of protein structure is that the difficult problem of crystallising

the protein sample is avoided. However due to the decrease in the molecular tumbling rate, which is related to the molecular correlation time, with increasing molecular weight it is impossible to investigate the structures of large proteins (approximately 200 amino acids or larger) using this solution technique. This limits the use of n.m.r. to the structural investigation of small proteins such as modules or pairs of modules. Frequently n.m.r. techniques are employed to study specific domains of larger proteins which have not been successfully crystallised. Yet once the structures of all of the individual domains of a protein have been determined, the question remains how these subunits fit together to form the intact protein. In the absence of other high resolution structural data the only method available to address this question is protein modelling.

Intact t-PA is an ideal candidate for protein modelling for a number of reasons. It is a modular protein composed of five independently folding domains: a finger (F1), an EGF-like module (G), two kringles (K1, K2) and a serine protease domain (SP). There are few amino acids linking the domains which implies relatively little guesswork is involved in modelling the regions connecting them. The structures of the F1 and K2 modules of t-PA have been determined experimentally, and consensus structures which can be used to model the remaining three domains are known. In addition, due to the important biological role that this protein plays, a wealth of experimental data has been published which gives insight into the relative orientation of the domains. Homology modelling methods have been described in detail (reviewed in Swindells & Thornton, 1991) and will only be referred to in the context of construction of the model of intact t-PA.

The model of intact t-PA was used to explore the possible spatial orientation of carbohydrate side chains in wild type t-PA and to explain observed differences in biological activity between the two naturally

abundant glycosylated variants of the molecule. These are referred to as type I and type II. Both types are glycosylated at positions 117 and 448, but only type I has an additional carbohydrate at position 184. The oligosaccharides of t-PA have been studied in detail, and it has been shown that the glycan at residue 117 is a high-mannose one and the other two are complex (Pohl *et al.*, 1984; Vehar *et al.*, 1986). Furthermore, t-PA is expressed as a mixture of N-glycosylated variants, i.e., the carbohydrate side chains occupying the three sites are chemically variable. In order to clarify the following discussion of the modelling of intact t-PA the complete sequence, disulphide bonding pattern and glycosylation sites are presented schematically in Figure 5.1.

5.2 Comparison of the t-PA F1 and Fibronectin F1 structures

A stereoview comparison of the structure of the t-PA F1 module and the seventh type 1 module from human fibronectin is shown in Figure 5.2. The structural similarity of the two fingers is striking, particularly in light of their limited sequence homology. The fold for both molecules is the same with a short region of double-stranded β-sheet connected by a tight turn to a triple-stranded β-sheet. Wide turns connect the three strands of antiparallel β-sheet. When the two average minimised structures are overlaid using the C^{α}, C and N atoms of the 22 residues found in β-sheet regions, the atomic r.m.s. deviation is 1.1 Å. In addition, the positions and orientations of the side chains of consensus residues are virtually identical. Figure 5.3 illustrates two comparisons of side chain conformations in the two molecules. Of the three hydrophobic residues forming the hydrophobic core of the fibronectin finger module (tyr 17, trp 23 and trp 45) only two are conserved in t-PA F1, tyr 15 and trp 21. Figure 5.3 shows that the stacking interaction between the two

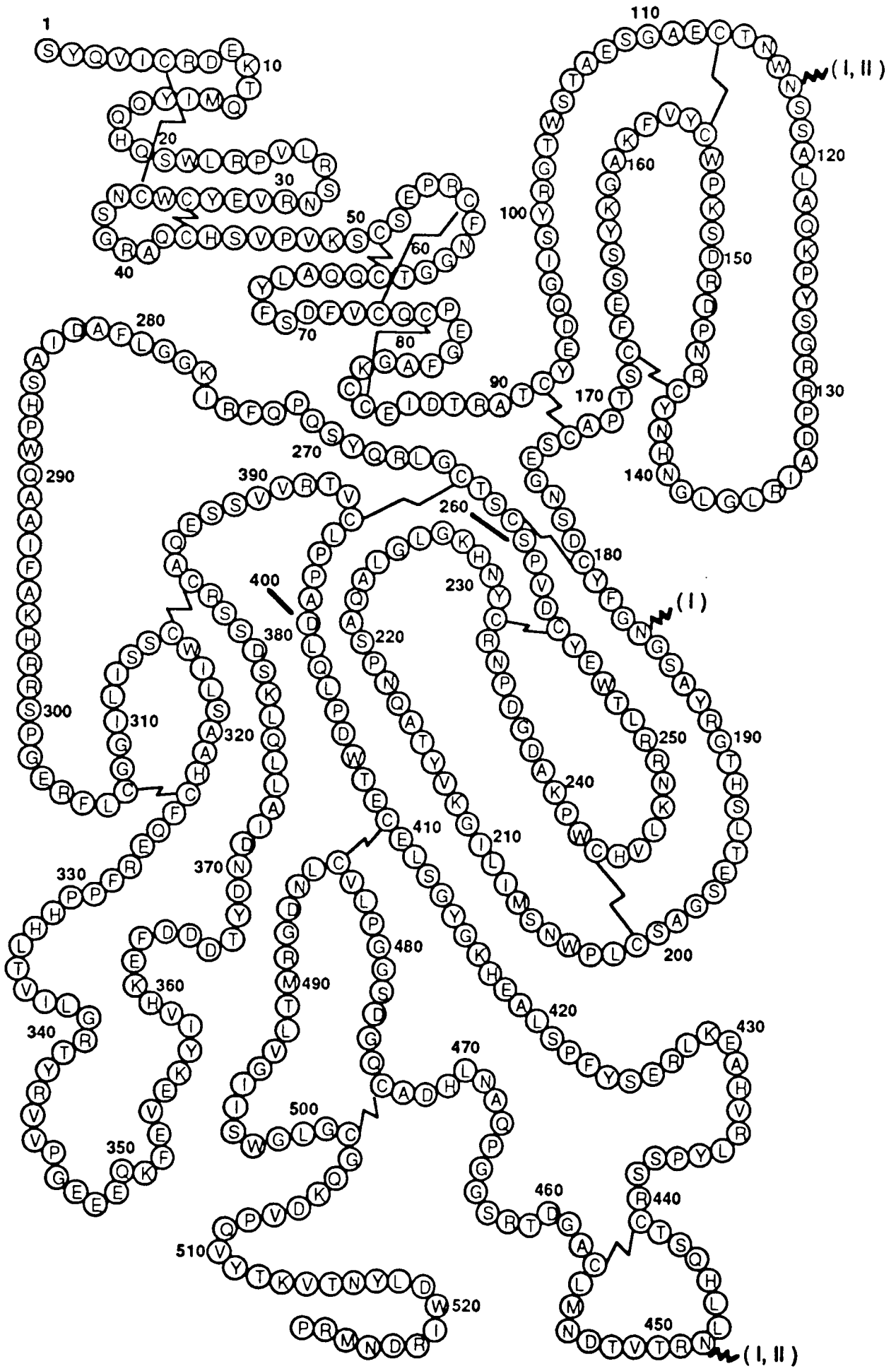


Figure 5.1

Complete sequence and disulphide bonding pattern of intact tissue-type plasminogen activator. Glycosylation sites and occupancy for the two glycosylated forms of the protein, types I and II, are indicated at positions 117, 184 and 448.

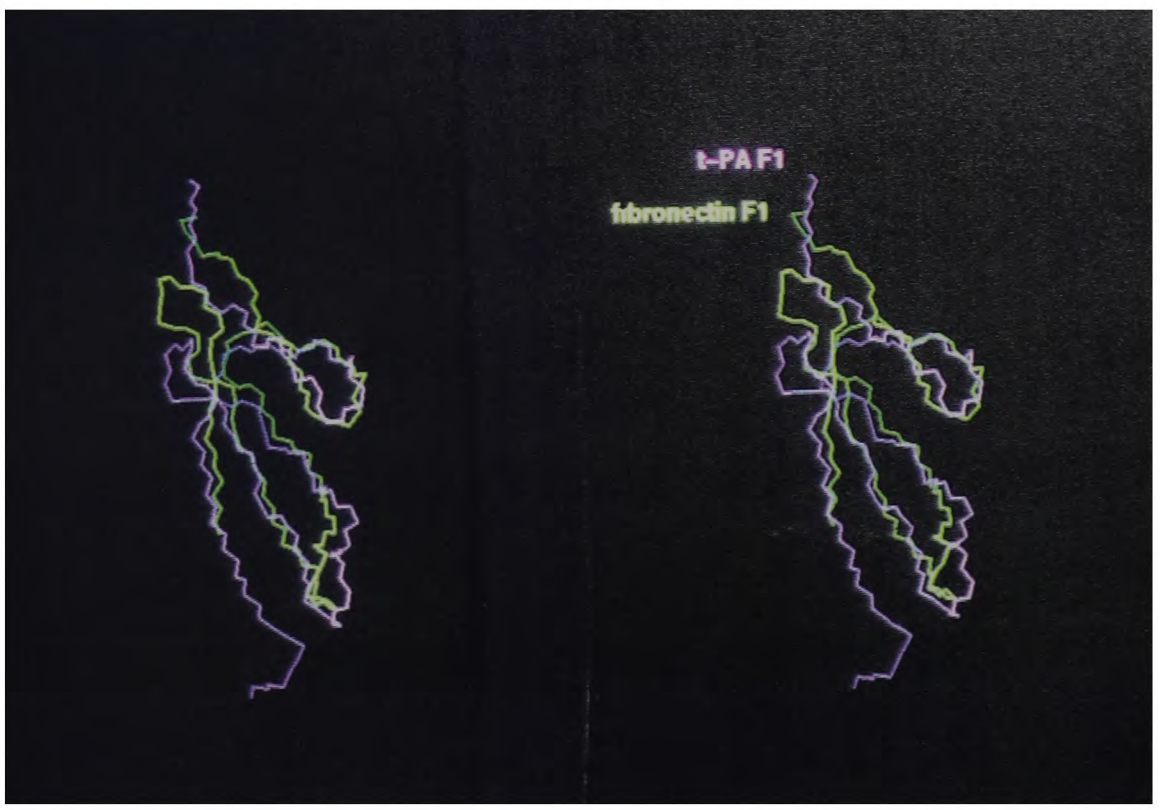


Figure 5.2

Stereoview superposition of the structures of the t-PA finger module and the seventh type 1 module from human fibronectin (Baron *et al.*, 1990a). The structures have been fit on the backbone (C, C^{α}, N) atoms of the 22 residues in conserved regions of β-sheet with an atomic r.m.s. deviation of 1.1 Å.

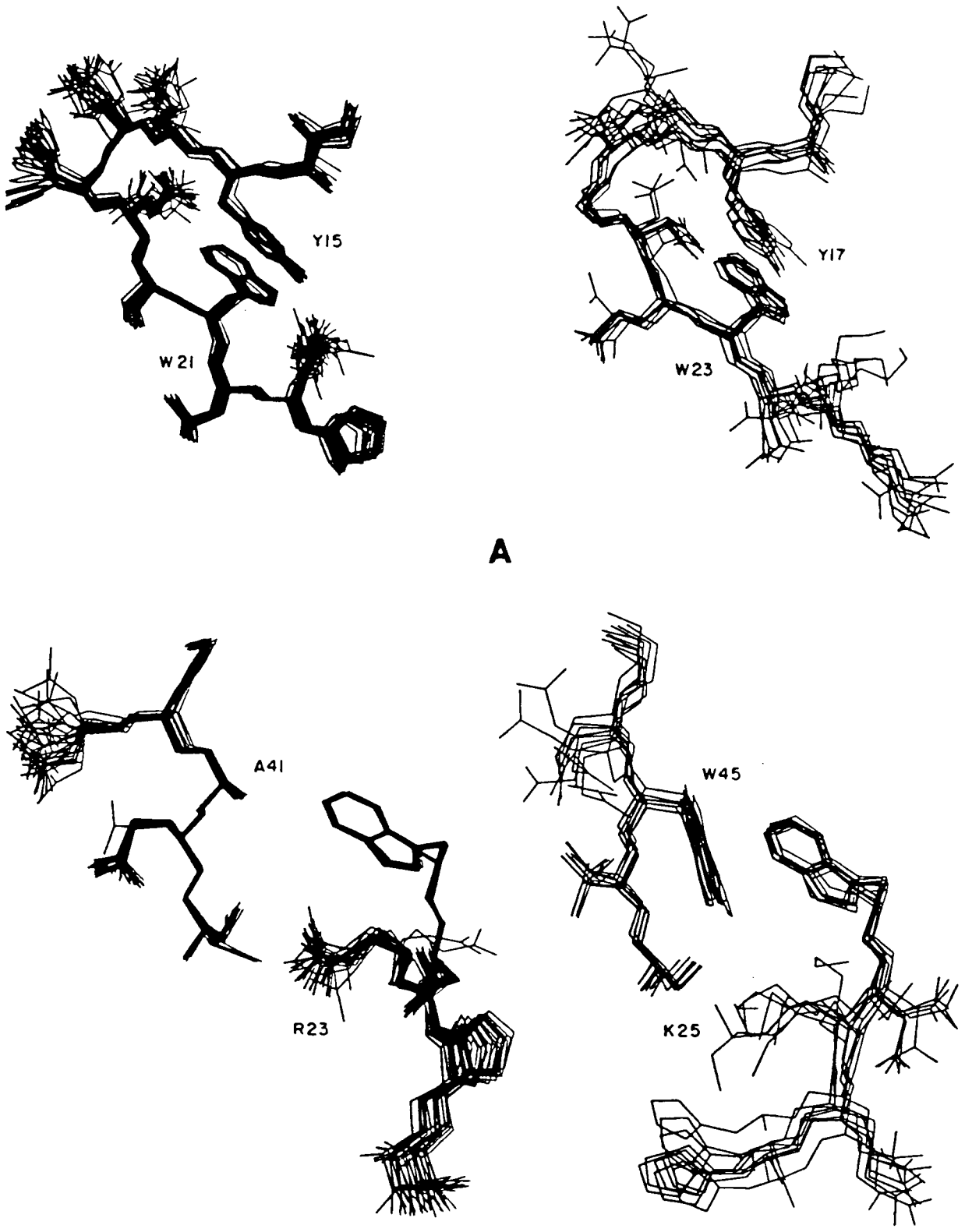


Figure 5.3

Selected regions of the 28 final t-PA F1 structures and the 10 final fibronectin type 1 module structures (Baron *et al.*, 1990a) highlighting structural similarities. The 2 molecules are best fit to each other based on conserved regions of secondary structure as in Figure 5.2. (A) Residues 14 to 24 of t-PA (left) and 16 to 26 of fibronectin (right) showing the similarity of the stacking interaction of the aromatic residues in the core of the structures. (B) Residues 19 to 23 and 39 to 43 of t-PA (left) and 21 to 25 and 43 to 47 of fibronectin (right) illustrating the similarity in side chain conformation of t-PA arg 23, ala 41 and fibronectin lys 25, trp 45.

residues is the same. The tryptophan in fibronectin that is not conserved in t-PA is replaced by an alanine. In spite of the size discrepancy between these two residues, their side chains are in the same orientation in the two molecules (Figure 5.3). This fact suggests that while trp 45 is well-defined in the fibronectin finger module, its position is not structurally critical.

Another residue of interest is lys 25 of the fibronectin F1 module, which is conservatively substituted by arg in t-PA F1. These hydrophilic side chains are directed towards the interior of the module, rather than directly into solution as would be expected (Figure 5.3). A plausible explanation for the anomalous orientation of these side chains might be that they form salt bridges with other residues. Both asp 8 and glu 32 are in close proximity to arg 23 in the t-PA finger. Similarly glu 13 is a likely candidate to form a salt bridge with lys 25 in the fibronectin finger. Since asp 8 in t-PA and glu 13 in fibronectin are in similar positions, if this ionic interaction is maintained in both fingers, it is likely that asp 8 will bridge with arg 23 in t-PA.

It has been suggested previously that the residues that are conserved in the finger consensus sequence tend to be those that are buried in the hydrophobic core, while those exposed to solvent are both variable and predominantly hydrophilic (Baron *et al.*, 1990a). However, the sequence of t-PA F1 is exceptional in both respects. The distribution of charged side chains in t-PA differs from that in fibronectin, with a high concentration of charged residues surrounding the cavity filled by trp 45 in the fibronectin finger structure. Conversely, the exterior surface of the triple-stranded β-sheet of t-PA has a large hydrophobic area, shown in Figure 5.4.

The difference in the topology of the two structures correlates well with experimental observations roughly defining the dimensions of the

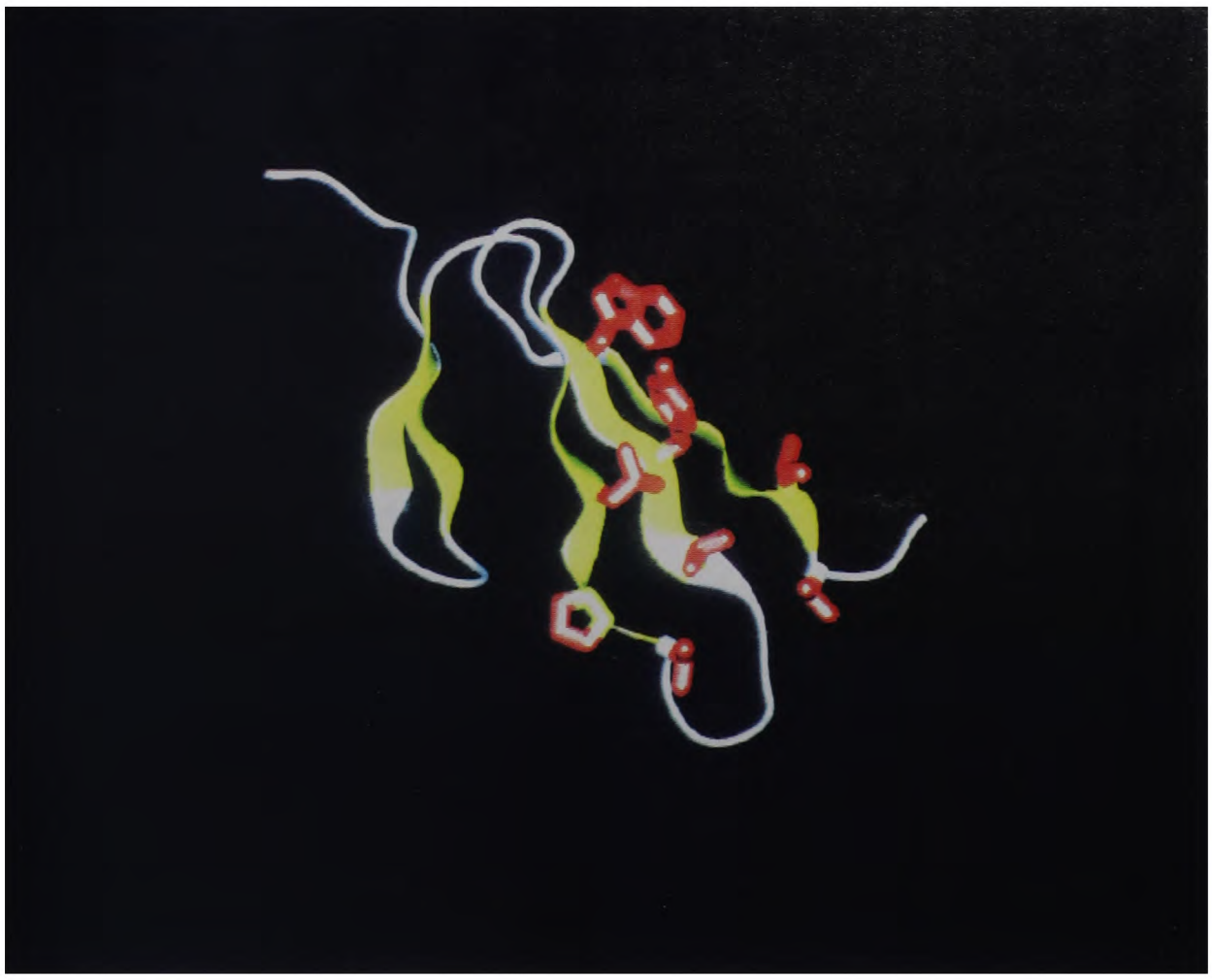


Figure 5.4

Schematic illustration of the t-PA F1 module displaying the exterior hydrophobic face of the triple-stranded β-sheet. Residues highlighted in red include leu 22, pro 24, val 25, val 31, tyr 33, trp 35, val 46 and val 48.

two intact proteins, human fibronectin and tissue-type plasminogen activator. The dimensions of a single F1 module are 1.7 nm x 1.6 nm x 3.2 nm (Baron *et al.*, 1990a). Dimensions of ~2-2.5 nm x 120 nm have been obtained by electron microscopy for the intact fibronectin dimer (Odermat *et al.*, 1982), which are consistent with an 'end-to-end' model for linking the type 1 structures together. Small angle x-ray scattering experiments performed on glycosylated two chain t-PA indicate that the maximum distance within the protein moiety of the molecule must be $\leq$ 89 Å which is consistent with compact arrangement of the five domains (Ponting, 1991). Therefore one may logically postulate that the reason for which the t-PA finger has a hydrophobic surface and the fibronectin finger does not is because the t-PA finger associates with other domains in the intact protein while the fibronectin finger does not engage in such interactions.

5.3 Modelling the Structure of Intact t-PA

The first step in construction of the model of intact t-PA was to predict structures of the domains which had not been determined experimentally. The modelling of the individual domains involved sequence alignment with homologous structures, amino acid mutation, and adjustment of insertions and deletions in the t-PA sequence relative to the sequences of the known structures. Energy minimisation was then performed on each module to refine the adjustments and to remove bad nonbonded contacts created by the amino acid mutations. All structural manipulations were performed using the program Insight (Biosym Technologies), and structures were energy minimised in a full CHARMM empirical energy potential (Brooks *et al.*, 1983) within the program XPLOR (Brünger *et al.*, 1990). The domains were subsequently fit

together based on a large number of experimental observations and subjected to energy minimisation.

5.3.1 Homology modelling of the G, K2 and SP domains

The structure of the EGF module from human epidermal growth factor (hEGF), which was previously determined in our laboratory (Campbell *et al.*, 1989), was selected as the structure upon which to base the model of the t-PA EGF-like module. An alignment of the two sequences and the consensus secondary structure for EGF-like modules is shown in Figure 5.5. Based on this alignment only one three-residue deletion in a loop of the hEGF structure was necessary. In modelling the t-PA G module from the hEGF structure, first all non-identical residues in the hEGF sequence corresponding to residues 48 to 90 in the t-PA sequence were mutated. Then the three amino acid deletion was made, and the gap was closed by energy minimisation.

A new sequence alignment for kringle modules was proposed by de Vos *et al.* (1992) based on the structure of the second kringle module of human t-PA. The alignment shown in Figure 5.5 gives evidence of the extensive sequence homology of the two kringles and that there are no insertions or deletions in the sequences relative to each other. Consequently the K1 module (residues 91 to 175) of human t-PA was easily modelled by amino acid mutation of the K2 structure and energy minimisation.

Modelling of the serine protease domain of t-PA proved to be challenging because a search of the protein sequence data bank identified several serine proteases with varying degrees of homology over the length of the sequence. From this list of serine proteases, three with known high-resolution ($\leq$ 2.0 Å) x-ray structures were chosen upon

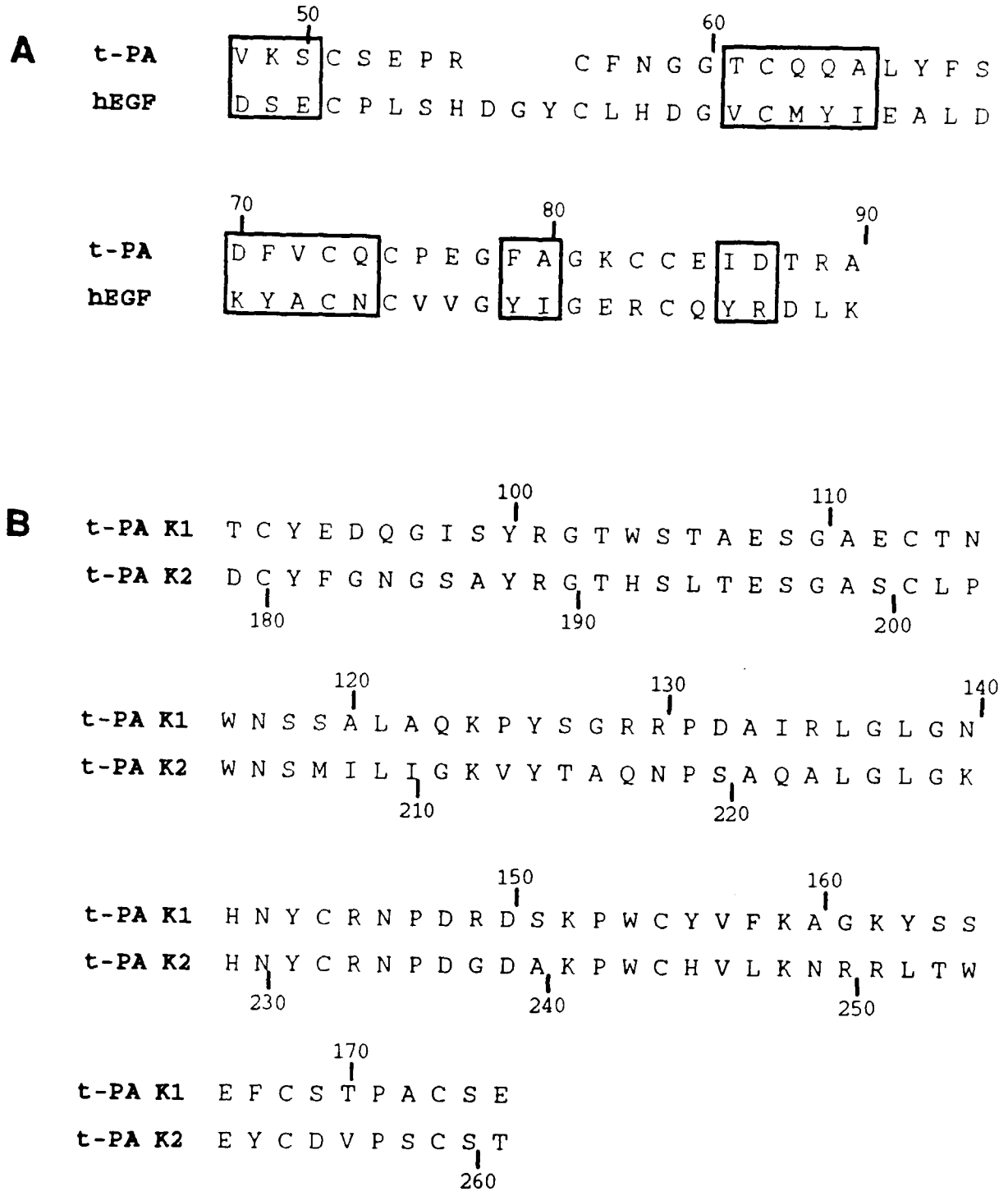


Figure 5.5

(A) Sequence alignment of the EGF-like module of tissue-plasminogen activator with the EGF module of human epidermal growth factor. Regions of β-strand thought to be conserved in homologous molecules are boxed. Based on this alignment, only one 3 residue insertion was necessary to model the t-PA EGF-like module. Alignment of the sequences of the two kringle modules from t-PA (B) manifesting their evident similarity (de Vos *et al.*, 1992). No insertions or deletions were required in modelling the K1 module.

which to base the model of the t-PA SP domain. The alignment of these three serine proteases from bovine chymotrypsin A (Blevins & Tulinsky, 1985), porcine pancreatic kallikrein A (Bode *et al.*, 1983) and bovine trypsinogen (Kossiakof *et al.*, 1977) with the t-PA serine protease sequence is given in Figure 5.6. The regions of each homologous structure chosen for incorporation into the t-PA model are highlighted as well as the six necessary insertions, which all fall into loop regions of the consensus structure. These insertions were modelled using loop searches of the Brookhaven database and refined during energy minimisation.

5.3.2 Relative orientation of the domains of intact t-PA

Having assembled the complete set of t-PA domains the next step was to fit them together in a conformation consistent with experimental data. All of the undefined link regions connecting the domains were short (less than 4 amino acids). Therefore the domains were arranged such that the distance limits imposed by the links could be satisfied, and these regions were modelled afterwards. As mentioned previously, small angle x-ray scattering data indicated that the largest dimension of intact t-PA must be $\leq$ 89 Å (Ponting, 1991). The dimensions of the extended model of the molecule illustrated in Figure 5.7 are ~100 Å x 100 Å x 50 Å. Therefore, these data are only consistent with a compact arrangement of the domains.

Several independently performed biological studies provided evidence that specific residues should be confined to lie on the surface of the intact structure. These residues include the catalytic triad of the serine protease: his 322, asp 371 and ser 478, the three glycosylation sites: asn 117, asn 184 and asn 448 (Pohl *et al.*, 1984), the K2 lysine

```
                                270                       280                       290
t-PA                  C G L R Q Y S Q [P Q F R I] K G G L F A D I A S H P W Q A A I F [A K]
chymotrypsin          C G V P A I Q P       i v n g e e a v p g s w p w q v s l     q
kallikrein                          i I G G R E C E K N S H P W Q V A I Y
trypsinogen                         i v g g y t c g a n t v p y q v s l     n

                    300                       310                       320
t-PA                [H R R S P G E R] F L C G G I L I S S C W I L S A A H C F [Q E R F P]
chymotrypsin        d k t g f       h f c g g s l i n e n w v v t a a h c g v t     t
kallikrein          h y s s         F Q C G G V L V N P K W V L T A A H C K n d     n
trypsinogen           s g y         h f c g g s l i n s q w v v s a a h c y k s     g

          330                       340                       350                       360
t-PA      [P H H] L T V I L G R T   Y R V V P G E E E Q K F E V E K Y I V H K
chymotrypsin          s d v v v a g e f d q g s s s e k i q k l k i a k v f k n s
kallikrein            y e v g w l r h   n l f e n e n t a q f f g v t a d f p h p
trypsinogen           I Q V R L G E D   N I N V V E G N E Q F I S A S K S I V H P

                                  370                       380                       390
t-PA                E F D       D D T Y D N D I A L L Q L K S D S S P [C A Q E S S] V
chymotrypsin        k y n       s l t i n n d i t l l k l s t a a s f       s q t
kallikrein          g f n l s a d g k d y s h d l m l l r l q s p a k i       t d a
trypsinogen         S Y N       S N T L N N D I M L I K L K S A A S L       n s R

                                  400                       410                       420
t-PA                V R T V C L P P A D L Q L P D W T E C E L S G Y G K H E A L S P F
chymotrypsin        v s a v c l p S A S D D f a a g t t c v t t g w g l t r y t n a n
kallikrein          v k v l e l p t q e   p e l g s t c e a s g w g s i e p g p d d
trypsinogen         V A S I S L P t s c   A S A G T Q C L I S G W G N T K S S G T S

                            430                       440                       450
t-PA                Y   S E R L K E A H V R L Y P S S R C [T S Q H L L] N R T V T D N
chymotrypsin        t   p d R L Q Q A S L P L L S N T N C k k   y w G T K I K D A
kallikrein          f e f p d e i q c v q l t l l q n t f c a h   a b p b k v t e s
trypsinogen         Y   P D v l k c l k a p i l s d s s c k s   a y p g q i t s n

                    460                       470                       480
t-PA                M L C A G D [T R S G G P Q A N] L H D A C Q G D S G G P L V C L N D
chymotrypsin        M I C A G   a s           g v s S C M G D S G G P L V C K K N
kallikrein          m l c a g y l p g         g k d t c m g d s g g p l i c     n
trypsinogen         m f c a g Y l e g         G K D s c q g d s g g p v v c     s

                      490                       500                       510
t-PA                G R M T L V G I I S W G   L G C G Q K D V P G V Y T K V T N Y L D
chymotrypsin        G A W T L V G I V s w g   s s t c s t s t p g v y a r v t a l v n
kallikrein          g m w     q g i t s w g h t p c g s a n k p s i y t k l i f y l d
trypsinogen         g     k l q g i v S W G   S G C A Q K N K P G V Y T K V C N Y V S

          520
t-PA      W I R D N M R P
chymotrypsin          w v q q t l a n
kallikrein            w i b b t i t e
trypsinogen           W I K Q T I A S
```

Figure 5.6

Sequence alignment used for homology modelling of the t-PA serine protease based on the serine proteases of chymotrypsin, kallikrein and trypsinogen. The regions of each homologous serine protease used to construct the model are shown in capital letters. Boxed regions of the t-PA sequence delineate loop insertions.

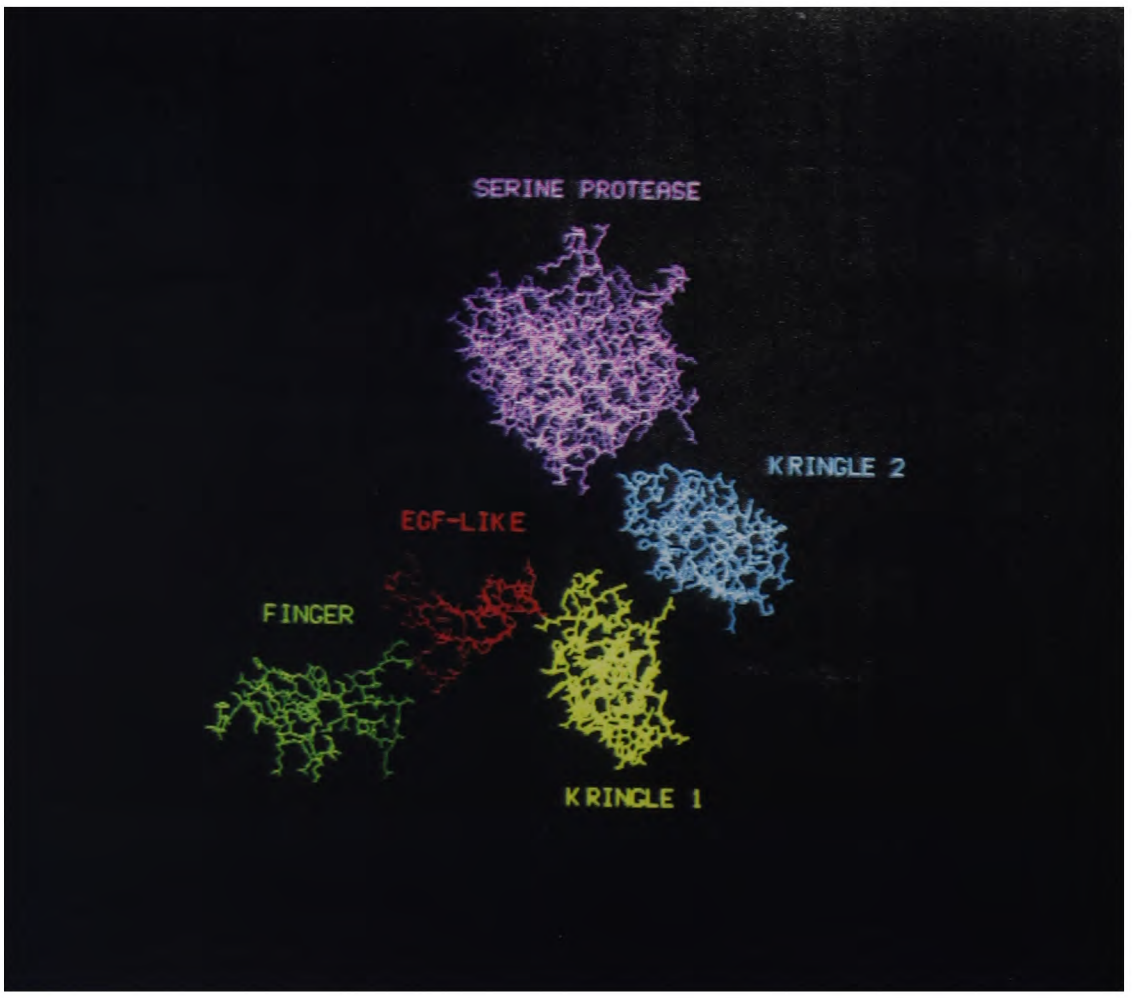


Figure 5.7

Extended model of intact t-PA. The dimensions of this model are approximately 100 Å x 100Å x 50 Å, which is not consistent with the results of small angle x-ray scattering data. Consequently it was concluded that the 5 domains must adopt a compact arrangement.

binding site: lys 212, asp 236, asp 238, trp 242 and trp 253 (de Vos *et al.*, 1992) and the cleavage point for the conversion of sc-tPA to tc-tPA which lies between arg 275 and ile 276 (Wallen *et al.*, 1983).

This information was supplemented by the identification of other residues likely to be found on the surface of the molecule from studies of mutant t-PAs. Specifically, Browne *et al.* (1990) reported a significant decrease in the clearance rate of t-PA upon removal of the tripeptide tyr 67-phe 68-ser 69 from a loop in the growth factor module. Mutations in the region his 44-ser 50 also manifested prolonged *in vivo* half life (Ahern *et al.*, 1990). Based on these studies it was concluded that these residues most probably lie on the surface of intact t-PA such that they are accessible by t-PA receptors. Also inferred was the possibility that these residues might be close together in space.

Two studies of t-PA identified the residues likely to be involved in the stimulation of t-PA activity. In the investigation of functionally significant residues by Bennett *et al.* (1991), charged residues were converted to alanine in clusters of one to four charges per variant. Of all the variants screened, only the mutant of sc-tPA in which residues 296 to 299 were mutated to alanine, i.e., KHRR to AAAA, displayed significantly reduced activity in the presence of fibrin without a simultaneous decrease in amidolytic activity. Eastman *et al.* (1992) extended the study of this mutant and confirmed the proposal that residues in this loop of the serine protease domain are involved in t-PA stimulation. Specifically, it was observed that in the presence of less than ideal stimulators, such as fibrinogen or plasmin degraded fibrin, this mutant formed the t-PA/plasminogen/stimulator complex equally well as wild type t-PA but failed to hydrolyse the plasminogen bond as efficiently. These observations led to the assumption that this loop should also lie exposed on the t-PA surface.

Analysis of those residues probably found on the surface of intact t-PA was complemented by speculations regarding those residues possibly involved in domain-domain interactions. Based on the results of Bennett *et al.* (1991), de Vos *et al.* (1992) remarked that the mutation of specific residues, including arg 189, lys 228 and his 229, far removed from the kringle 2 lysine binding pocket, resulted in decreased fibrin binding affinity but unchanged lysine binding. From these data de Vos *et al.* (1992) postulated that the decreased binding affinity was likely to be due to an indirect effect, possibly the disruption of intramolecular domain-domain interactions. Bennett *et al.* (1991) also reported that fibrin binding was significantly affected by mutations in the region of residues 125 to 150 of kringle 1. It should be noted that the N and C termini of each kringle structure are disulphide bridged, and only six residues separate the two (see Figure 5.1). Consequently, the two t-PA kringles are confined to lie close together spatially. Consideration of these observations led to the hypothesis that residues 125 to 150 of kringle 1 might interface with residues 189, 228 and 229 of kringle 2, and that the decrease in fibrin binding when these residues are mutated could be explained by a disruption of this domain-domain interaction. No data have been published which suggest any form of interaction between the kringles and the other domains of the molecule.

A clue to the packing of the domains is provided by the investigation of the melting of recombinant t-PA by differential scanning calorimetry and fluorescence spectroscopy performed by Novokhatny *et al.* (1991) which suggested that the serine protease domain interacts strongly with and is stabilised by the finger and growth factor modules. This theory is substantiated by the existence of a monoclonal antibody which recognises the finger and serine protease domains in the intact molecule but neither of the two independently (Keyt, B., *personal*

communication). Therefore, given that the finger and growth factor modules interact with the serine protease domain, the final remaining question was the orientation of these domains with respect to each other. Comparison of the t-PA finger and fibronectin finger structures highlighted a patch of hydrophobic residues on the surface of the triple stranded β-sheet of t-PA F1 which is thought to participate in domain-domain interactions. The residues comprising this patch include leu 22, pro 24, val 25, val 31, tyr 33, trp 35, val 46 and val 48. The nature of the possible interaction is not clearly defined, however, because it could involve the growth factor module and/or the serine protease domain. The decision to model the finger and growth factor modules in an extended conformation which packs against the serine protease domain was based on several pieces of information. Baron *et al.* (1990a) noted with reference to the finger and growth factor consensus structures that the type 1 module could link into EGF-like modules via a common β-strand. This postulation is in agreement with assigned short range n.m.r. data collected on the F1-G pair of modules from human t-PA (B. O. Smith, *personal communication*). It seems unlikely that a kink in the common strand would allow the two modules to pack together. Furthermore, in this conformation the hydrophobic patch on the t-PA F1 may be extended to include residues tyr 67, phe 68 and val 72 in the G module as shown in Figure 5.8. A hydrophobic patch, including residues tyr 269, pro 272, phe 274, phe 281, ile 284, tyr 341, val 344, pro 345, ile 420, pro 422, phe 423 and tyr 424, was also identified on the surface of the serine protease domain. Interaction of the finger and growth factor modules via these two hydrophobic surfaces seemed a likely explanation for the stabilisation of the serine protease by the finger and growth factor observed by Novokhatny *et al.* (1991).

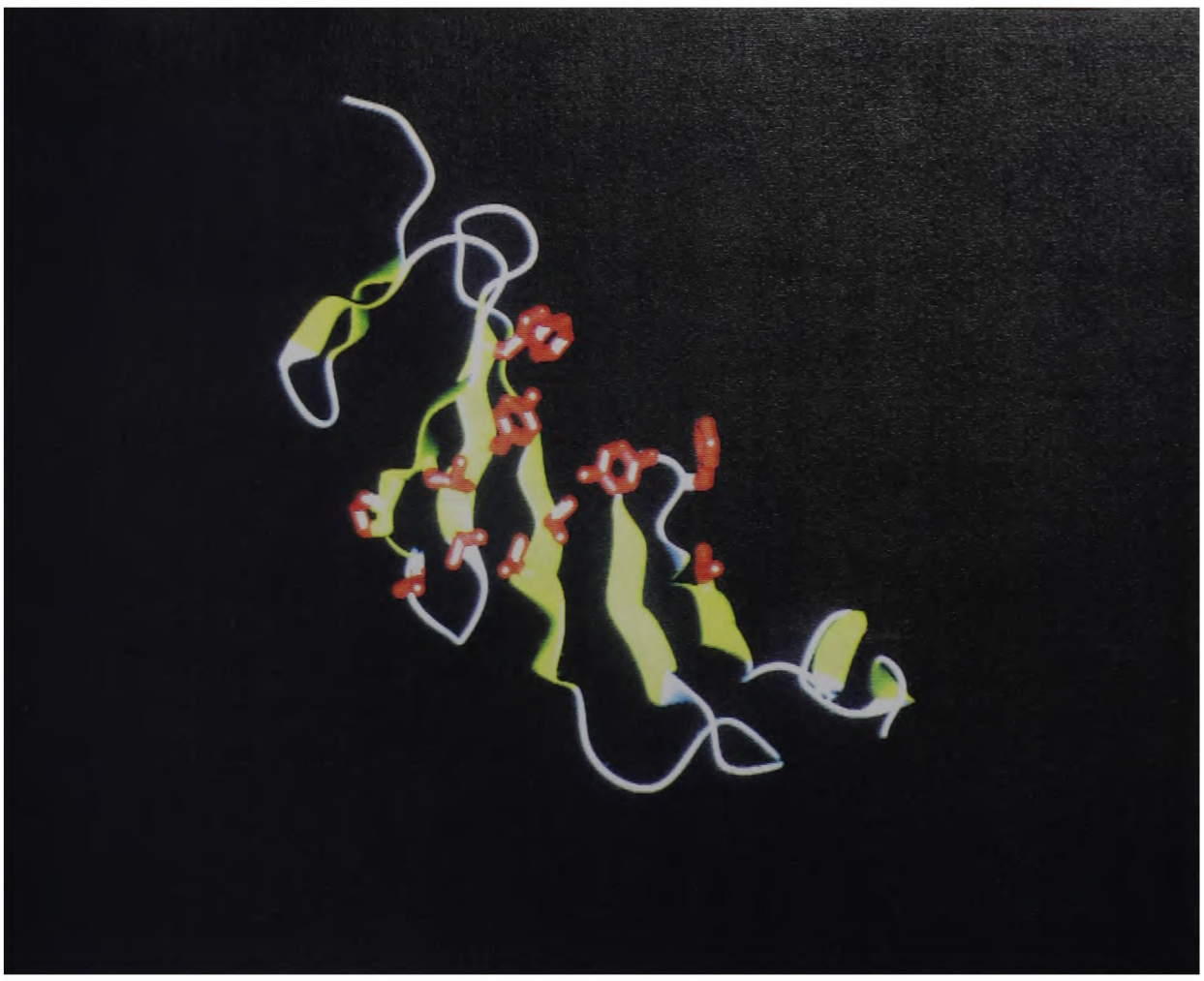


Figure 5.8

Model of the conformation of the finger and EGF-like pair of modules from t-PA. In this extended conformation in which the two modules share a common β-strand, the hydrophobic patch on the exterior surface of the triple-stranded sheet of finger is extended to include residues in the EGF-like module including tyr 67, phe 68 and val 72. Hydrophobic residues are highlighted in red.

In this orientation of the heavy chain (defined as the F, G, K1 and K2 modules) and the light chain (SP domain) of t-PA the hydrophobic interface is beside the loop thought to participate in stimulation of t-PA activity (Figure 5.9). This observation might explain differences in the stimulated activity of wild type t-PA and the light chain documented by Rijken & Groeneveld (1986). The amidolytic activities of wild type t-PA and the SP domain are very similar. However, unlike the parent tc- tPA, the activity of the serine protease is not stimulated by fibrinogen or fibrin degradation products. The conclusion drawn from these findings is that stimulated activity involves the heavy chain, but Bennett *et al.* (1991) showed that only mutations in the light chain affected t-PA stimulation. Therefore, mutations in the t-PA stimulation dependent loop must affect the interaction of the serine protease with the heavy chain, which might be explained structurally by the close proximity of the loop to the hydrophobic interface between the two chains.

In summary, the five domains of t-PA were oriented with respect to each other based on the following criteria (residue numbers given in parentheses):

(i) Residues known to participate in intermolecular interactions, directly or indirectly via carbohydrate moieties, were confined to lie on the surface of the intact structure. These residues included:

- the catalytic triad of the serine protease (322, 371,478)
- the three glycosylation sites (117, 184, 448)
- the K2 lysine binding site (212, 236, 238, 242, 253)
- the cleavage point for conversion of sc-tPA to tc-tPA (275, 276)

(ii) Residues involved in clearance of t-PA from circulation were positioned on the exterior of the molecule and in close proximity (44-50, 67-69).

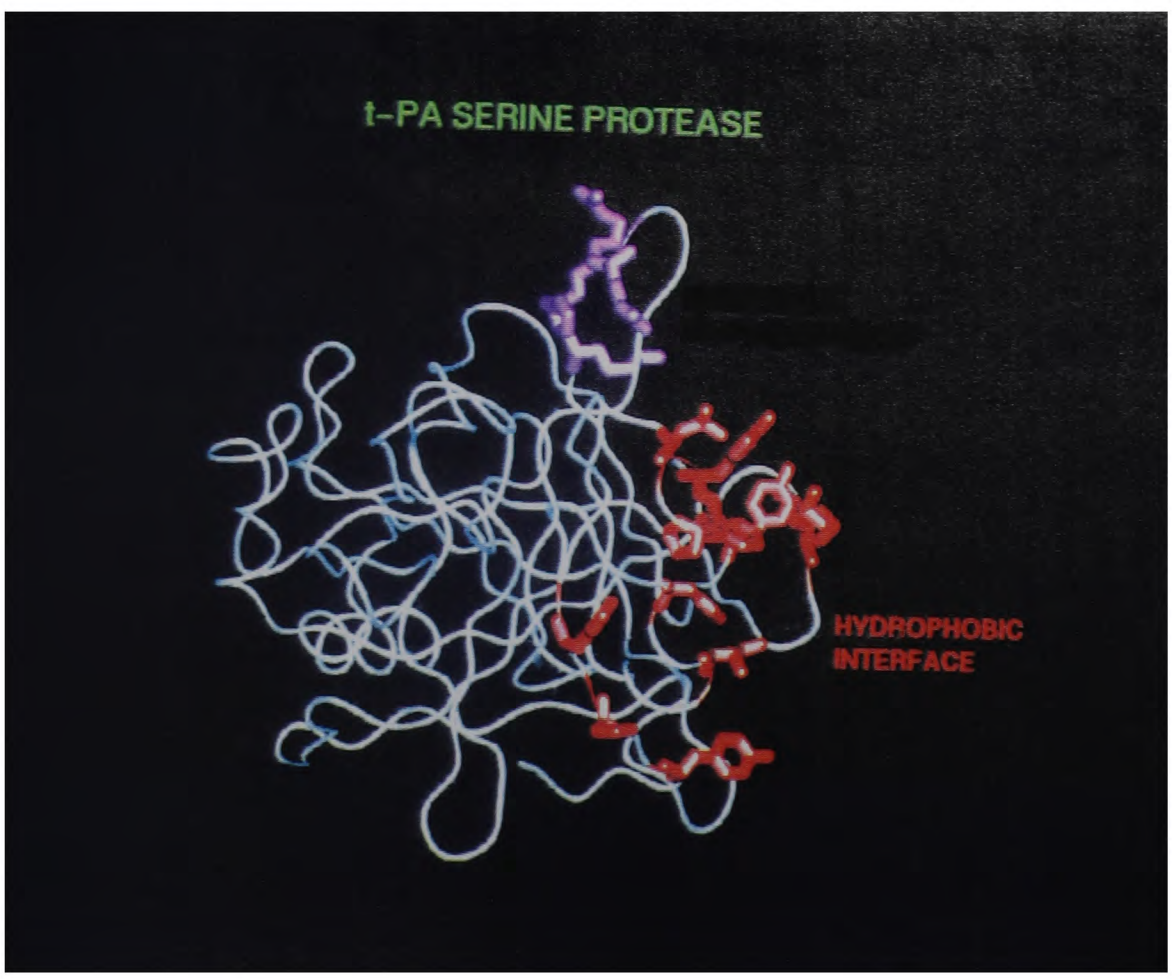


Figure 5.9

Schematic illustration of the t-PA serine protease domain highlighting the close proximity of the hydrophobic patch on the surface of this domain (red) to the loop (magenta). reported to be involved in stimulation of t-PA activity (Eastman *et al.*, 1992).

(iii) The loop containing residues identified as critical to t-PA stimulation was situated on the t-PA surface (296-299).

(iv) Residues proposed to participate in fibrin binding affinity via intramolecular domain-domain interaction were located at the interface of the two kringles (125-150, 189, 228, 229).

(v) The finger and growth factor modules were modelled in an extended conformation in order to form a contiguous hydrophobic surface (22, 24, 25, 31, 33, 35, 46, 48, 67, 68, 72)*.

(vi) The hydrophobic patch identified on the surface of the serine protease was placed in contact with the above hydrophobic surface (269, 272, 274, 281, 284, 341, 344, 345, 420, 422, 423, 424).

A model of intact t-PA consistent with these considerations is presented in Figure 5.10. The dimensions of the model as shown are ~85Å x 50 Å x 50 Å.

5.4 Glycosylation of t-PA: Correlation of Structure and Function

When modelling glycosylated t-PA it was necessary to select specific representative structures for each of the carbohydrates. It was decided that the smallest high mannose type and complex type oligosaccharides would be used in the model to ensure that predicted

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* It should be noted that residues 67 and 68 in the G module are also thought to be involved in the t-PA clearance mechanism, and that in the extended conformation of the finger and growth factor pair, the participation of these residues in hydrophobic interactions with the serine protease requires that their side chains are not solvent exposed. This region of the molecule has been modelled to the highest degree of accuracy permitted by presently available information, and the model will be revised when calculation of the F1-G pair of modules from multidimensional n.m.r. data is completed.

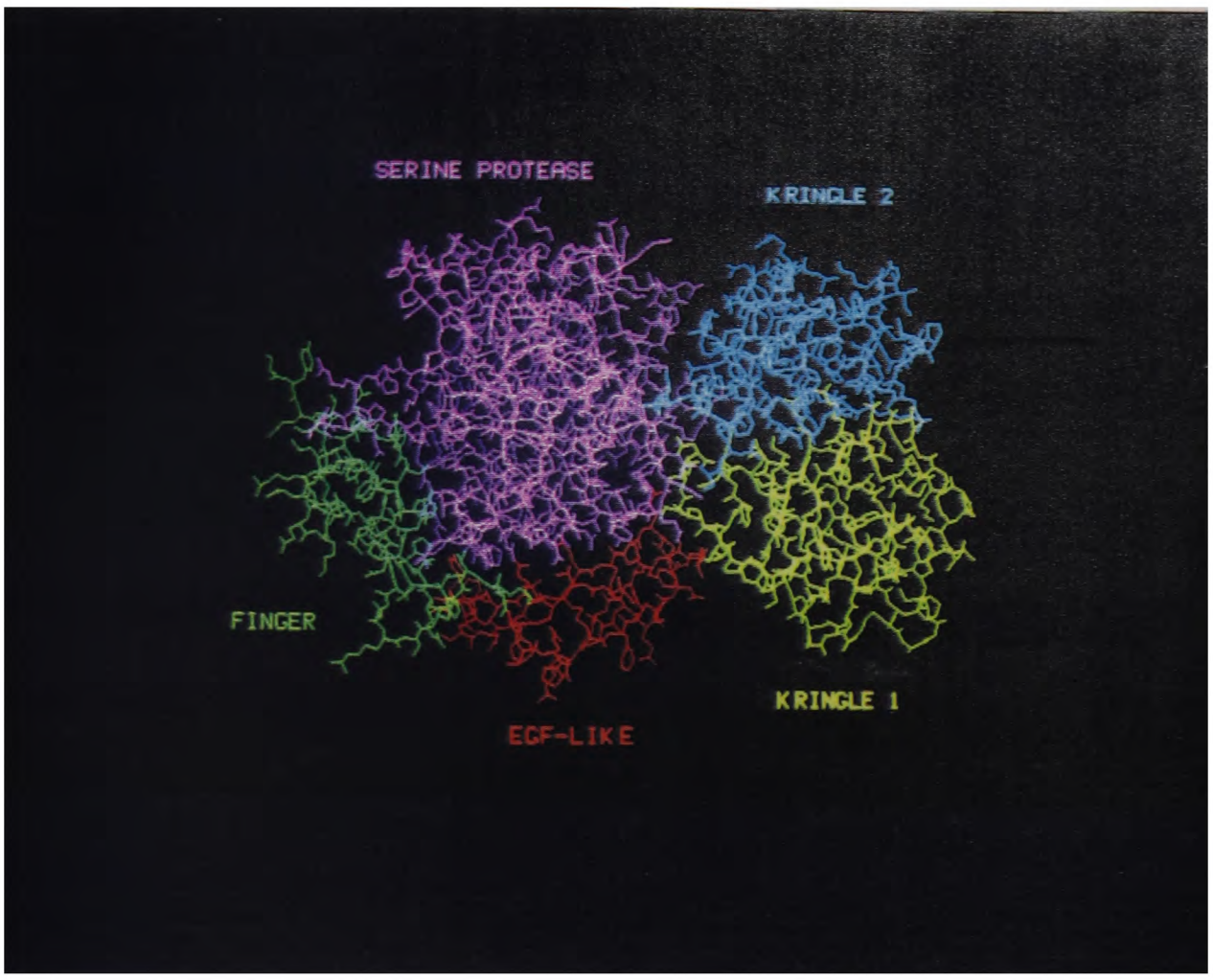


Figure 5.10

Compact model of intact tissue-plasminogen activator derived from cumulative biological data. The finger and EGF-like modules pack against and structurally stabilise the serine protease (Novokhatny *et al.*, 1991). The dimensions of this model, ~85 Å x 50 Å x 50 Å, are consistent with the results of low resolution small angle x-ray scattering experiments (Ponting, 1991).

steric interactions between the carbohydrate side chains and the protein moiety could exist in all variants of wild type t-PA. The side chains used in the model are shown in Figure 5.11. Types I and II t-PA are displayed in Figure 5.12. The dimensions are ~95Å x 60 Å x 50 Å for both molecules; these dimensions provide an approximation of the smallest size of a wild type t-PA molecule. These models were used to explain functional properties of glycosylated variants of wild type and mutant t-PAs in terms of the spatial orientation of the carbohydrate side chains.

In order to probe the relationship between glycosylation and t-PA function, Haigwood *et al.* (1989) constructed mutants with altered glycosylation sites. A t-PA mutant lacking the site at position 117 (N117Q) was measured as having slightly reduced fibrin binding. Figure 5.13 presents a possible structural rationalisation of this observation: asn 117 is located close to the interface modelled between the two kringles and may stabilise the association of the two modules. Mutation of residues located in this region also results in reduced fibrin binding (Bennett *et al.*, 1991), an effect suggested as the result of destabilisation of the domain-domain interaction. Therefore, the observed decrease in fibrin binding for mutant N117Q might be explained by glycosylation at sequon 117 similarly affecting this interaction.

Haigwood *et al.* (1989) also reported slightly enhanced fibrin binding for the mutant lacking a glycosylation site at position 184 (N184Q). This finding is corroborated by the study of Wittwer *et al.* (1989) in which the fibrin affinity of two glycosylated variants each of type I and type II t-PA was compared. Significantly higher fibrin binding was observed for the type II forms. Hence glycosylation at sequon 184 decreases the fibrin binding of t-PA. A structural interpretation of this result is facilitated by knowledge of the kinetics of t-PA activity. When t-PA is stimulated, biphasic kinetics are observed (Norrman *et al.*, 1985;

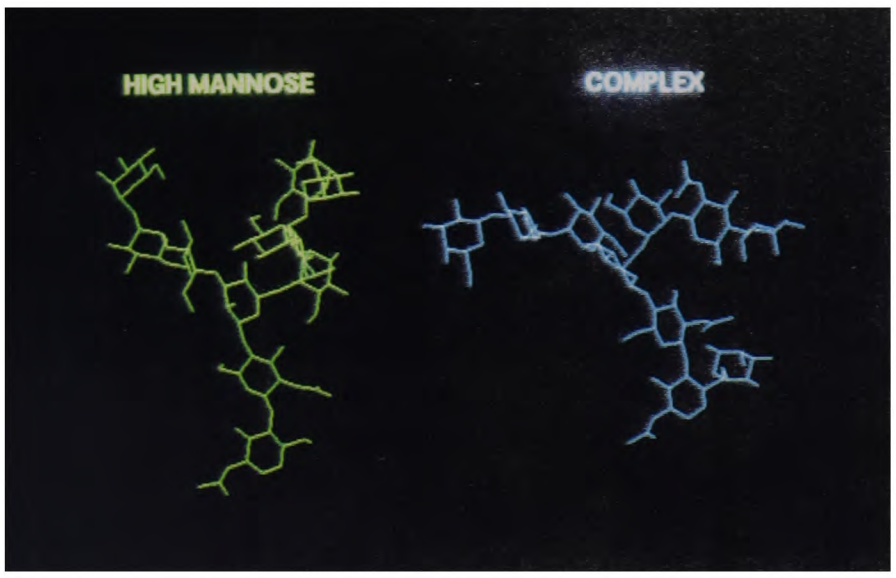


Figure 5.11

Structures of the high mannose and complex carbohydrate side chains used in modelling glycosylated intact t-PA. The coordinates for these oligosaccharides were kindly provided by C. Edge.

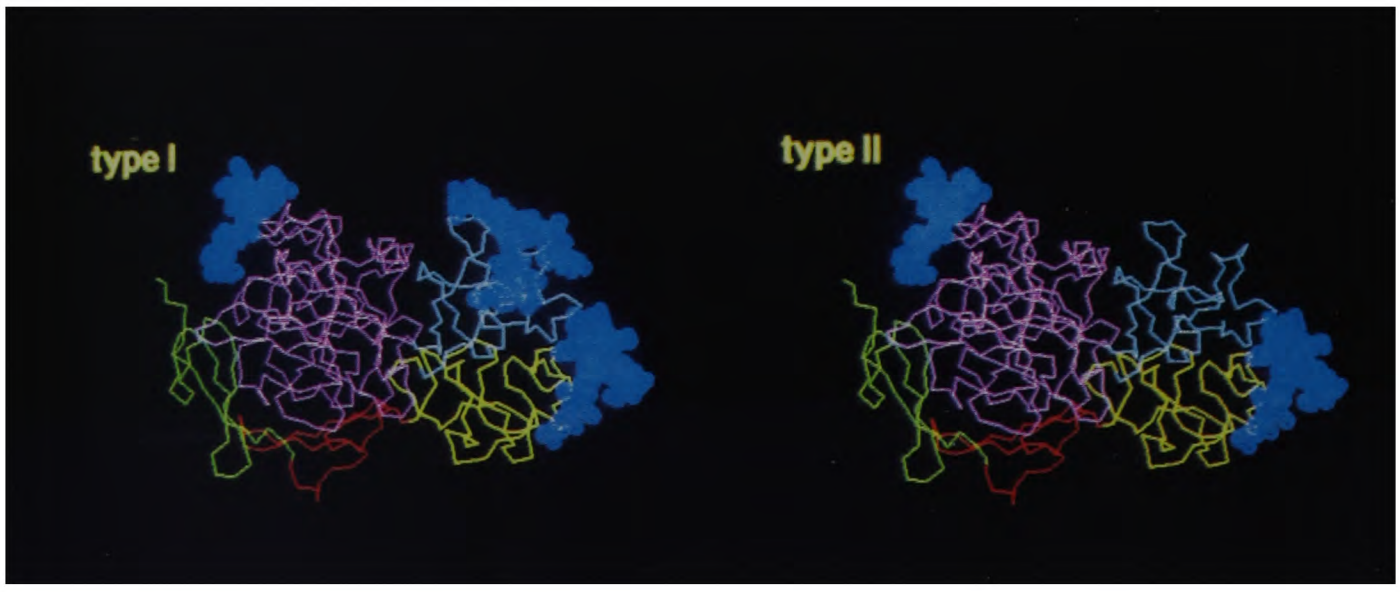


Figure 5.12

Models of types I and II t-PA. The C^{α} atoms of the model of the intact protein are shown coloured by domain as in Figure 5.10, and the carbohydrates are surfaced in blue. Type I is glycosylated at sites 117, 184 and 448, while type II lacks the oligosaccharide at site 184. The approximate dimensions of both of these molecules, 95 Å x 60 Å x 50 Å, provide a size estimate of the smallest wild type t-PA molecule.

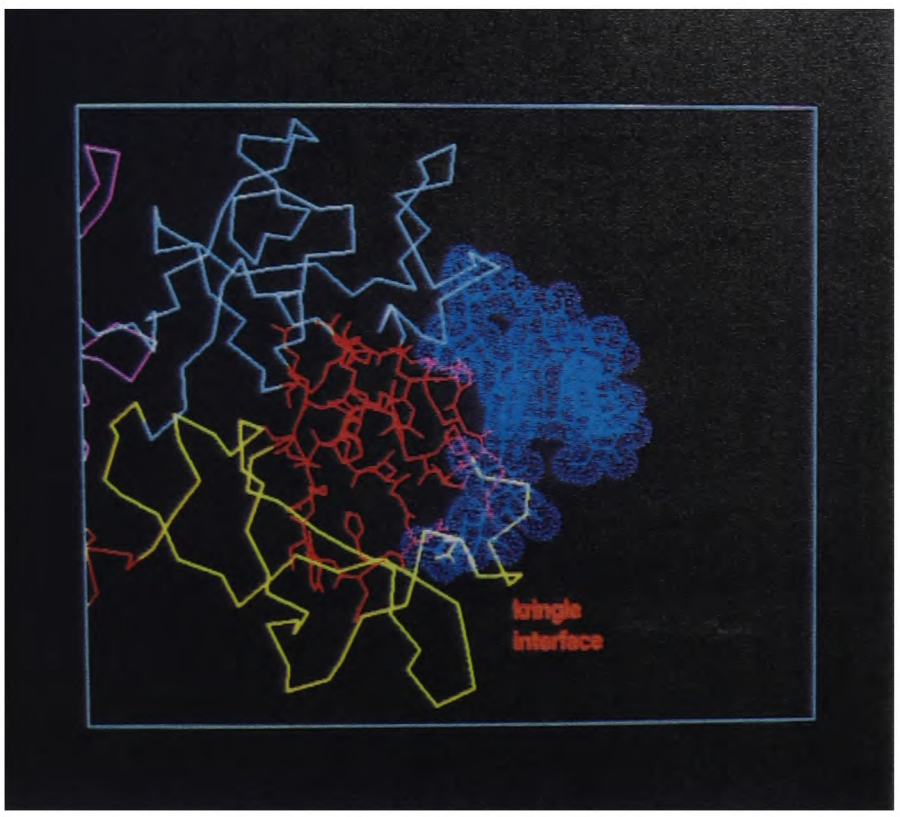


Figure 5.13

Proposed stabilisation of the interface between kringles 1 and 2 by the high mannose oligosaccharide at asn 117 (blue). Mutation of residues highlighted in red results in reduced fibrin binding which is thought to be due to disruption of interdomain interactions (Bennett *et al.*, 1991; de Vos *et al.*, 1992). Removal of the glycosylation site at asn 117 also results in decreased fibrin affinity (Haigwood *et al.*, 1989) and may affect the stability of the kringle interface.

Beckmann *et al.*, 1988). van Zonneveld *et al.* (1986b) suggested that the first, slower phase is rate limited by the binding of fibrin to the finger module, while in the second, faster phase, plasmin-degraded fibrin interacts with the lysine binding site on the second kringle module. If the second kringle is involved in fibrin binding by such a mechanism, then a likely explanation of reduced fibrin binding in type I t-PA is that the oligosaccharide at sequon 184 shields the lysine binding site as depicted in Figure 5.14.

Stimulated and unstimulated amidolytic activity measured indirectly for aglycosyl t-PA was found to be significantly greater than that of wild type t-PA (Wittwer *et al.*, 1989). However, direct amidolytic assays showed no differences between aglycosyl and wild type t-PA. The indirect amidolytic assay measures the activation of plasminogen indirectly, whereas the direct amidolytic assay measures the activity of t-PA on a chromogenic peptide substrate which is cleaved (Rijken & Collen, 1981). Therefore, comparison of the results of these two assays suggests that glycosylation of tPA might sterically hinder access of large substrates to the active site. Specifically, the oligosaccharide at asn 448 may shield the catalytic triad of the serine protease as depicted in Figure 5.15.

5.5 Conclusions

Comparison of the structures of the finger modules from t-PA and fibronectin demonstrated that sequences with very little homology apart from a common consensus sequence adopt strikingly similar structures. In t-PA one of the aromatic residues that is highly conserved in fibronectin F1 sequences is substituted by an alanine. This residue forms part of the hydrophobic core of the seventh type 1 module from



Figure 5.14

Postulated shielding of the kringle 2 lysine binding site (red) by the complex oligosaccharide at asn 184 (blue). If the lysine binding site contributes to t-PA fibrin binding via association with plasmin-degraded fibrin, then the decreased fibrin affinity of molecules glycosylated at this position might be explained by the suggested protein-carbohydrate interaction.

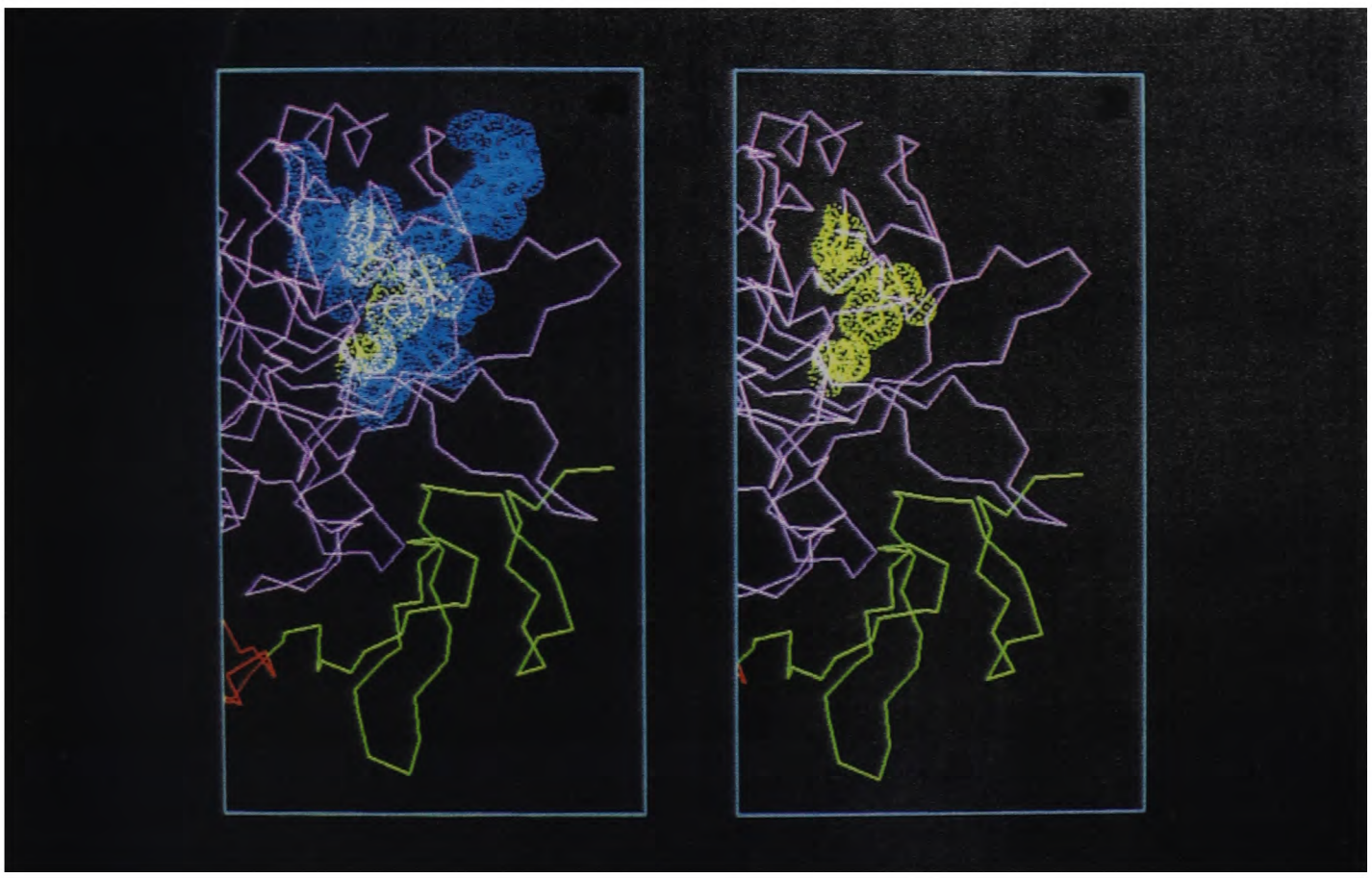


Figure 5.15

Steric hindrance of access to the catalytic triad (yellow) by the complex carbohydrate at asn 448 (blue). The amidolytic activity of aglycosyl t-PA on large substrates is significantly greater than that of wild type t-PA, however they are equally active on small peptide substrates. This effect might be explained by shielding of the catalytic triad by the oligosaccharide at position 448.

human fibronectin, however comparison of the two F1 modules showed that a hydrophobic residue at this position is not critical for stabilisation of the F1 structure. Based on this observation the consensus sequence which defines the F1 consensus structure may be refined to include only the eight residues conserved in t-PA F1 (Figure 3.8). It was noted that the side chain of one charged residue of the consensus sequence is directed toward the core of the molecule in the fibronectin F1 structure. Comparison of the t-PA F1 structure verified this anomalous side chain orientation and suggested a plausible explanation for this observation, that this residue may form a salt bridge. A topological difference between the two F1 modules was also highlighted by the comparison: one surface of the t-PA F1 is covered with hydrophobic residues. It was deduced that this region of the molecule might engage in domain-domain interactions in the intact protein.

The goal of structural studies of intact proteins is to gain a physical understanding of their functions. This will be difficult in the case of t-PA, however, because thus far attempts to crystallise the protein have proven unsuccessful. Using the solution structure of the t-PA F1 module, the K2 structure from human t-PA (de Vos *et al.*, 1992) and models of the G, K1 and SP domains, a model of the intact protein was generated that is consistent with its known biological properties.

The function of wild type t-PA is mediated at least in part by glycosylation. The problem is further complicated by the variation of the carbohydrate side chains in the two types of t-PA. Models of glycosylated types I and II t-PA were used as a basis for theoretical explanation of the biological properties of the glycosylated protein. The size of the oligosaccharides with relationship to the protein moiety of the molecule was illustrated, and specific interactions between the carbohydrate side

chains and the functional regions of the protein were postulated based on the model.

Chapter 6

Structure and Significance of the N-terminal SH2 Module of the p85α Subunit of Phosphatidylinositol-3-OH Kinase

6.1 Introduction

High affinity binding of SH2 modules to receptor tyrosine kinases is critical to control of cell signalling pathways (see Chapter 1). This chapter describes the n.m.r. spectral assignment and structure determination of the N-terminal SH2 module of the p85α subunit (p85α-N SH2) of phosphatidylinositol 3-OH kinase*. Conclusions drawn from the spatial proximity of residues thought to be involved in phosphopeptide binding of the module are also discussed. A comparison of this structure to the crystal structure of the *v-src* SH2 module complexed with low affinity phosphopeptides (Waksman *et al.*,

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* The expression and purification of the N-terminal SH2 module of the p85α-N SH2 module was performed by G. Panayotou and I. Gout. Although residues 1-120 of the p85α-N SH2 module were expressed, residues 1 to 16 failed to provide any structurally significant n.m.r. data. These residues lie outside of the SH2 consensus sequence and for this reason will not be further discussed.

1992) is presented. Then the modelling of high affinity SH2-peptide interactions based on these two structures is described, and the results are compared with interactions subsequently observed in the structures of two high affinity SH2-peptide complexes (Eck *et al.*, 1993; Waksman *et al.*, 1993).

6.2 Sequential Assignment of the p85α-N SH2 Module Using 3-Dimensional NOESY Data

The assignment of the p85α-N SH2 n.m.r. spectra was considerably more difficult than for the t-PA F1 module for a number of reasons. Foremostly, the SH2 consensus sequence defined by multiple sequence alignment shares no obvious sequence similarity to any domains of known 3-dimensional structure. Therefore a consensus structure was not available to assist spectral assignment. In addition, the expressed p85α-N SH2 module was more than twice the molecular weight of the t-PA F1 module. Consequently 2-dimensional n.m.r. spectra were extensively overlapped and relatively difficult to interpret. In order to resolve assignment ambiguities, 3-dimensional heteronuclear n.m.r. techniques were employed. Due to the limited availability of spectrometer time, the resolution (in terms of data points per Hz) of 3-dimensional n.m.r. data is typically inferior to 2-dimensional n.m.r. data. Also, these experiments require the use of a ^{15}N-labelled protein sample which was both difficult and expensive to prepare. These complications led to use of a dilute (1mM) sample in the 3-dimensional experiments which further reduced the quality of the data.

Spectral assignment of the p85α-N SH2 module was completed in collaboration with G. W. Booker and A. L. Breeze. Three-dimensional n.m.r. techniques have been described in detail by Clore & Gronenborn

(1991b) and are now briefly reviewed. Understanding how the use of 3-dimensional data alleviates spectral complexity is conceptually straightforward. Figure 6.1 illustrates the simple relationship between 3-dimensional ^{15}N-edited and 2-dimensional H_2O NOESY spectra. Considering only the amide region of the spectra, the 2-dimensional data are spread into a third dimension according to the chemical shift of the ^{15}N nucleus attached to the amide proton. Therefore crosspeaks that are overlapped in the 2-dimensional data set are likely to be resolvable in different planes of the 3-dimensional spectrum.

The separation of the planes in the 3-dimensional spectrum is small enough that often NOEs appear in more than one plane with different intensities. This idea is more easily comprehended if each NOE is thought of as a sphere. Figure 6.2 illustrates that a plane passing through the centre of a column of NOEs will contain crosspeaks of the greatest intensity. Consequently the first step in analysis of a 3-dimensional data set is identification of the ^{15}N-edited plane with maximum signal for each proton. As shown in Figure 6.3, one strip is extracted for each amide proton which contains all the NOEs involving this proton. Then the strips are ordered by alignment of sequential NOE connectivities. This method was used for sequential assignment of the p85α-N SH2 module. Representative strips demonstrating sequential assignment of residues 29 to 33 and 43 to 47 are shown in Figure 6.4.

6.3 Secondary Structure of the p85α-N SH2 Module

After sequential assignment of the p85α-N SH2 a prediction of secondary structure was made from calculated secondary $C^{\alpha}H$ chemical shifts (Wishart *et al.*, 1992) and used as a guide in the assignment of short range NOEs. Then the secondary structure of the p85α-N SH2

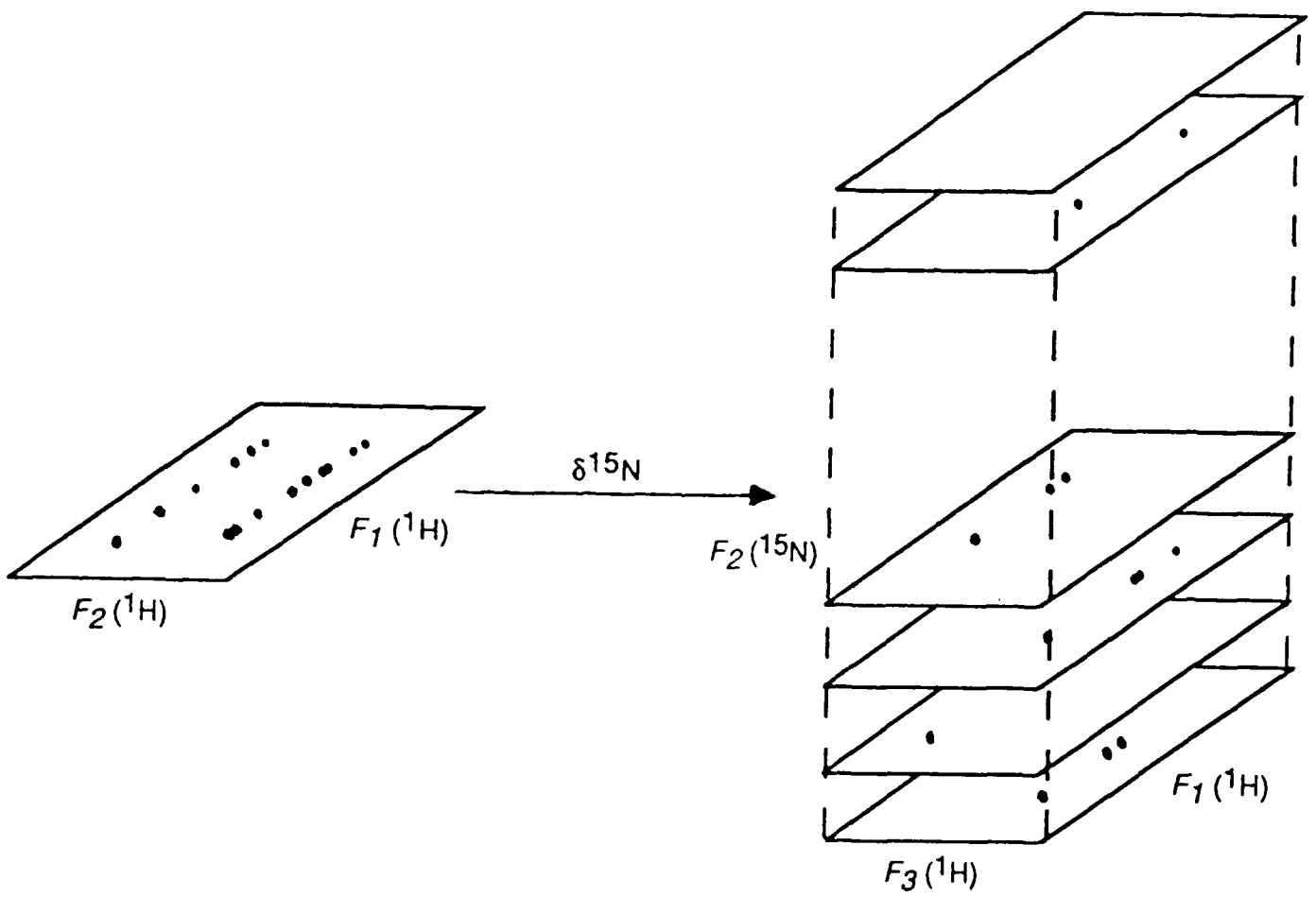


Figure 6.1

Schematic illustration of the relationship between 2-dimensional and 3-dimensional ^{15}N-edited NOESY spectra. The closed circles represent NOE crosspeaks. Amide protons with degenerate chemical shift in the F_2 dimension are resolved in different planes of the ^{15}N-edited spectrum. Thus the NOEs associated with different amide protons are better resolved in the 3-dimensional data set.

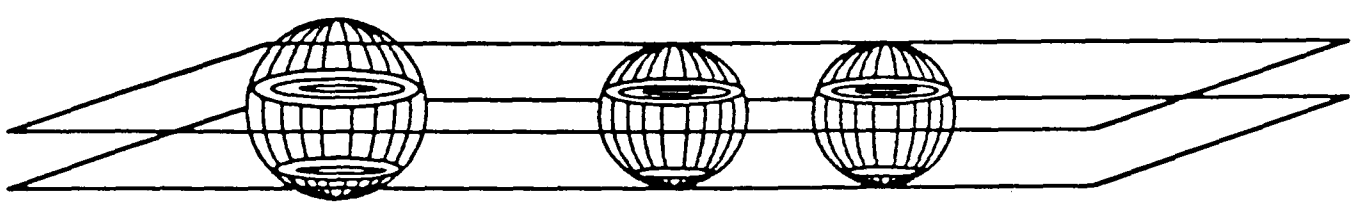


Figure 6.2

Schematic illustration of variation of NOE intensity in closely spaced planes of a 3-dimensional n.m.r. spectrum. Each NOE is represented by a sphere. The upper plane intersects a greater cross-section of each sphere than the lower plane, thus the NOEs will appear larger in the upper plane. It is clear that a plane intersecting the midpoint of the line of spheres would contain crosspeaks of the greatest intensity.

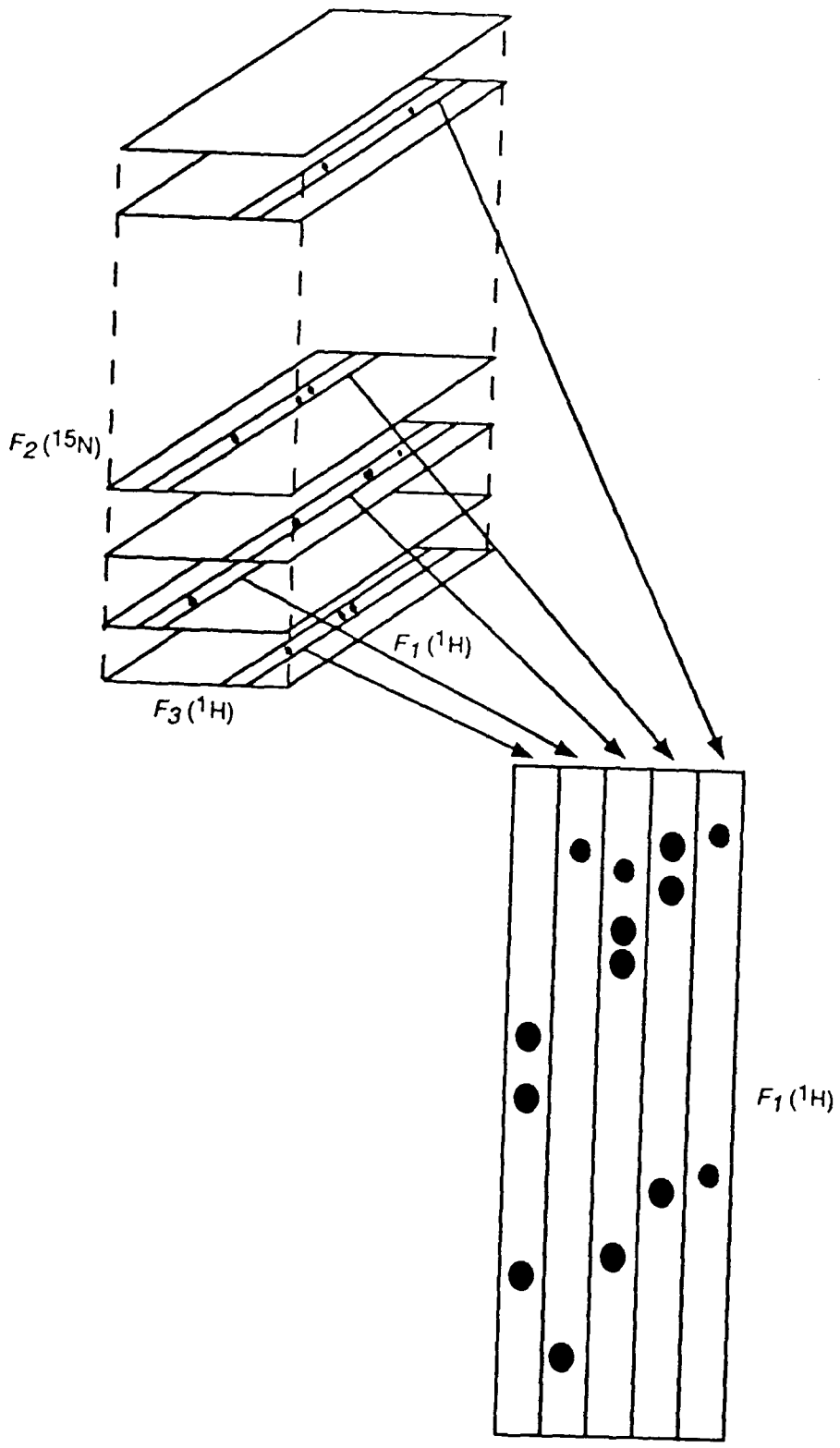


Figure 6.3

Schematic illustration of the extraction of strips from a 3-dimensional n.m.r. spectrum. NOEs are represented by filled circles. One plane containing the highest signal is identified for each resolvable amide proton, then the strip containing the NOEs involving this proton is extracted and plotted in 2-dimensions. Alignment of the NOEs in the F_1 dimension leads to sequential assignment of the strips.

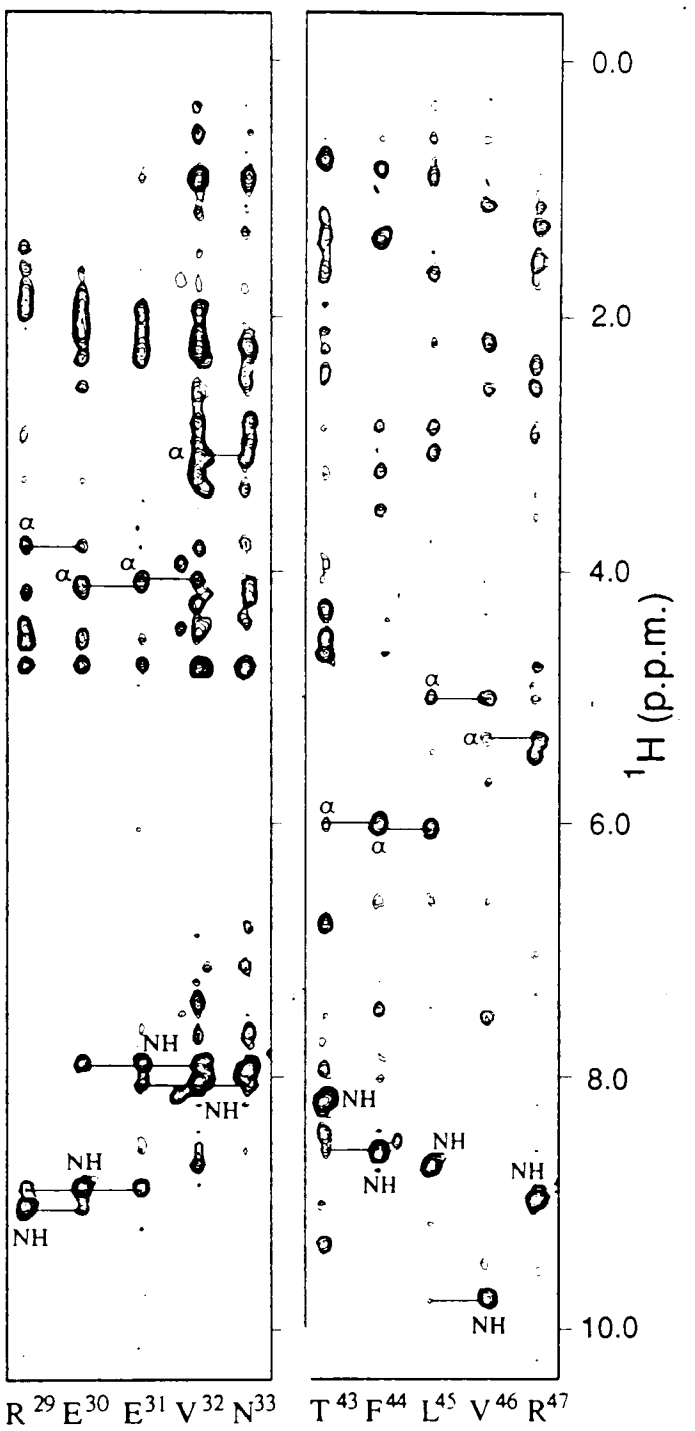


Figure 6.4

Strips taken from the amide region of selected ^{15}N planes of a three dimensional ^{15}N-^{1}H NOESY-HMQC spectrum illustrating sequential assignment. Horizontal lines indicate the presence of sequential NOEs involving C$^{\alpha}$H and NH protons used to substantiate the assignments (reproduced from Booker *et al.*, 1992).

module was derived from n.m.r. data based on the cumulative interpretation of many independent observations. Prior to discussion of the secondary structure determination of the p85α-N SH2 module, the characteristics of n.m.r. data associated with loop, α-helix and β-strand motifs are briefly reviewed.

6.3.1 Correlation of secondary structure with n.m.r. data

Comparison of NOESY spectra reveals two general correlations between the qualitative appearance of the data and the precision with which the coordinates of a residue can be determined. Firstly, since fewer NOEs are observed in regions without well-defined structure, the crosspeak density in the strips taken from the amide region of a 3-dimensional ^{15}N-edited NOESY spectrum is lower in the consecutive strips corresponding to a loop region. Secondly, crosspeaks for residues which are relatively mobile in solution are often relatively sharp and intense. In addition, the chemical shifts of the protons for these residues are likely to be close to random coil.

Helical regions have characteristic patterns of short range interactions in a NOESY spectrum (see Figure 3.14). Weak NOEs are commonly observed corresponding to $C^{\alpha}H/HN_{(i,\ i+1)}$, $HN/HN_{(i,\ i+2)}$, and $C^{\alpha}H/HN_{(i,\ i+4)}$ connectivities, and medium for $HN/HN_{(i,\ i+1)}$, $C^{\alpha}H/HN_{(i,\ i+3)}$ and $C^{\alpha}H/HB_{(i,\ i+3)}$ connectivities. An α-helix has a backbone ϕ torsion angle of approximately -60°, which gives rise to consecutive small $^{3}J_{HN\alpha}$ coupling constants (< 5 Hz) for these regions. Definition of the termini of a helical region can be assisted by analysis of amide proton exchange. The hydrogen bonding pattern in the core of α-helix (CO_i-HN_{i+4}) is detected by observation of slowly exchanging amide protons at the fourth position from the beginning of the helix and for every successive residue

up to the end of the helix. Finally, a more qualitative indication of an α-helix is negative secondary $C^{\alpha}H$ chemical shift displacement from random coil positions (Wishart *et al.*, 1991).

Characteristic n.m.r. data for parallel and antiparallel β-strands comprise strong $C^{\alpha}H/HN_{(i, i+1)}$ and weak $HN/HN_{(i, i+1)}$ NOEs, large $^{3}J_{HN\alpha}$ coupling constants and positive $C^{\alpha}H$ chemical shift displacement from random coil (Wishart *et al.*, 1991). Also, an alternating pattern of slowly exchanging amide protons corresponds to a surface β-strand, whereas a buried β-strand is likely to have a higher number of hydrogen bonded amide protons.

6.3.2 Derivation of the p85α-N SH2 secondary structure

A secondary structure prediction based solely on the chemical shifts of $C^{\alpha}H$ protons (Wishart *et al.*, 1992) was generated to assist in the assignment of short range NOEs. In this method random coil chemical shift values are subtracted from the $C^{\alpha}H$ chemical shifts of the protein of interest. Any residue with a chemical shift deviation of greater than 0.1 is marked "1" while those residues negatively deviating by more than -0.1 are marked "-1". All remaining residues are marked "0". A series of four or more "-1's" which is uninterrupted by a "1" is predicted to be a helix, and a series of three or more "1's" is predicted to be a β-strand. All of the remaining undefined regions are designated as coil. The prediction of secondary structure generated for the p85α-N SH2 according to the method of Wishart *et al.* (1992) is shown in Figure 6.5.

A graphical summary of the short range NOEs and slowly exchanging amide protons for the p85α-N SH2 is presented in Figure 6.6. $^{3}J_{HN\alpha}$ coupling constants are tabulated in Figure 6.7. It should be noted that the low signal to noise of the data made accurate

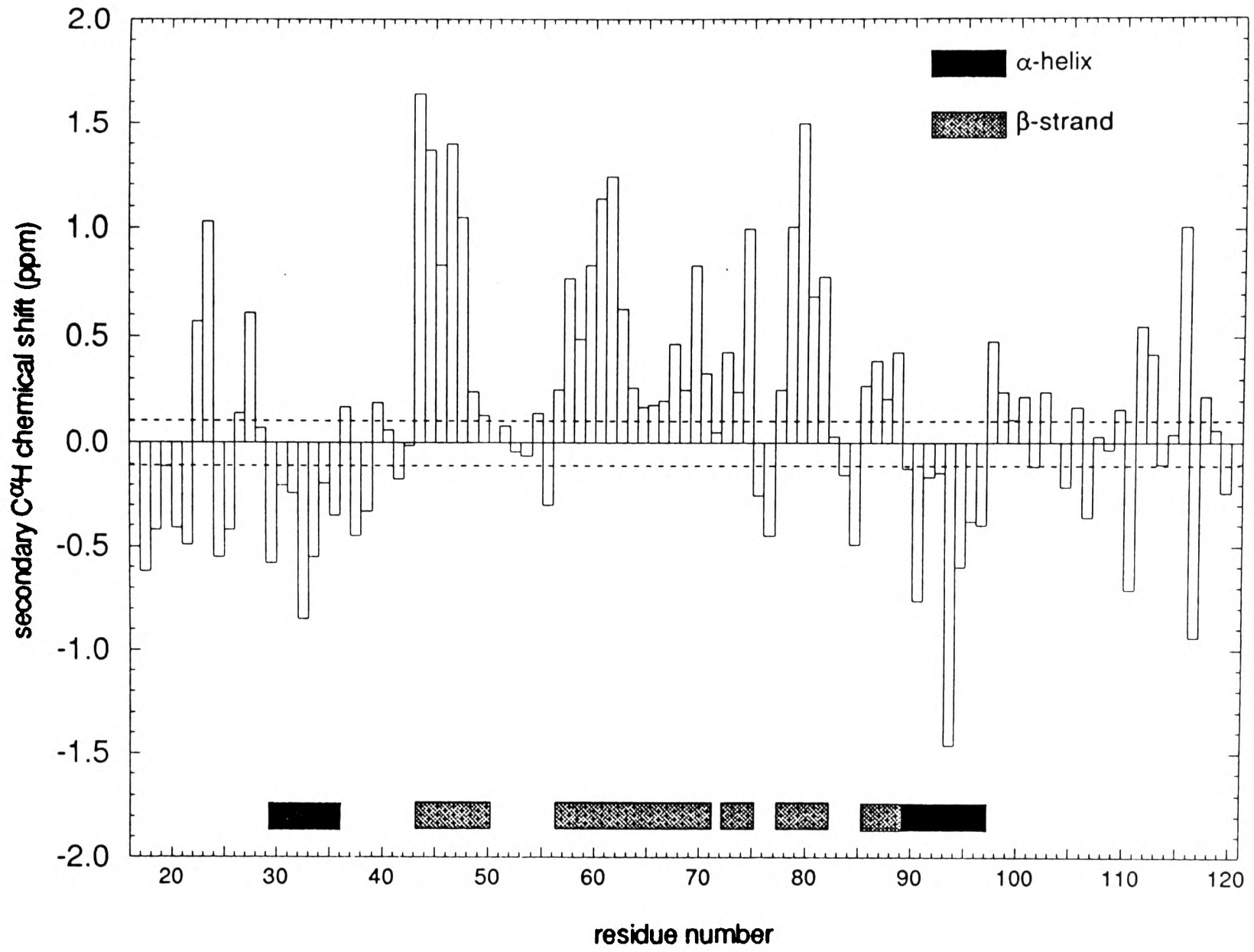
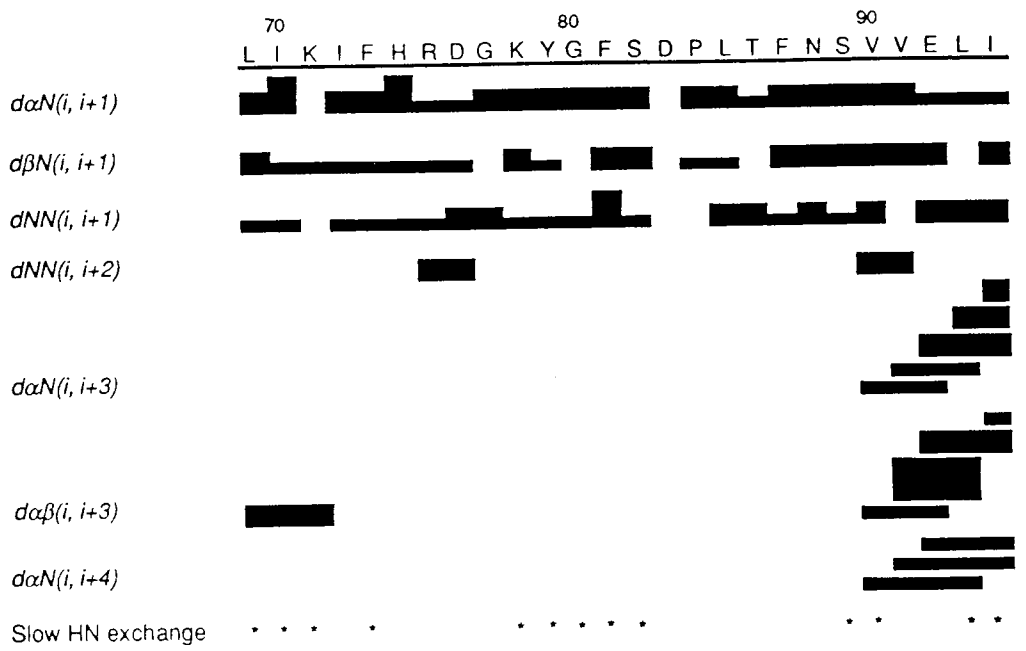
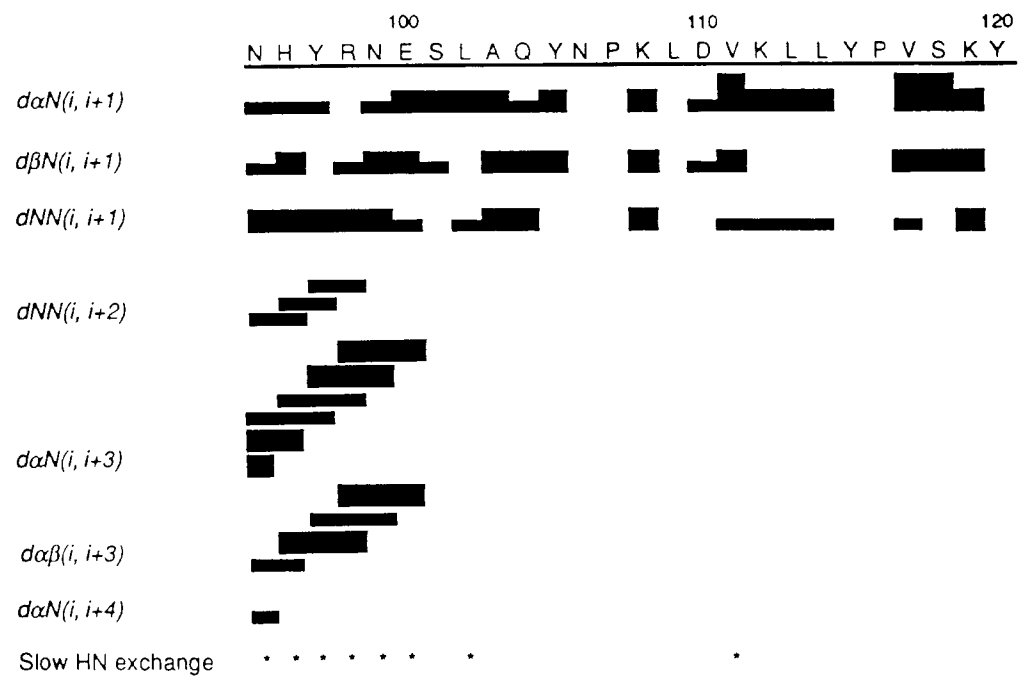
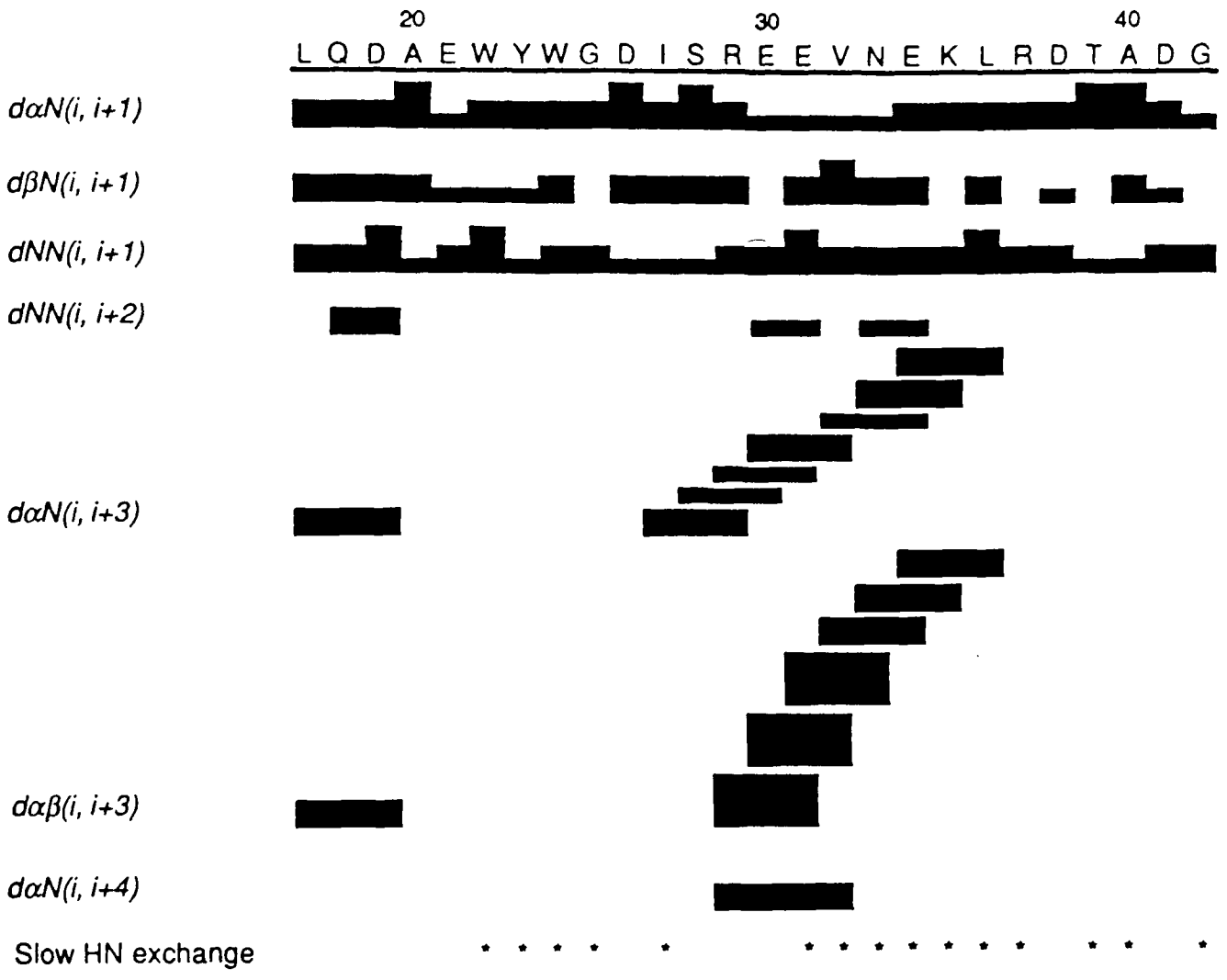


Figure 6.5

Secondary structure prediction of the p85α-N SH2 based on secondary CαH chemical shifts (Wishart *et al.*, 1992). The dashed lines are drawn at ± 0.1 ppm. Chemical shift perturbation greater than this threshold is considered significant. Four or more consecutive negative secondary shifts are predicted to be a helix, and a series three or more positive secondary shifts is predicted to be a strand. The prediction made on this basis is also shown, with strands and helices designated by shaded and opaque boxes respectively.


70
80
90
L I K I F H R D G K Y G F S D P L T F N S V V E L I
dαN(i, i+1)
dβN(i, i+1)
dNN(i, i+1)
dNN(i, i+2)
dαN(i, i+3)
dαβ(i, i+3)
dαN(i, i+4)
Slow HN exchange


100
110
120
N H Y R N E S L A Q Y N P K L D V K L L Y P V S K Y
dαN(i, i+1)
dβN(i, i+1)
dNN(i, i+1)
dNN(i, i+2)
dαN(i, i+3)
dαβ(i, i+3)
dαN(i, i+4)
Slow HN exchange



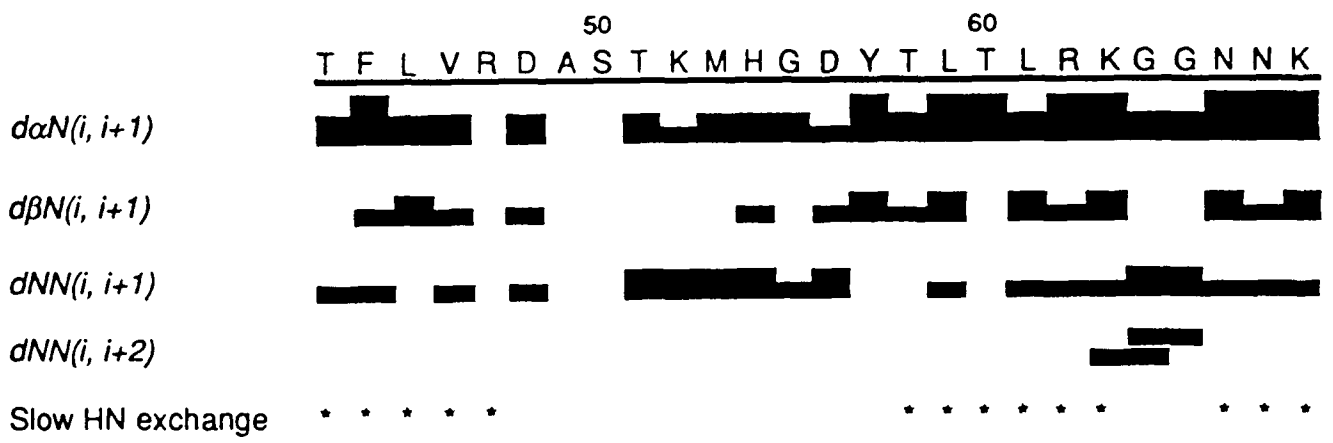


Figure 6.6

Summary of the short range NOE connectivities and slowly exchanging amide protons for the p85α–N SH2 module. Short interproton distances are indicated by lines linking the residues that contain the connected hydrogen atoms. The thickness of the lines is proportional to the NOESY crosspeak intensities.

			20										30					
leu	gln	asp	ala	glu	trp	tyr	trp	gly	asp	ile	ser	arg	glu	glu	val	asn	glu	lys
4.4	4.5	7.6	4.5	4.0	8.3	8.1	8.9	ND	7.4	6.2	ND	3.5	4.3	5.5	ND	3.8	ND	5.2
				40										50				
leu	arg	asp	thr	ala	asp	gly	thr	phe	leu	val	arg	asp	ala	ser	thr	lys	met	his
ND	ND	6.6	7.5	4.1	3.1	7.5	7.6	7.8	5.6	8.7	8.3	4.7	ND	ND	7.7	7.5	6.2	6.0
					60										70			
gly	asp	tyr	thr	leu	thr	leu	arg	lys	gly	gly	asn	asn	lys	leu	ile	lys	ile	phe
8.6	8.2	8.4	8.9	ND	9.1	8.6	ND	6.4	5.5	ND	8.8	7.8	8.5	7.9	9.0	5.1	7.6	8.6
						80										90		
his	arg	asp	gly	lys	tyr	gly	phe	ser	asp	pro	leu	thr	phe	asn	ser	val	val	glu
8.8	6.3	8.4	ND	ND	9.3	ND	7.3	5.9	4.4	ND	6.4	9.6	7.8	6.2	5.6	5.2	4.8	5.0

							100										110	
leu	ile	asn	his	tyr	arg	asn	glu	ser	leu	ala	gln	tyr	asn	pro	lys	leu	asp	val
4.0	5.3	4.0	ND	ND	ND	ND	7.9	5.0	6.3	4.8	5.3	ND	ND	ND	ND	8.4	ND	ND
								120										
lys	leu	leu	tyr	pro	val	ser	lys	tyr										
8.7	8.9	8.4	9.6	ND	7.3	5.6	6.0	8.6										

Figure 6.7

Summary of the $^{3}J_{HN\alpha}$ coupling constants for the p85α–N SH2 module which were determined by line shape fitting traces of peaks from a 2-dimensional ^{15}N-^{1}H HMQC-J experiment (Kay & Bax, 1990). Due to the low signal to noise of the data, the values shown here should be regarded as approximate. ND, not determinable.

measurement of coupling constants difficult. For this reason, the values in Figure 6.7 were treated as approximate when used in secondary structure assignment.

The n.m.r.-derived secondary structure pattern of the p85α-N SH2 module is shown in Figure 6.8. Helices αA and αB were easily recognised from the striking pattern of short range NOEs for residues 29 to 37 and 90 to 100. This conclusion was reinforced by slow amide proton exchange and coupling constant information except for the relatively high $^{3}J_{HN\alpha}$ value of 7.9 Hz for the C-terminal residue of helix αB, glu 100. These data were interpreted as a slight loosening of the helix at this junction. Seven regions corresponding to β-strand were identified which are labelled βA to βF in Figure 6.8. Specific features of the n.m.r. data for these regions are indicative of the environments of the strands and will be discussed with reference to determination of the tertiary fold of the module.

Aside from the regions of regular secondary structure identified in the p85α-N SH2 module the conformations of two other regions were suggested by the n.m.r. data. Predominantly small $^{3}J_{HN\alpha}$ coupling constants and negative secondary $C^{\alpha}H$ chemical shifts for residues 17 to 21 indicated that these amino acids have a helix-like configuration in the 3-dimensional structure. While this region seemed to be well-defined based on qualitative inspection of the ^{15}N-edited NOESY data, the loop spanning residues 50 to 54 appeared more flexible. The sharpness and scarcity of crosspeaks involving these amino protons contrasted the data for the rest of the sequence. In fact, ser 50 was not resolvable in any of the spectra, and all of the crosspeaks in strips 51 to 54 correspond to intraresidue or sequential connectivities. The $^{3}J_{HN\alpha}$ coupling constants for residues 51 to 54, lying in the range of 6 to 7 Hz, could reflect motional averaging, and the secondary chemical shifts for these amino

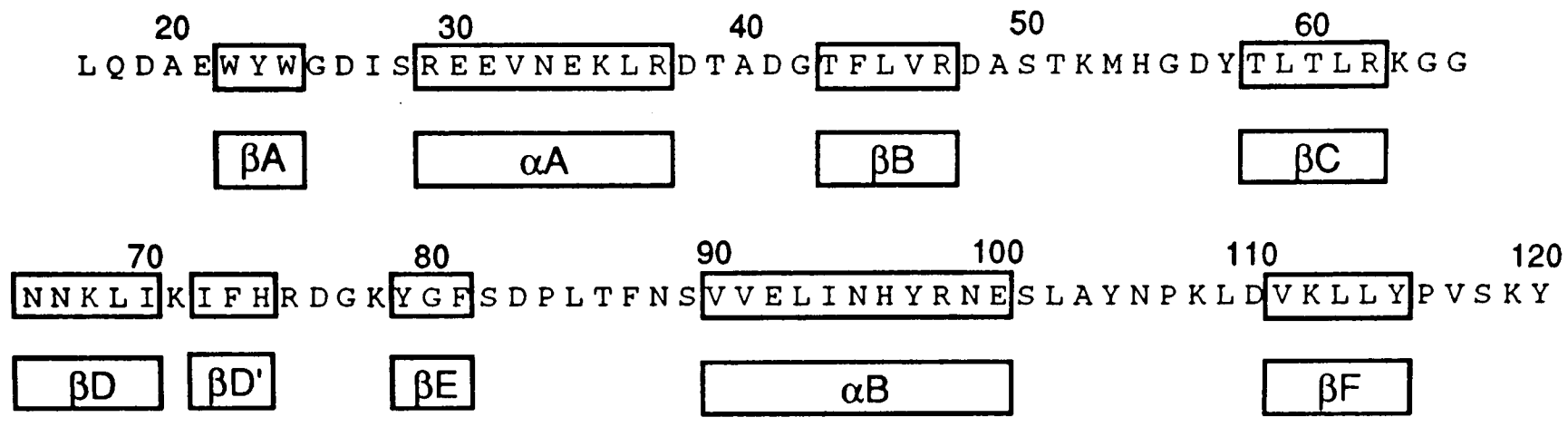


Figure 6.8

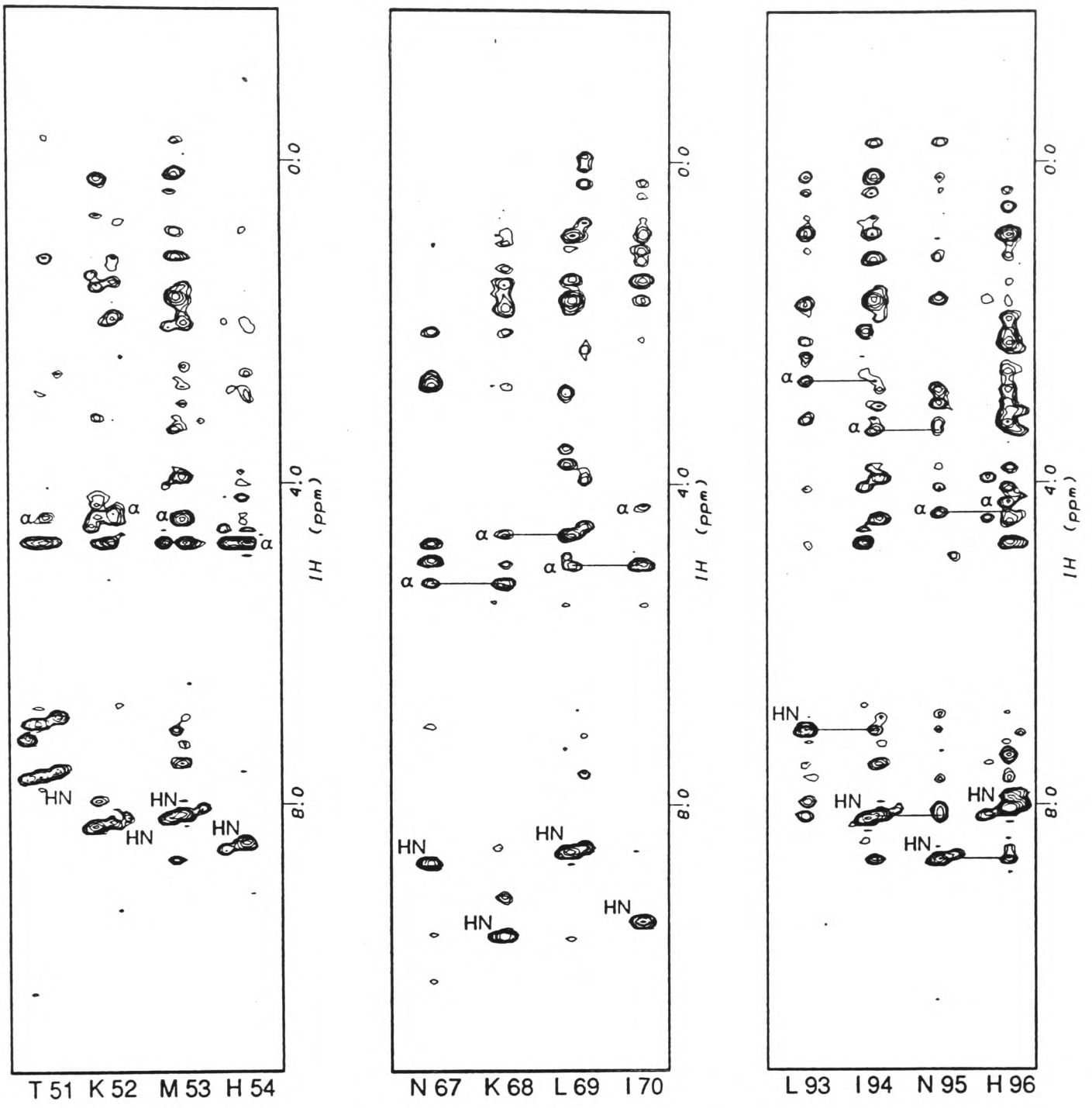
Summary of the secondary structure of the p85α–N SH2 module derived from NOESY, $^3J_{HN\alpha}$ coupling constant, amide proton exchange and chemical shift data. Seven regions corresponding to β-strand are labelled βA to βF. Inspection of the n.m.r. data also suggested that residues 17 to 21 would have a helix-like conformation, and that residues 51 to 54 would not have well-defined structure.

acids are all near random coil positions. All of these data are consistent with these residues forming a structurally disordered loop. Sequential ^{15}N-edited strips corresponding to this loop and to regions of β-strand and α-helix are presented in Figure 6.9 for qualitative comparison.

6.4 Tertiary Structure of the p85α-N SH2 Module

The secondary structure components of the p85α-N SH2 module which required assembly are summarised in Figure 6.10. Hydrophobic and charged residues are highlighted such that the character of each of these structural elements can be easily interpreted. The amino acid composition and n.m.r. data for each of these segments provided clues to their environments in the 3-dimensional structure. Hydrophobic residues at positions i, i + 3, i + 4, i +7, i + 8,... in an α-helix are likely to participate in a hydrophobic packing interaction of the helix with the core of the protein. Likewise an identical pattern of charged residues, such as that found in helix αA, is likely to be on the surface of a 3-dimensional structure. As a result, packing interactions between this helix and the core were predicted to involve val 32, asn 33 and leu 35. The pattern of hydrophobic residues in helix αB suggested that leu 93, ile 94 and tyr 97 would also form part of the core (Russell *et al.*, 1992), however the positive charge of arg 98 made strong hydrophobic interaction at the C-terminal end of the helix less probable.

Considering the first of the seven strands in Figure 6.10, all of the residues are hydrophobic and have slowly exchanging amide protons which suggested a buried environment. However, residue 22 has a strong sequential HN/HN connectivity and residue 24 has negative secondary $C^{\alpha}H$ chemical shift. Therefore, it seemed that these three residues would comprise an extended region but not part of a β-sheet.


HN
HN
HN

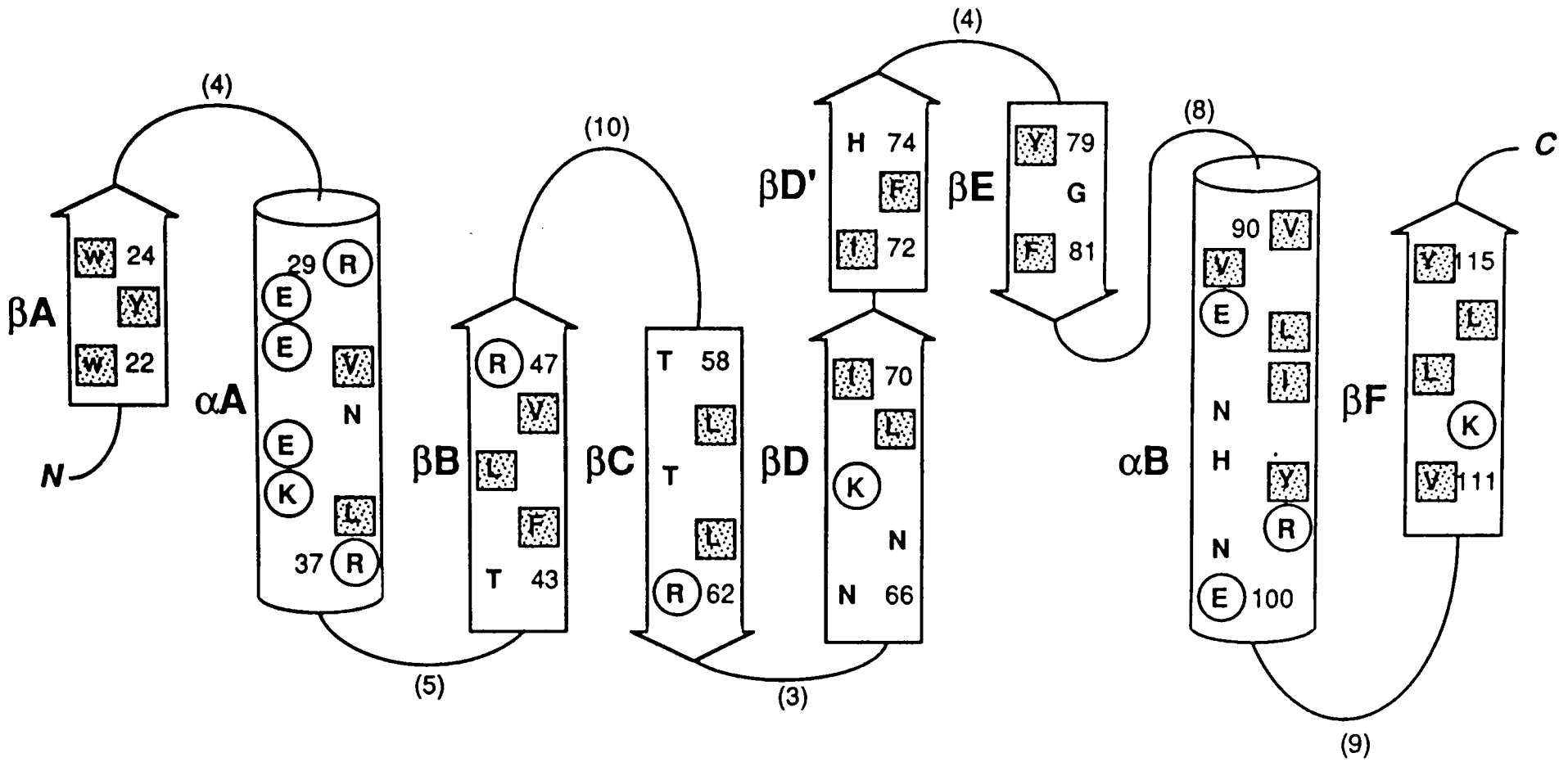


Figure 6.10

Schematic summary of the secondary structure elements of the p85α–N SH2 module which required assembly. The two α-helices (αA and αB) and seven β-strands (βA to βF) are represented by cylinders and arrows respectively. Hydrophobic residues are enclosed by shaded boxes, while those with charged side chains are circled. The number of the first and last residue for each component is shown, and the number of amino acids in each loop connecting the subunits is given in parentheses.

All of the residues in strand βB also have slowly exchanging amide protons, and their hydrophobic side chains (with the exception of arg 48) suggested that this region is a buried β-strand. It was concluded that this strand might form a part of the core of the structure, with the side chain of the last residue, arg 48, extending into solution.

Russell *et al.* (1992) predicted that residues 57 to 61 would form a surface β-strand because in the multiple sequence alignment the amino acids in this region alternate conservatively between hydrophobics and polar residues. However, in strand βC of the p85α-N SH2 two of the conserved polar residues are substituted by threonines. Hence it seemed likely that this strand is less exposed in this particular SH2 module. Based on the secondary $C^{\alpha}H$ chemical shift and the anomalously small $^{3}J_{HN\alpha}$ coupling constant of lys 71, it was thought that strands βD and βD' were separated by a kink at this position. All of the amide protons in strand βD were observed to be slowly exchanging, yet the characteristics of the amino acids comprising this strand did not strongly suggest either an exposed or buried environment. Of the three residues in strand βD', only the central amino acid has a slowly exchanging backbone amide proton; this is consistent with this region corresponding to a surface β-strand. The three residues in strand βE, on the other hand, all have slowly exchanging backbone HNs, so this strand was assumed to be buried. Finally, inconsistencies in the data defining strand βF (see Figure 6.6) led to the conclusion that these amino acids were likely to lie in an extended conformation.

The strategy for determination of the tertiary structure of this module was to first assemble the β-strands into sheets by assignment of interstrand NOE connectivities. Then long range contacts between the α-helices and the sheets were sought in order to ascertain their relative orientation. Since the 3-dimensional fold of the SH2 module was

unknown, there was a greater likelihood of NOE misassignment. The probability of identifying incorrect restraints increases with the total number of assignments. Hence the number of long range NOEs was maximised prior to commencement of structure calculations.

6.4.1 Assembly of the β-sheets of the p85-α N SH2

The assembly of the β-strands was a relatively straightforward process. A search for interstrand $C^{\alpha}H/C^{\alpha}H$ NOEs involving strand βA yielded only a single weak connectivity between residues 22 and 45. This finding was consistent with the notion that strand βA was extended and not part of a β-sheet. Strands βB, βC and βD, on the other hand, were found to form a triple-stranded antiparallel sheet with alternating $C^{\alpha}H/C^{\alpha}H$ close contacts and inferred hydrogen bonds. However, the three strands did not align as might have been predicted from Figure 6.10. Specifically, the first $C^{\alpha}H/C^{\alpha}H$ connectivity between strands βC and βD is thr 58 to lys 71. This NOE was of lower intensity than the others found in the triple-stranded sheet , supporting the theory that a kink existed at residue 71. The short loop from residue 75 to 78 made obvious the pairing of strands βD' and βE. A strong α-proton interstrand NOE was found between residues 74 and 79 and a weak one between residues 72 and 81. The "lock and key" fit of these two strands is worthy of mention: strand βD' has one aromatic residue in the centre while strand βE has two flanking a central glycine. The small side chain of the glycine ensures that the two strands can lock together with the phenylalanine ring of strand βD' neatly sandwiched between the tyrosine and phenylalanine of strand βE.

Surprisingly, when $C^{\alpha}H/C^{\alpha}H$ NOE connectivities were sought for strand βF, a strong interstrand NOE was found linking residues 112 and

101, and a medium one between residues 110 and 103. Based on the additional observation that both residue 102 and residue 111 have slowly exchanging amide protons, it was concluded that the conformation of residues 101 to 103 must be strand-like, and that they form a double-stranded sheet with 110 to 112. Obviously, the helical conformation of residues 90 to 100 prohibits the extension of the sheet beyond residue 112 of strand βF, which is in accord with an extended conformation for residues 113 to 115. Figure 6.11 presents the β-sheet pattern of the p85α-N SH2.

6.4.2 Orientation of the α-helices of the p85α-N SH2

Packing of the two α-helices against the β-sheets was more difficult than anticipated. In general, assignment of long range NOEs to aromatic ring protons, which usually play a large role in defining the hydrophobic core of the structure, was greatly complicated by a high degree of chemical shift degeneracy in the aromatic region of the 2-dimensional H_2O NOESY spectrum. Furthermore, exhaustive search yielded very few NOEs involving residues in the helices. For helix αA long range NOE connectivities from val 32/asn 33 to leu 45/thr 60, lys 35 to leu 45 and leu 36 to thr 43/phe 44 were found. NOEs were tabulated for helix αB involving contacts between val 90 to leu17/trp 22/tyr 79, val 91 to leu 17/trp 22/ser 20, leu 93 to gly 80/phe 81, ile 94 to trp 22/leu 102, tyr 97 to leu 113 and glu 100 to leu 114. A schematic summary of the NOE connectivities is shown in Figure 6.12. The small number of residues involved in long range helix packing interactions is striking.

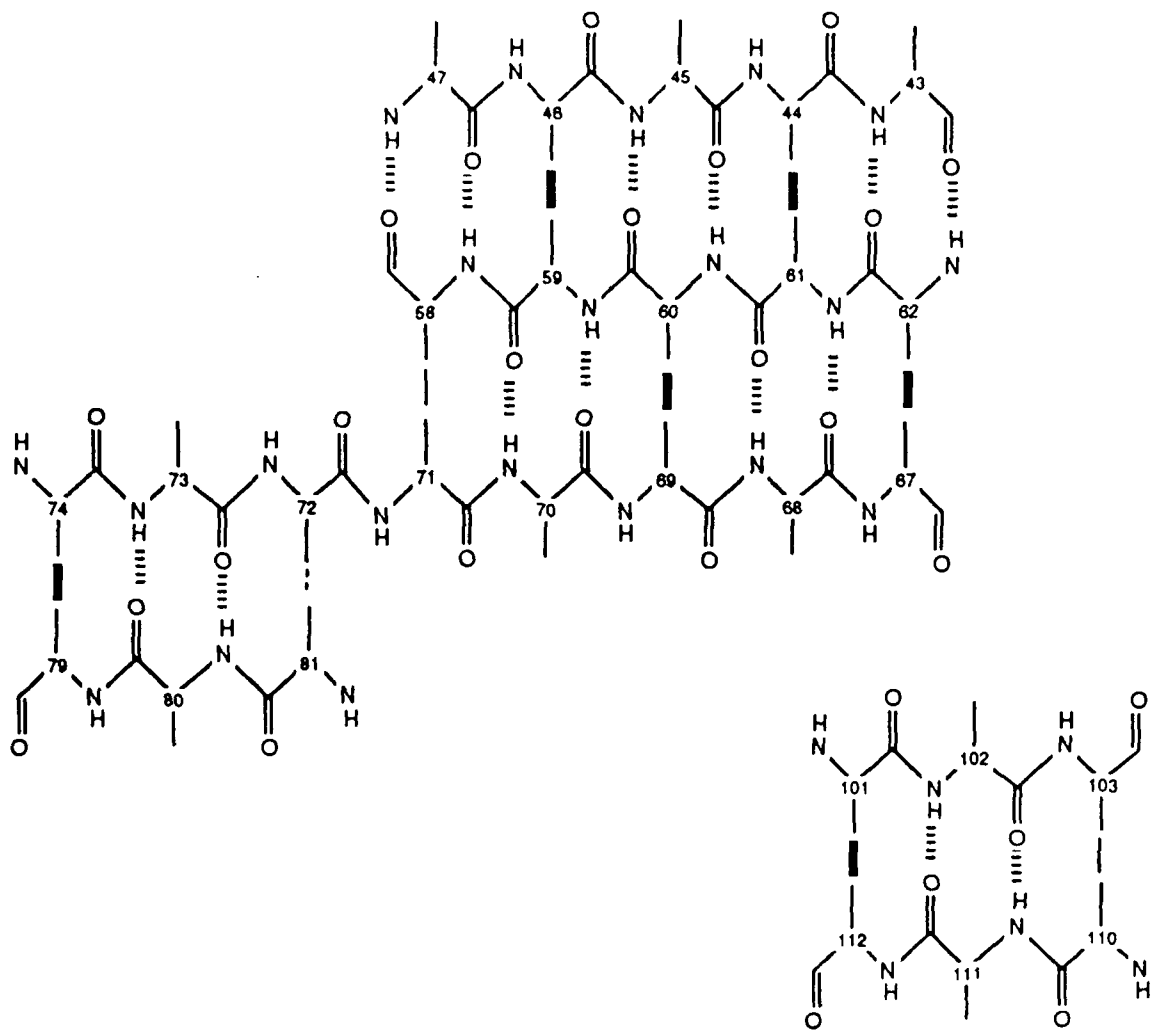


Figure 6.11

Summary of the antiparallel β-sheets of the p85α–N SH2 module. The hydrogen bonds connecting the strands (which were inferred from the observation of slowly exchanging amide protons) are indicated by horizontally dashed lines. Strong, medium and weak interstrand $C^{\alpha}H/C^{\alpha}H$ NOE connectivities are denoted by thick, thin and dashed lines respectively.

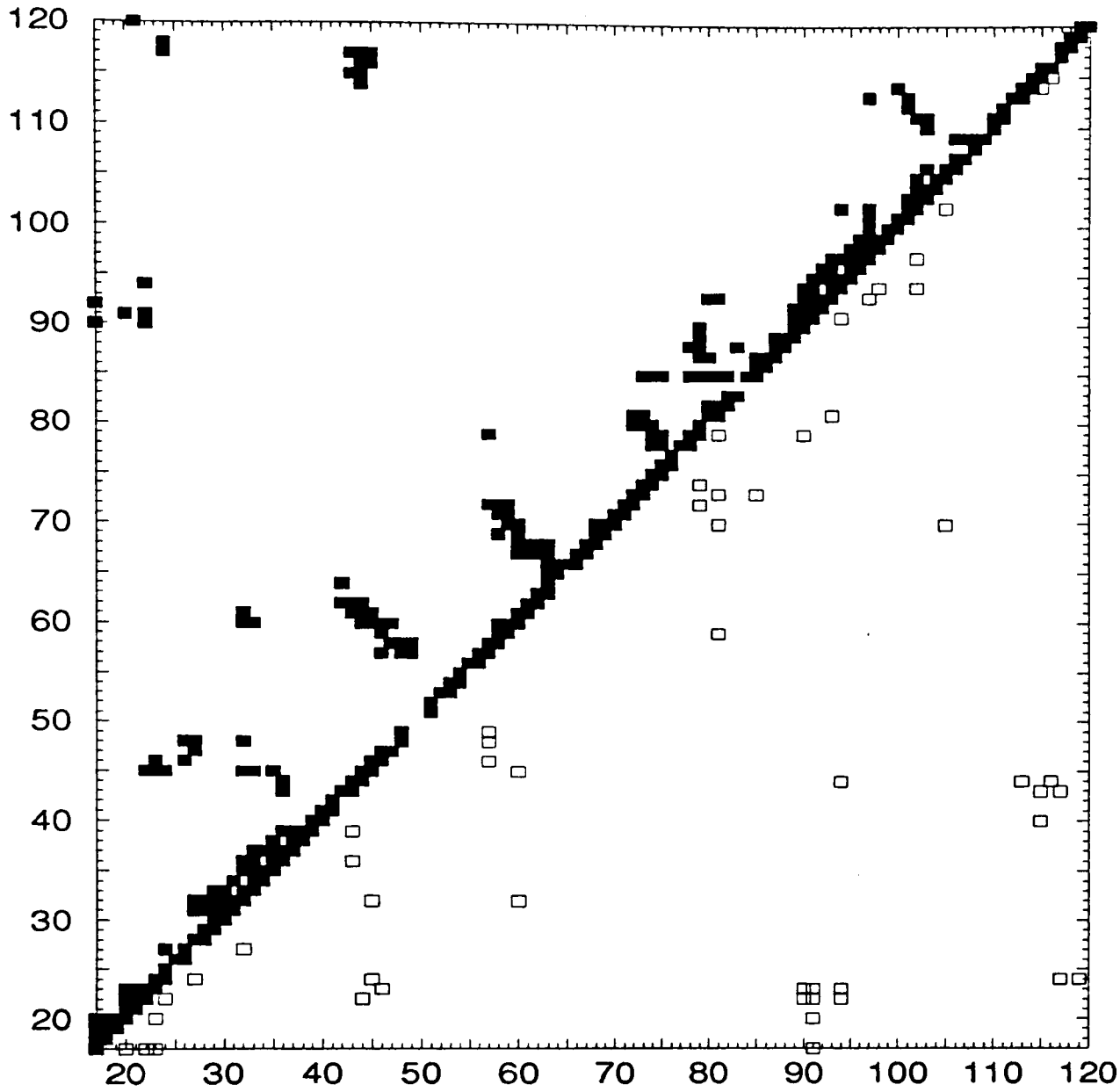


Figure 6.12

Schematic summary of the NOE connectivities defining the p85α–N SH2 structure. The two axes are numbered by residue from 17 to 120. Filled squares pair residues for which an NOE involving at least one backbone proton ($C^{\alpha}H$ or HN) was assigned. Open boxes connect residues for which side chain to side chain NOEs were tabulated. Note the low number of long range interactions involving residues in the α-helices (29 to 37 and 90 to 100).

6.4.3 Structure Calculations

The calculation of the p85α-N SH2 structure was performed analogously to that of the t-PA F1 module. Initial structures were calculated using dynamical simulated annealing (Nilges *et al.*, 1988a; Brünger, 1990). The starting backbone configurations were randomly generated with extended side chains. These structures were refined using the method described by Downing *et al.* (1992).

If an NOE is affected by spin diffusion, often the restraint associated with the NOE will be consistently violated by a small amount during the course of structure refinement. Such violated restraints are commonly relaxed, i.e., set to the next weaker category of distance constraint. Thus the list of experimental restraints is constantly refined during structure calculations. At the same time, incorrectly assigned NOEs must be identified and removed.

Normally a high number of NOEs used in structure calculations ensures a cooperative effect which helps pinpoint misassigned NOEs. Minor changes to the list of restraints do not usually change the structure globally. However, in the case of the p85α-N SH2 structure, the low number of restraints used to define the positions of the α-helices meant that such changes radically affected the tertiary fold and greatly complicated refinement.

Since every long range NOE weighed heavily in the calculations, when ambiguous restraints (particularly those involving aromatic ring protons) were alternately included, different 3-dimensional folds were calculated and it was not entirely clear which was more accurate. Consequently initial structures were calculated using a subset of the NOEs which could be assigned with a high level of confidence. Then the distances separating the protons involved in each ambiguous restraint

were measured in the crude structures to see which assignment was more likely to be correct. As the table of distance constraints was appended structures were frequently recalculated to verify new assignments. Use of this method was effective, although the high number of iterative structure calculations was computationally time consuming.

Final structure calculations were based on 1,481 interproton distance constraints including 581 intraresidue, 388 sequential ($|i - j| = 1$), 150 short range ($1 < |i - j| \leq 4$) and 362 long range ($|i - j| > 4$) restraints. Three categories of distance restraints were used in calculations corresponding the estimated intensities of NOEs: strong $\leq$ 2.8 Å, medium $\leq$ 3.3 Å and weak $\leq$ 5.0 Å. The lower limit for all distance constraints was the sum of the van der Waals radii, and the appropriate pseudoatom corrections were made to distances involving methyl or methylene protons (Wüthrich *et al.*, 1983).

In addition to the NOE derived distances, 54 restraints corresponding to 27 hydrogen bonds in the β-sheet and α-helical regions of the molecule that could be unambiguously assigned, based on the qualitative analysis of amide exchange rates, were included in the structure calculations as well as thirty dihedral angle constraints derived from measurement of $^3J_{HN\alpha}$ coupling constants. The complete list of experimental restraints used in the calculation of the p85α-N SH2 structures is included in Appendix II.

6.4.4 Description of the p85α-N SH2 structure

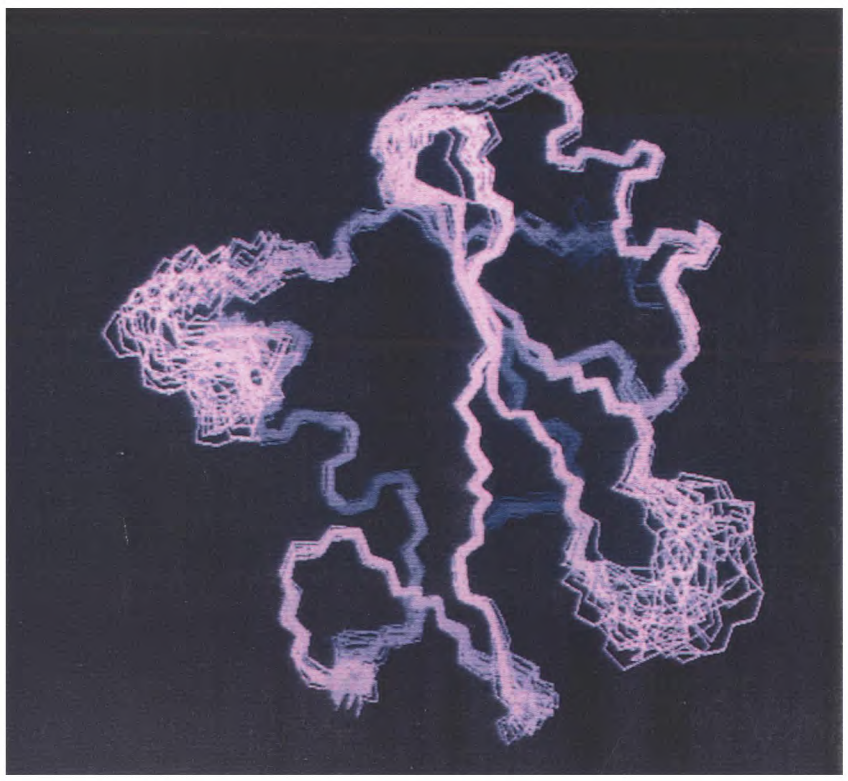
Using the final restraints lists a total of 83 refined simulated annealing structures converged from 100 random coil configurations. Of these, 22 were selected on the basis of agreement with the experimental

data, with the cut-off taken at F_{NOE} < 490 kJ mol^{-1}. All of the chosen structures satisfy the n.m.r. constraints within the experimental error, with no constraint violations over 0.5 Å and no torsion angle violations greater than 3°. Deviations from idealised geometry are small, and the negative value of -1131 kJ mol^{-1} for the Lennard-Jones energy (which is not used in the target function for the SA calculations) indicates that nonbonded contacts are good .

The 22 structures are shown in Figure 6.13 superposed with best-fit on the backbone atoms of residues 17-49, 56-102 and 110-118. A histogram of the number of NOE distance constraints and r.m.s. deviation values plotted for each residue number for the structures is also presented. The overall atomic r.m.s. difference between the individual structures and the mean coordinate positions is 0.48 ± 0.08 Å for the backbone atoms (residues 17-49, 56-102 and 110-118) and 1.17 ± 0.13 Å for all heavy atoms (residues 17-50 and 55-120) indicating that the structures are of medium resolution. Residues excluded from these calculations were considered to be disordered on the basis of the available n.m.r. data. A mean structure was calculated from the 22 individual best-fitted structures by coordinate averaging. The covalent geometry of the mean structure was regularised by restrained minimisation as in the last stage of the refinement. This restrained minimised average structure satisfies the experimental constraint data as well as any of the individual SA structures. A summary of the structural statistics for structures is presented in Table 6.1.

In Figure 6.14 the schematic representation of the average structure of the p85α-N SH2 module shows the relative orientation of the β-sheets and α-helices. If a coordinate axis is superimposed on the structure as shown, the molecule is spherical but slightly flattened along the *z*-axis with dimensions of approximately 37 Å x 37 Å x 30 Å. It can be

Figure 6.13 A



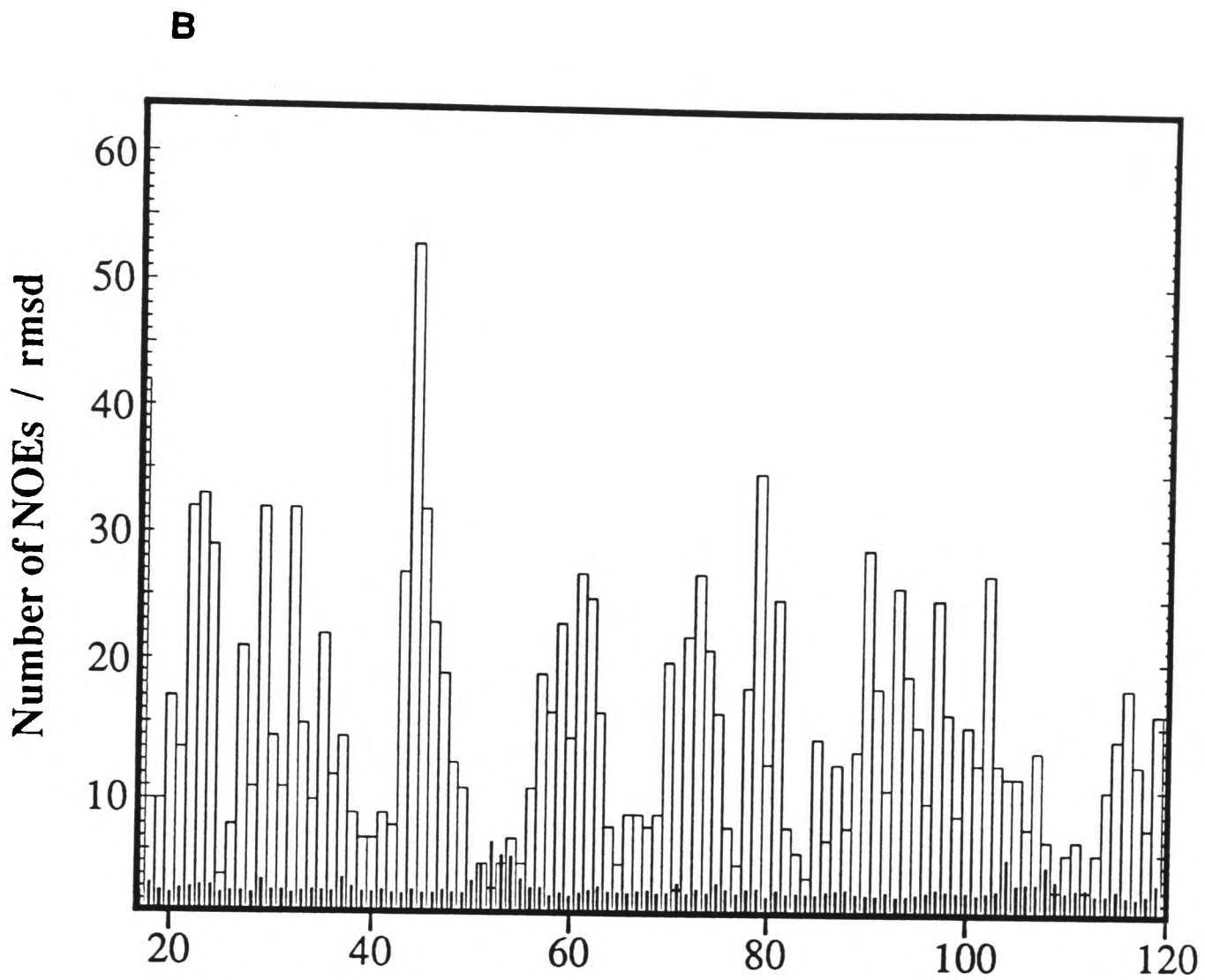


Figure 6.13

Superposition of the 22 final structures of the p85α–N SH2 module (A) and histogram of the number of NOE distance constraints (open bars) and r.m.s. deviation values (closed bars) plotted against residue number for the structures shown (B). The structures in A are superposed with best fit on the backbone atoms (C, C^{α}, N) of residues 17-49, 56-102 and 110-118.

Table 6.1

Structural Statistics for the p85α-N SH2 Module[a]

	<SA>	$(SA)_r$
RMS deviations from exptl distance restraints (Å)[b]		
all (1481)	0.047±0.002	0.040
interproton distances		
intraresidue (581)	0.045±0.004	0.037
sequential (388)	0.045±0.004	0.039
interresidue short range ($1 < \|i-j\| \leq 4$) (150)	0.047±0.005	0.036
interresidue long range ($\|i-j\| > 4$) (362)	0.050±0.004	0.044
H-bonds (54)[c]	0.062±0.009	0.046
RMS deviations from exptl dihedral restraints (deg) (30)[b,d]	0.48±0.13	0.30
Deviations from idealized covalent geometry		
bonds (Å)	0.009±0.0	0.009
angles (deg)	2.161±0.007	2.439
impropers (deg)[e]	0.970±0.013	0.950
F_{NOE} ($kJ.mol^{-1}$)[f]	426.5±41.0	303.7
F_{repel} ($kJ.mol^{-1}$)[g]	236.1±22.1	190.2
F_{VDW} ($kJ.mol^{-1}$)[h]	-1131.5±52.5	-972.0

[a] The notation of the structures is as follows: <SA> are the 22 refined simulated annealing structures; $(SA)_r$ is the restrained minimised mean structure, where the mean structure was obtained by averaging the coordinates of the individual <SA > structures best fitted to each other. The number of terms for the various restraints is given in parentheses.

[b] None of the structures exhibit distance violations greater than 0.5 Å or dihedral violations greater than 3^o.

[c] Each hydrogen bond is characterized by two distance restraints: r_{NH-O}, 2.1-2.3 Å and r_{N-O}, 2.1-3.3 Å. All hydrogen bonding restraints involve slowly exchanging backbone amide protons and were only included after they could be unambiguously assigned following initial structure calculations.

[d] The torsion angle restraints comprises 30 ϕ angles.

[e] The improper torsion restraints serve to maintain planarity and chirality.

[f] The value of the square-well NOE (F_{NOE}) is calculated with a force constant of 210 $kJ.mol^{-1}.Å^{-2}$.

[g] The value of the quartic van der Waals repulsion term F_{rep} is calculated with a force constant of 17 $kJ.mol^{-1}.Å^{-4}$ with the hard sphere van der Waals radius set to 0.8 times their standard values.

[h] The van der Waals energy F_{VDW} is calculated with the CHARMM (Brooks *et al.*, 1983) empirical energy function and is *not* included in the target function for simulated annealing or restrained minimisation.



Figure 6.14

Schematic representation of the structure of the p85α–N SH2 module. α-helices and β-sheets are shown as magenta coils and yellow ribbons respectively. The dimensions of the molecule as shown are approximately 37 Å x 37 Å x 30 Å.

described as a central triple-stranded antiparallel β-sheet with a kink at the end of the third strand (at lys 71) preceding a short double-stranded antiparallel β-sheet. The two helices pack onto either side of the central β-sheet, mainly through hydrophobic interactions, thus this structure has two divided regions of hydrophobic core. A second short two-stranded antiparallel β-sheet near the C terminus folds back to lie over one face of the central β-sheet, placing the N and C termini of the molecule (as defined by sequence homology) approximately 15 Å apart on one side of the molecule. The regions of secondary structure are linked by a number of turns and mobile loops. In particular, residues 50-56 in the loop joining the first two strands of the central β-sheet do not have well-defined conformation. This lack of structural definition agrees with the qualitative interpretation of the NOESY data for residues 50-54.

6.4.5 Comparison of the SH2 secondary structure with prediction

Multiple sequence alignment combined conservation analysis led to the secondary structure prediction of Russell *et al.* (1992) which is presented in Figure 6.15. The prediction of the first helix was considered weak because it was not based on any clear hydrophobic pattern. An alignment of the experimentally determined p85α-N SH2 secondary structure and this prediction are shown in Figure 6.16. In order to assess the accuracy of the prediction, the number of residues assigned to the correct secondary structure motif was computed. Considering only the 78 residues of the p85α-N SH2 exhibiting strong sequence similarity to other SH2 modules (residues 21 to 98), the prediction of Russell *et al.* (1992) scores 60 correct assignments. This score corresponds to 77% accuracy which is significantly higher than the previously established

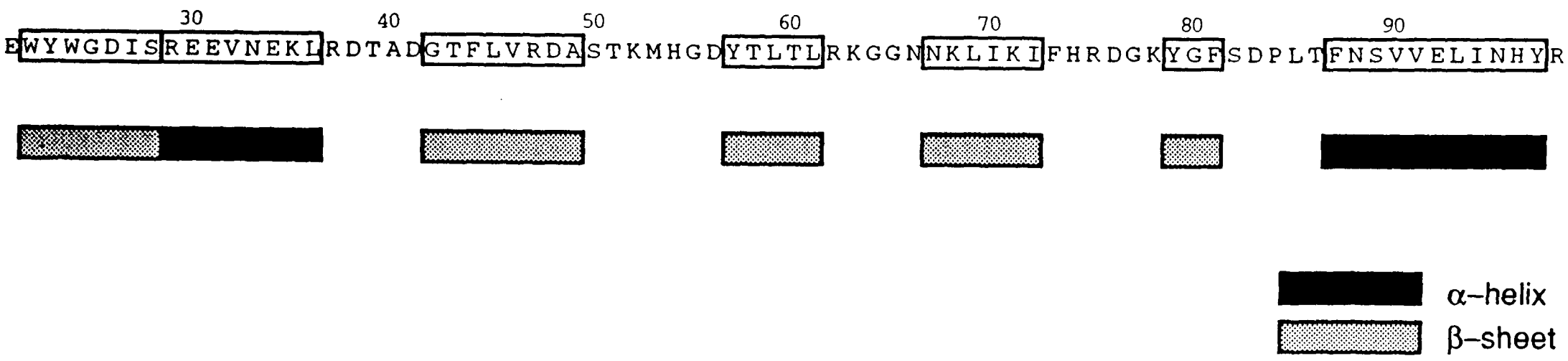


Figure 6.15

Secondary structure prediction of the p85α–N SH2 module according to Russell *et al.* (1992). Helical and strand regions of the sequence are boxed. Predicted β-strands and α-helices are aligned with shaded and opaque boxes respectively. All remaining regions were assigned as loop or non-core.

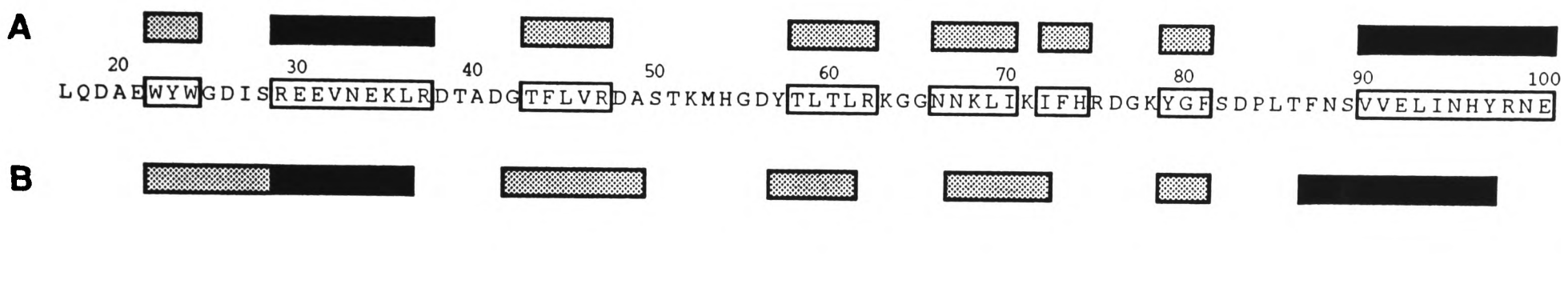


Figure 6.16

Alignment of the n.m.r.-derived secondary structure of the p85α–N SH2 module (A) with the prediction of Russell *et al.* (1992) (B). Regions of β-strand and α-helix are denoted by shaded and opaque boxes respectively.

upper limit of 65% accuracy for prediction of loop, α-helix and β-strand positions in a single protein (Sternberg, 1992).

6.5 Significance of the p85α-N SH2 Structure

Knowledge of the structure of the p85α-N SH2 module allowed the results of mutagenesis and sequence comparison studies to be interpreted more fully. In addition, a comparison was made between the solution structure of the p85α–N SH2 and the *v-src* SH2 co-crystallised with phosphopeptides (Waksman *et al.*, 1992) to estimate the degree of conformational change associated with binding and to gain insight into the mechanism of phosphopeptide recognition.

6.5.1 Spatial proximity of functionally important residues

Russell *et al.* (1992) observed that in the alignment of 67 SH2 module sequences for maximum homology, 14 residues are either absolutely conserved or represented by only two alternative amino acids. Of these 14, 10 (22, 23, 36, 42, 44-46, 72, 87 and 93) correspond to residues that have hydrophobic side chains buried within the molecule and are most probably conserved because they maintain the structural integrity of the module. The remaining four strongly conserved residues (arg 29, arg 47, thr 58 and leu 69) were speculated to be critical to the function of the SH2 module (Mayer & Hanafusa, 1990; Booker *et al.*, 1992), i.e., binding to tyrosine phosphorylated proteins. Figure 6.17 highlights the close proximity of these residues in the p85α-N SH2 structure. Other residues identified by site directed mutagenesis as important for binding activity that are not highly conserved correspond to ala 49 and thr 51 in the p85α-N SH2 sequence (Mayer *et al.*,



Figure 6.17

Schematic illustration of the p85α–N SH2 module highlighting the close proximity of proposed phosphotyrosine binding residues. These amino acids (arg 29, arg 47, thr 58 and leu 69) are strongly conserved (Russell *et al.*, 1992), and residues 47 and 69 have been identified as critical for binding from site directed mutagenesis experiments (Mayer & Hanafusa, 1990). Residues in the flexible loop connecting strands βA and βB are also likely to be important.

1992), both of which are found in the highly mobile loop connecting strands βA and βB. In light of this it was proposed that the loop also participates in the phosphopeptide binding interaction.

6.5.2 Comparison with the *v-src* SH2 module

The crystal structure of the *v-src* SH2 module complexed with tyrosine-phosphorylated peptides (Waksman *et al.*, 1992) was reported concurrently with the solution structure of the p85α-N SH2 module. The two peptides used in the crystallographic structure determination both bound to the *v-src* SH2 weakly, with millimolar concentrations of peptide required to compete for SH2 binding to the phosphorylated epidermal growth factor receptor (Waksman *et al.*, 1992). Consequently, although several highly specific contacts were observed between the SH2 module and the phosphotyrosine, very few interactions were seen between the rest of the peptide and the protein. Thus the mechanism of sequence specific binding was not revealed by these structures.

Comparison of the *v-src* and p85α-N SH2 structures confirmed the biological role of residues corresponding to positions 29, 47, 49-52 and 69 in the p85α-N SH2 sequence. All of these residues make direct contact with the phosphotyrosine with the exception of 69. The side chain of leu 69 forms part of the small cleft in which the phosphotyrosine binds. The histidine at this position in the p85α–N SH2 is probably not charged in the ligand-bound structure, hence the hydrophobic interaction of its side chain with the tyrosine ring is most likely conserved. The only residue predicted from sequence alignment to be involved in the phosphopeptide binding of the p85α-N SH2 which was not accounted for by Waksman *et al.* (1992) is thr 58. This residue is nonconservatively substituted by a cysteine in the *v-src* SH2 which might explain why this

position was not observed to be critical to the binding interaction of this particular SH2 module. Alternatively, this residue may be engaged in highly specific protein-peptide interactions not observed in the *v-src* complexes.

The backbone atoms of the p85α-N and *v-src* SH2 structures are shown superposed in Figure 6.18. The structures have been overlaid based on the coordinate positions of the 42 backbone atoms in conserved regions of secondary structure with a r.m.s. deviation of 1.71 Å. When only the backbone atoms of residues in the central β-sheet are overlaid the r.m.s. deviation is 0.70 Å. The overlaid structures of the two molecules provided an improved sequence alignment which is also shown in Figure 6.18.

The structures of the two SH2 modules, one with and one without the phosphopeptide bound, are remarkably similar. The secondary structure of the two molecules is virtually identical with the exception of insertions and deletions in the loop regions. In the *v-src* SH2 structure the side chains of two conserved arginines (155 and 175) were observed to make specific contacts to the phosphate group of the phosphotyrosine. A comparison of the positions of the corresponding residues (arg 29 and arg 47) in the p85α-N SH2 structure gives an indication of the degree of conformational change induced by ligand binding. Figure 6.19 presents a magnified superposition of this region of the two structures. It should be borne in mind that in solution the side chain conformations of the two arginines are not as well-defined without the ligand bound. Therefore the most enlightening comparison is made between the positions of the C^{α} backbone atoms of these residues. It appears that upon binding, the C^{α} atom of arg 29 is displaced by approximately 2.4 Å in order for the side chain to swing forward into position. Similarly, the C^{α} of arg 47 moves backwards by about 3 Å to accommodate the phosphotyrosine.

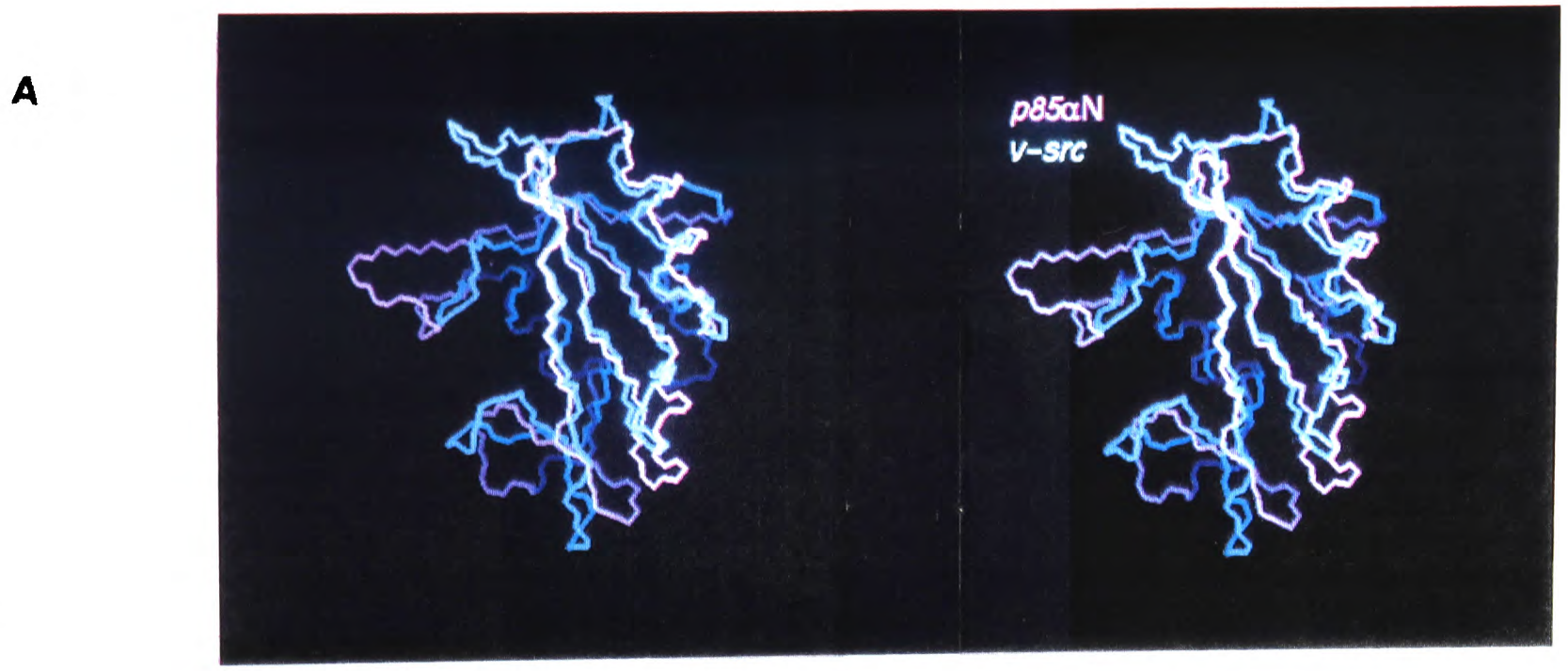


Figure 6.18

Stereoview superposition of the structures of the p85α–N and *v-src* (Waksman *et al.*, 1992) SH2 modules (A) and revised sequence alignment (B). The p85α–N SH2 (magenta) and the *v-src* SH2 (cyan) have been fit on the backbone (C, C^{α}, N) atoms of the 42 residues in conserved regions of secondary structure, which are shown boxed in B, with an atomic r.m.s. deviation of 1.71 Å.

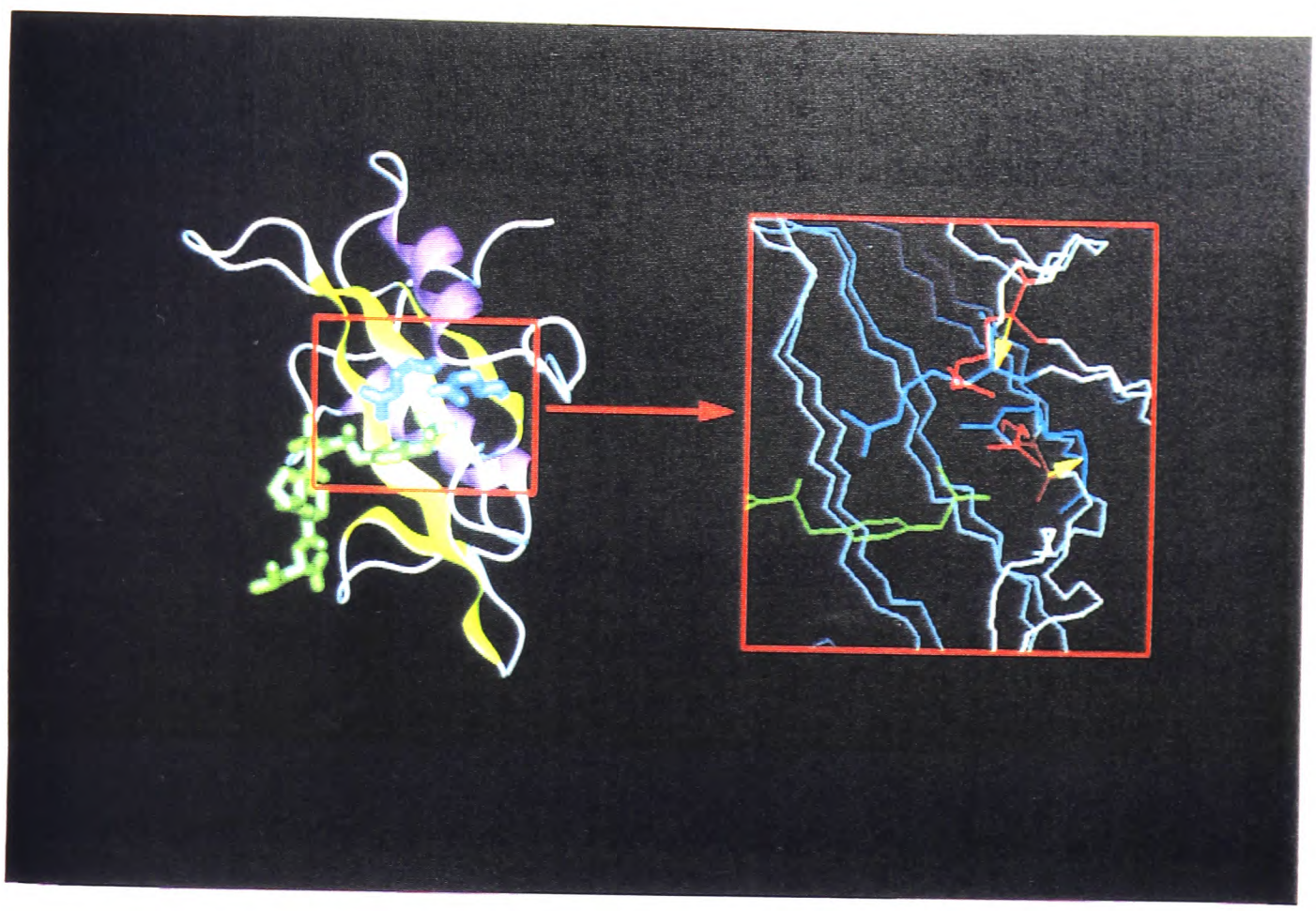


Figure 6.19

Illustration of the displacement of the $C^{\alpha}H$ atoms of the two highly conserved arginine residues (arg 29 and 47 of the p85α–N SH2 sequence) upon phosphopeptide binding. On the left, a schematic drawing of the *v-src* SH2 module complexed with a phosphopeptide (green) highlights the two arginines (cyan) that contact the phosphotyrosine (Waksman *et al.*, 1992). A magnified view of the phosphotyrosine binding region of the superposed structures of the p85α–N and *v-src* SH2 modules is shown on the right. The conserved arginine side chains are shown for the uncomplexed (p85α–N) and complexed (*v-src*) structures in red and cyan respectively. The displacement of the $C^{\alpha}H$ atoms of these residues upon binding is indicated by yellow arrows.

These speculations lead to the conclusion that the conformational change of the SH2 module when a phosphopeptide is bound is very subtle.

Subsequent comparison of the crystal structures of *Src* in the presence and absence of a high affinity peptide confirmed that only highly localised and relatively small conformational changes occur upon peptide binding (Waksman *et al.*, 1993). However, it should be noted that spectroscopic evidence suggests that the p85α-N SH2 undergoes a large conformational change upon ligation of specific peptides (Panayotou *et al.*, 1992; Shoelson *et al.*, 1993). The most significant structural difference between the p85α-N and *v-src* SH2 structures is the relative insertion of a short stretch of antiparallel β-sheet C-terminal to the second α-helix. Hence it was postulated that a larger conformational change in the p85α-N SH2 might be associated with this loop clamping down on bound peptides (Booker *et al.*, 1992; Shoelson *et al.*, 1993). Nevertheless, it was concluded from the *Src* results that extensive conformational changes are not likely to be a general feature of SH2-peptide interactions.

6.6 Modelling of SH2 Binding Interactions

The comparison of the two SH2 modules laid the foundation for interpretation of the results of several independent studies aimed at dissecting the specificity of phosphopeptide binding by SH2 modules. Analysis of the specific protein-peptide interactions of *v-src* highlighted the residues involved in phosphopeptide recognition. The structures of the C-terminal SH2 of PI3-kinase (p85α-C SH2) and the N-terminal SH2 of the GTPase activating protein of p21-ras (GAP-N SH2) were modelled. Then these models were used to pinpoint amino acids likely to be critical

for binding interactions and to explain known differential binding affinities of the two SH2s from PI3-kinase and the GAP-N SH2.

6.6.1 Specificity of phosphopeptide binding interactions

Mutagenesis studies have established that both PI3-kinase and GAP bind to phosphorylated tyrosines in the kinase insert region of the human platelet-derived growth factor receptor β-subunit (βPDGFR) (Kaplan *et al.*, 1990; Kazlauskas & Cooper, 1989; Kazlauskas & Cooper, 1990). The kinase insert, a region of approximately 70 amino acids which interrupts the kinase domain of the βPDGFR, contains three major phosphorylation sites which were mapped by Kashishian *et al.* (1992) to tyrosines 740, 751 and 771 (which correspond to tyrosines 708, 719 and 739 of the mouse βPDGFR). Furthermore, it was shown that elimination of the tyrosine phosphorylation sites at positions 740 and 751 abolishes association of PI3-kinase, while high affinity binding of GAP requires the site at tyrosine 771. Deletions of more than 10 amino acids adjacent to residues 740 and 771 had little effect on the binding of PI3-kinase or GAP, which is consistent with the primary determinant of binding specificity being the local sequence surrounding the phosphotyrosine (Escobedo *et al.*, 1991) and not its position in the overall 3-dimensional structure of the receptor.

The requirements for high affinity interactions of PI3-kinase and GAP with the receptor have been elucidated by competitive binding studies. Fantl *et al.* (1992) synthesised short phosphotyrosine-containing peptides representing βPDGFR sequences and tested their ability to compete with the association of PI3-kinase and GAP to the receptor in vitro. With respect to the binding specificity of PI3-kinase it was found that 5 amino acid peptides corresponding to positions 708 (Y^{P}MDMS)

and 719 (Y^{P}VPML) of the mouse βPDGFR blocked PI3-kinase enzyme activity. However neither of these peptides inhibited association of GAP to the receptor, even at high concentrations. In addition, only peptides containing a methionine at position +3 downstream of the phosphotyrosine efficiently blocked association of PI3-kinase, and the addition of amino acids from the receptor sequence on the amino-terminal side of the phosphotyrosine had no effect of the binding. Finally, a minimum of 5 amino acids was required for inhibitory activity.

In a similar search for receptor sequences that bind selectively to GAP, octameric peptides were synthesised representing each of the 29 cytoplasmic tyrosines of the PDGF receptor, each with 7 amino acids carboxy-terminal to the phosphotyrosine (Fantl *et al.*, 1992). Of the 29 peptides tested, only the one corresponding to position 739 of the mouse βPDGFR (Y^{P}MAPYDNY) could efficiently block association of GAP.

The results of the study of Fantl *et al.* (1992) were complemented and refined by a kinetic analysis of the binding of short peptides (9 to 17 amino acids in length) corresponding to sites 740 and 751 of the human βPDGFR to isolated SH2 modules (Panayotou *et al.*, 1993). The modules tested included the N- and C-terminal SH2 modules of PI3-kinase and the N-terminal SH2 of GAP. For modelling purposes, since the length of the peptides used in this study was not constant, the results will only be considered qualitatively as summarised in Figure 6.20. The N-terminal SH2 from PI3-kinase bound with significantly higher affinity to the peptide corresponding to the sequence surrounding tyr 751 than to the one encompassing residues surrounding tyr 740. The p85α–C SH2 associated equally tightly with both peptides. In contrast, the GAP-N SH2 was observed to adhere with an intermediate affinity to site 740 but not at all to site 751.

human βPDGFR position / SH2 module	740	751
p85αN	+	+++
p85αC	+++	+++
GAP-N	++	-

Figure 6.20

Summary of the results of the kinetic analysis of binding of short peptides to the p85α–N, p85α–C and GAP-N SH2 modules (Panayotou *et al.*, 1993). Each "+" corresponds to an order of magnitude in observed binding, i.e., the binding of the p85α–N SH2 to the peptide corresponding to position 751 of the human βPDGFR (DMSKDESYDY^{P}VPMLDMK) is 10^2 times stronger than its binding to the peptide representing position 740 (GESDGGY^{P}MDMSK). A "-" indicates that no significant protein-peptide interaction was observed.

6.6.2 Modelling of p85α-N, p85α-C and GAP-N SH2 modules and prediction of sequence-specific phosphopeptide interactions

The results of mutagenesis and competitive binding studies strongly suggested that the difference in binding affinity of a specific SH2 module for two different peptides is solely dependent on the peptide sequence and not on any tertiary structure of the sequence. In support of this theory, the conformations of the two peptides co-crystallised with *v-src* (Waksman *et al.*, 1992) are nearly identical in spite of the differences in the two sequences. An overlay of the peptides (Y^PVPML and Y^PLRVA) is shown in Figure 6.21. It is particularly interesting that the backbone atoms of the arginine and the less flexible proline in position +2 downstream of the phosphotyrosine are in virtually identical positions.

The binding of a phosphopeptide by a SH2 module can be likened to a hand slipping into a glove. Different hands may be inserted into the same glove with a varying degree of fit. Since the glove size is constant, only the sizes of the hands need to be considered in order to understand the relative fit. However, understanding why *different* gloves fit some hands and not others requires analysis of the glove and hand sizes simultaneously. If in this analogy the gloves represent SH2 modules and the hands are phosphopeptides, then it is clear that comparison of the binding affinities of different SH2 modules for various peptides requires consideration of the peptide sequences *and* of the residues contacting the peptides in the various modules.

Careful inspection of the peptide-protein contacts in the *v-src* complex highlighted amino acid positions which participate in binding interactions. Waksman *et al.* (1992) observed that of the five residues in each peptide, only the phosphotyrosine and amino acids in position +1

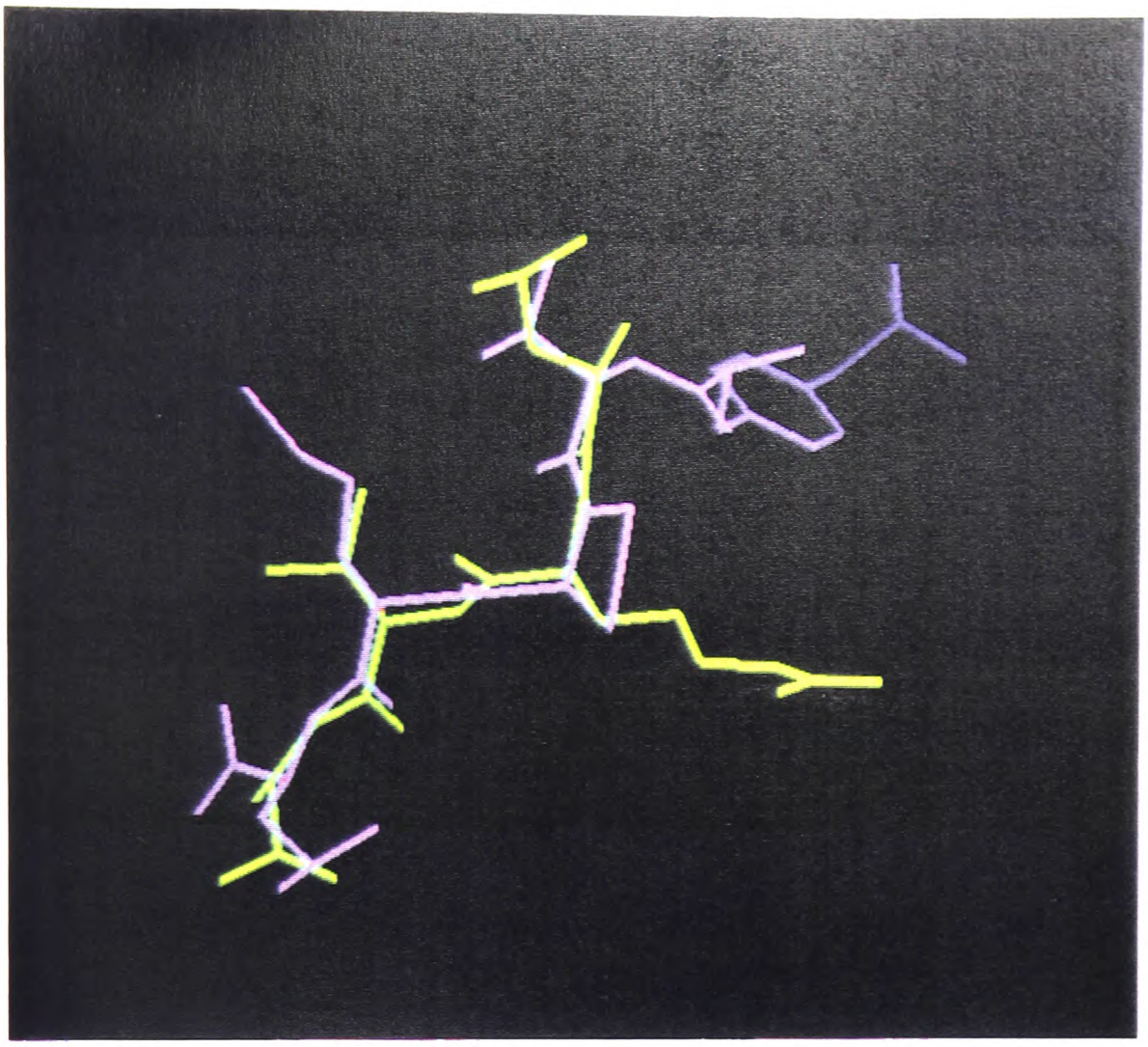


Figure 6.21

Superposition of the two peptides (Y^PVPML and Y^PLRVA) which were complexed with the *v-src* SH2 module (Waksman *et al.*, 1992). The positions of the backbone atoms are virtually identical.

and +3 make contacts with the SH2 module. Several protein-peptide interactions were noted at the phosphotyrosine binding site. However due to the weak nature of the binding, the mechanism of sequence specificity remained unclear. In the *v-src* complexes the hydrophobic side chain (val or leu) at position +1 packs against the hydrophobic surfaces of the side chains of lys 200 and tyr 202. The val or met at position +3 is situated in a cavity of mixed polarity and makes contacts with the side chains of tyr 202, ile 214 and the methyl group of thr 215. Figure 6.22 highlights residues 200, 202, 214 and 215 involved in peptide-protein interactions. From these data it was postulated that, generally speaking, positions 200 and 202 are important for the packing of the side chains in position +1 of the peptide and that a hydrophobic residue in position +3 nestles in a binding pocket which varies in character from one SH2 module to another. The hypothesis that the peptide by is anchored by insertion of the +3 residue into a hydrophobic binding pocket was confirmed by observation of this interaction in two high affinity SH2-peptide complexes (Eck *et al.*, 1993; Waksman *et al.*, 1993).

In order to structurally interpret the results of the binding assays conducted by Fantl *et al.* (1992) and Panayotou *et al.* (1993) it was first necessary to model the structures of the p85α-C and GAP-N SH2s. In deciding upon which SH2 structure the models should be based it was noted that the sequences of the p85α-C SH2 and GAP-N SH2 are more similar to the p85α-N SH2 than to the *v-src* SH2. Furthermore, comparison of the two known structures suggested that only minimal conformational change is associated with peptide binding. For these reasons, the model structures of the p85α-C and GAP-N SH2s were based on the solution structure of the p85α-N SH2. The structures were

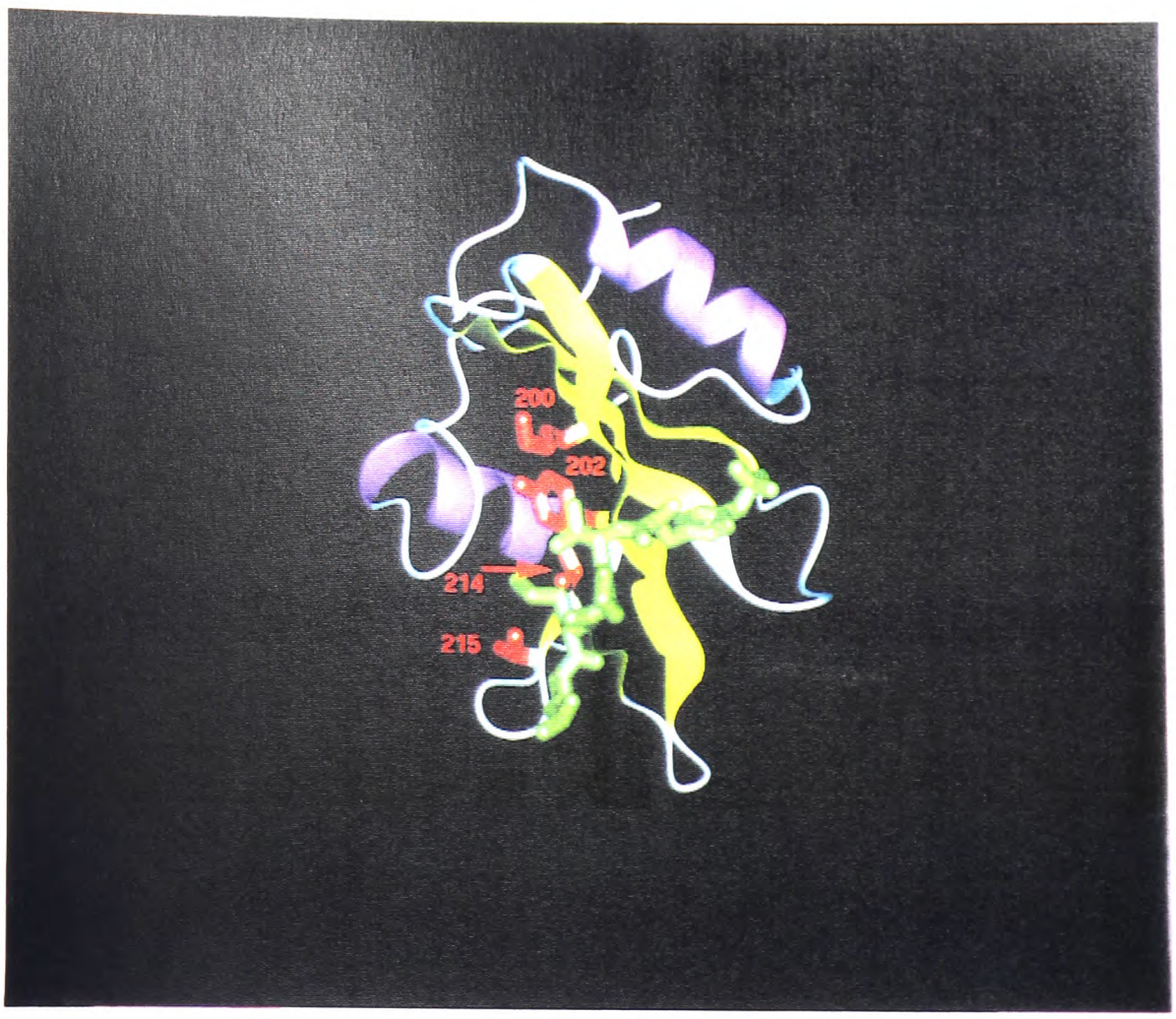


Figure 6.22

Schematic illustration of the *v-src* SH2 module complexed with a phosphopeptide (green) highlighting specific amino acids (cyan) which contact the side chains of peptide residues in the +1 and +3 positions downstream of the phosphotyrosine. Specifically, the amino acid at position +1 packs hydrophobically against the side chains of lys 200 and tyr 202, while the one at position +3 fits into a binding pocket of mixed polarity created by tyr 202, ile 214 and the methyl group of thr 215.

modelled by homology using the program QUANTA v3.2 based on the sequence alignment shown in Figure 6.23.

The *v-src* sequence is also shown in Figure 6.23 in order to clarify references to residue positions in later discussion. To facilitate comparison of SH2 domains, Eck *et al.* (1993) have proposed a notation for secondary-structure elements as follows: the strands are called βA-βG, and the helices αA and αB. Loops are named by the letters of the secondary structures they join, and residues are numbered sequentially within each structural element, e.g., αA1, αA2, etc. Standardisation of notation is recommended, however the notation proposed for SH2s by Eck *et al.* (1993) fails to achieve this goal for the simple reason that not all SH2 modules have identical secondary structure elements with the same number of residues. The boundaries of the secondary structure elements of the p85α-N SH2 are boxed by dashed lines in Figure 6.23. If the notation of Eck *et al.* (1993) was used for this module, then many inconsistencies in the labeling of the residues in the *v-src* SH2 relative to the p85α-N SH2 would be apparent. For example, αA1 of the p85α-N SH2 would correspond to αA2 of the *v-src* SH2. Therefore, in order to enable the reader to easily compare the results of the modelling presented in this thesis with published reports detailing specific residues involved in SH2-peptide interactions (Eck *et al.*, 1993; Waksman *et al.*, 1993), residues in all sequences shown in Figure 6.23 will be referenced using the notation of Eck *et al.* (1993) with respect to the *v-src* secondary structure shown.

The models of the p85α-C and GAP-N SH2 structures were energy minimised using a full CHARMM empirical energy potential (Brooks *et al.*, 1983) with the program XPLOR (Brünger, 1990) in order to alleviate bad nonbonded contacts of mutated side chains. Assessment

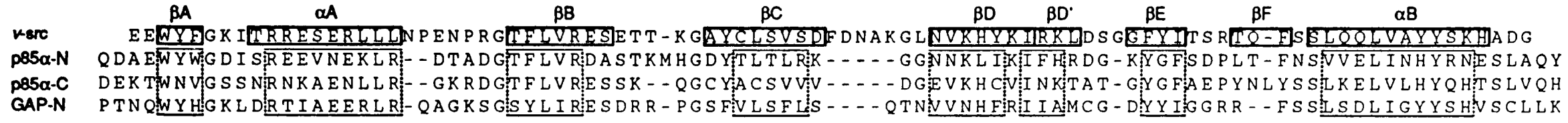


Figure 6.23

Alignment of the *v-src*, p85α-N, p85α-C and GAP-N SH2 sequences. The models of the p85α-C and GAP-N SH2 structures were modelled from the p85α-N SH2 based on the high level of sequence homology shared by these three domains. The *v-src* secondary structure elements are boxed by solid lines and labelled according to the notation of Eck *et al.* (1993). For comparison, the secondary structure elements of the p85α-N SH2 (and the models) are boxed by dashed lines. In subsequent discussion the residues in all of the aligned sequences will be referred to using the notation of Eck *et al.* (1993) with respect to the *v-src* secondary structure shown.

of the modelled structures using the program PROCHECK (Laskowski *et al.*, 1993) showed them to be of similar quality to the n.m.r. structure.

In order to model the binding of the five amino acid peptide corresponding to position 751 of the human βPDGFR (Y^PVPML), the backbone coordinates of residues in regions of conserved secondary structure in the p85α-N, p85α-C and GAP-N SH2s were superposed on the *v-src* SH2. Then the coordinates of this peptide, which was co-crystallised with the *v-src* SH2 module, were copied to each of the other coordinate files. The five residue peptide corresponding to position 740 of the human βPDGFR (Y^PMDMS) was modelled simply by mutation of residues +1, +2 and +4 of peptide 751.

In a comparison of the residues comprising the binding pockets of the p85α-N, p85α-C and GAP-N SH2 modules it was observed that the two SH2s from PI3-kinase have a phenylalanine at position βE4 which is likely to participate in peptide binding, while the GAP-N SH2 does not (Figure 6.24). Furthermore, an alignment of eleven closely related SH2 sequences (Panayotou *et al.*, 1992) revealed that the SH2s from PI3-kinase are the only two with a phenylalanine in this position. Therefore, the requirement of a methionine in position +3 of the phosphopeptide for strong binding to PI3-kinase (Cantley *et al.*, 1991; Fantl *et al.*, 1992) was postulated to be explained by interaction of the methionine side chain with this aromatic ring. Likewise the lower affinity of the GAP-N SH2 for peptides with methionine at the +3 position (Fantl *et al.*, 1992; Panayotou *et al.*, 1993) was surmised as the consequence of mutation of phe βE4 to an isoleucine. In support of these theories, interactions between residue βE4 and peptide position +3 have been observed in two high affinity SH2-peptide complexes (Eck *et al.*, 1993; Waksman *et al.*, 1993).

According to the results of the competitive binding studies it might be assumed that the substitution of met by val in position +1 enhances

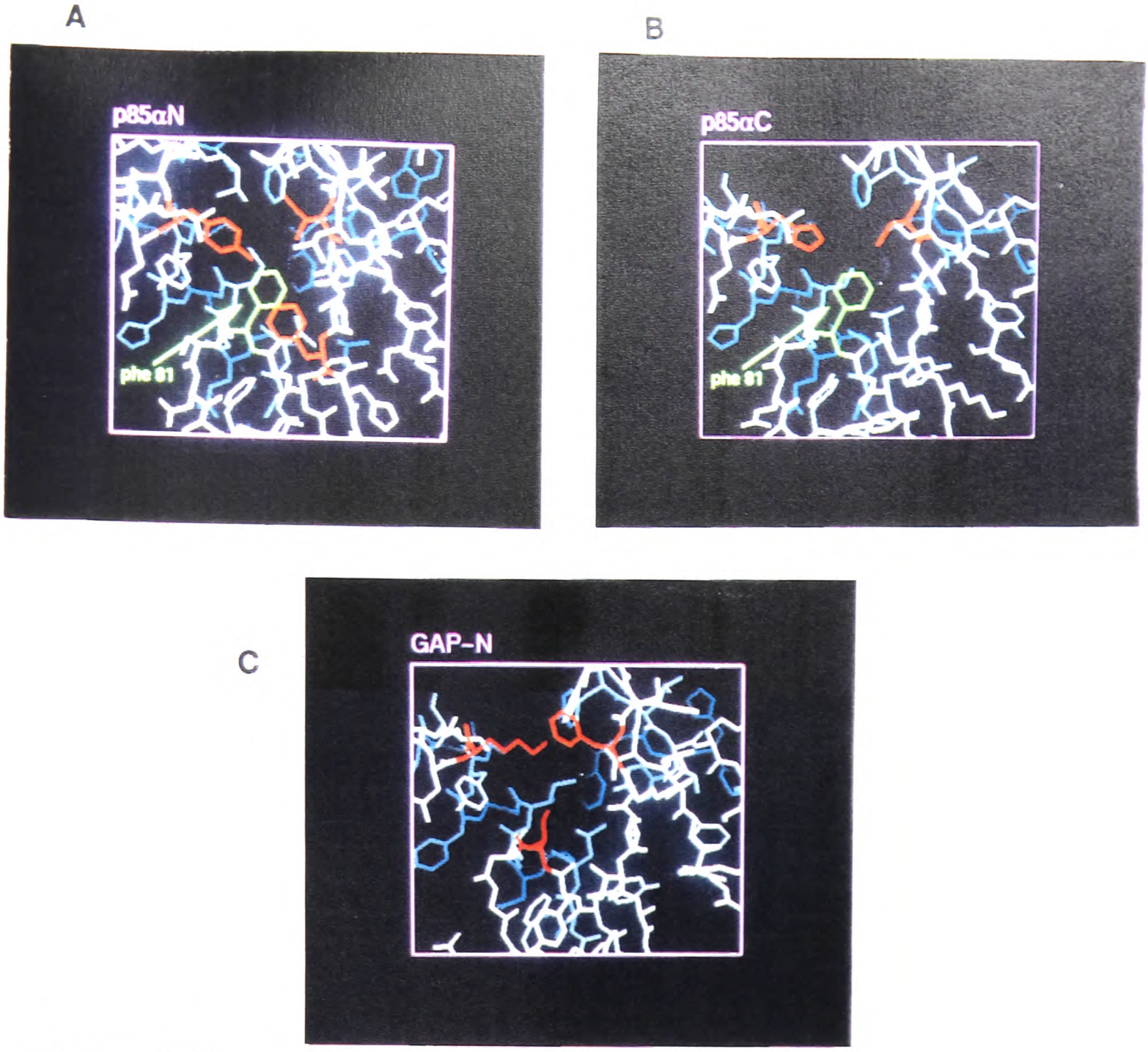


Figure 6.24

Residues found in the peptide +3 position binding pockets of the p85α–N (A), p85α-C (B) and GAP-N (C) SH2 modules. The coloured residues correspond to positions 70 (βD5), 81 (βE4) and 105 (in the insertion C-terminal to αB) of the p85α-N sequence. The phenylalanine in position βE4 of the two p85α SH2s (green) is replaced by an isoleucine in the GAP-N SH2. In an alignment of 11 closely related sequences, only the SH2 modules of PI3-kinase have a phenylalanine in position βE4 (Panayotou *et al.*, 1992), which may explain their observed affinity for phosphopeptides with methionine in the +3 position (Cantley *et al.*, 1991; Fantl *et al.*, 1992).

the interaction of the peptide and the p85α-N SH2 but has little effect on the association of the peptide with the p85α-C SH2. If this is true, then these differences must be explained in terms of the protein contacts with the peptide at position +1. One of the two residues in *v-src* identified as participating in hydrophobic packing interactions with the peptide at this position (Waksman *et al.*, 1992), ile βD5, is substituted by a cysteine in the p85α-C SH2. While no direct conclusion regarding peptide +1-βD5 interactions was drawn from this observation, further support for the critical importance of peptide interactions with residue 70 was suggested by the fact that the p85α-C SH2 does not bind to either peptide when the experiments are performed in buffer without DTT (Panayotou *et al.*, 1993). In the model of the p85α-C SH2 module, cys βC4 and cys βD5 are within close enough proximity spatially to form a disulphide bridge (Figure 6.25). Therefore, if in the absence of DTT this bridge forms and results in a loss of peptide binding, then it may be concluded that cys βD5 participates in essential protein-peptide contacts. The critical role of residue βD5 in determining phosphopeptide specificity has been verified by the structures of the two high resolution SH2-peptide complexes and by determination of the sequence specificity of the peptide-binding sites of several SH2 domains (Songyang *et al.*, 1993).

Perhaps then the fact that the GAP-N SH2 has a phenylalanine at position βD5 might explain why it binds only to the peptide with a methionine at position +1. Although the phenylalanine is even larger than the isoleucine at this position in the p85α-N SH2, the strength of the hydrophobic interaction between the met and phe probably causes a local adjustment in the structure of the SH2 module in order to accommodate the methionine side chain. Phe βD5 is conserved in the C-terminal SH2 of GAP, thus if this theory is correct, it could be postulated that one of the reasons why GAP associates with the

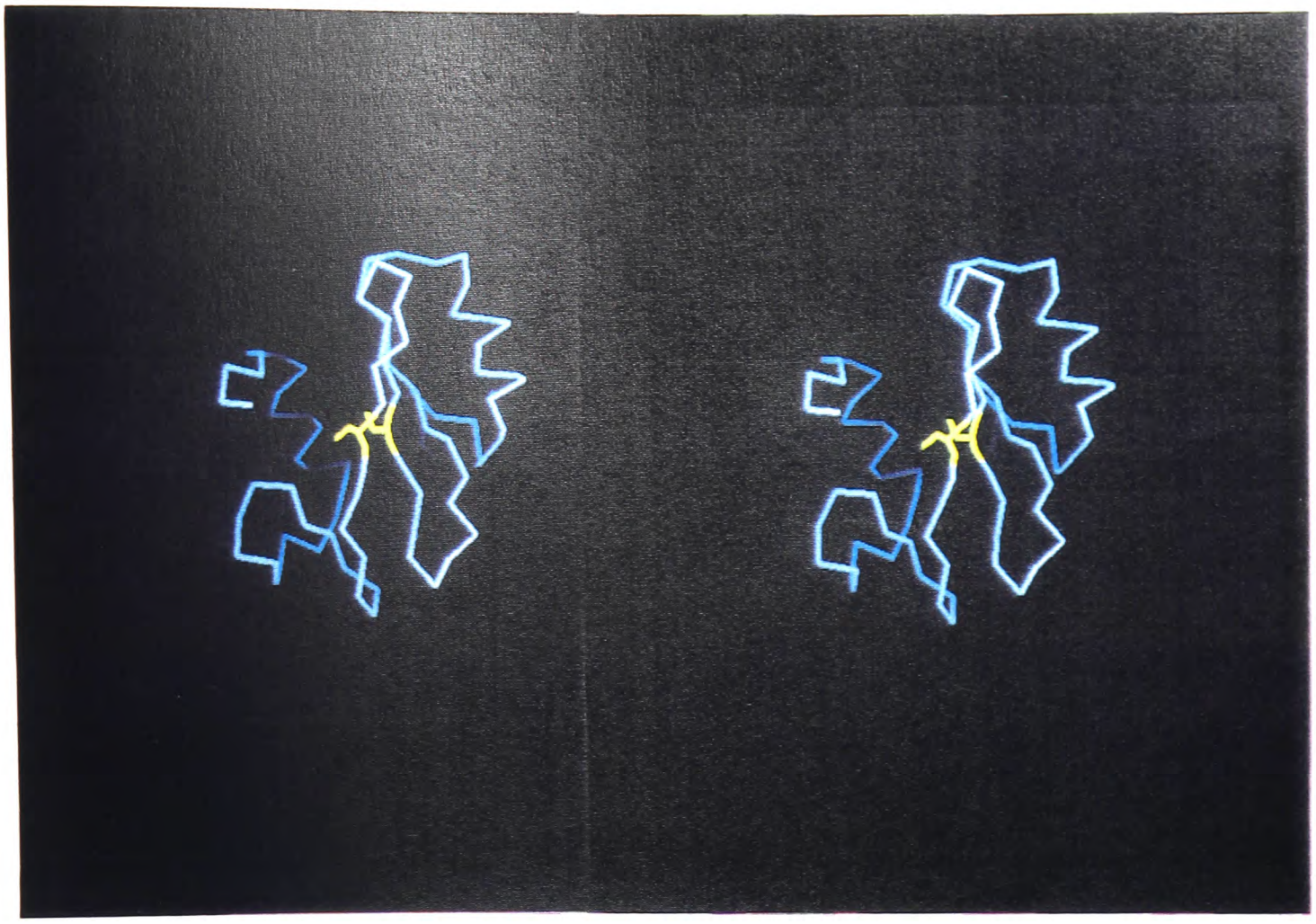


Figure 6.25

Stereoview of the modelled p85α-C SH2 module displaying the close proximity of cys βC4 and cys βD5 (yellow) which are proposed to disulphide bond in the absence of DTT.

octameric peptide corresponding to position 771 of the human βPDGFR (Fantl *et al.*, 1992) is because this sequence also includes a methionine +1 downstream of the phosphotyrosine.

6.6 Conclusions

After sequential assignments for the p85α-N SH2 module were made using 3-dimensional n.m.r. data, short range NOEs were assigned. NOESY, $^{3}J_{HN\alpha}$ coupling constant, amide proton exchange and chemical shift data were used in conjunction to experimentally define the secondary structure. The secondary structure elements of the p85α-N SH2 module were examined in order to deduce their relative orientation. The β-strands were assembled without great difficulty. However, few long range interactions between the helices and the core of the structure coupled with unusually extensive chemical shift degeneracy in the aromatic region of the H_2O NOESY spectrum complicated calculation of the tertiary fold of the molecule. Ultimately the structure was determined to be composed of a central region of β-sheet flanked by two α-helices. A comparison of the predicted secondary structure of SH2 modules (Russell *et al.*, 1992) with the pattern determined for the p85α-N SH2 showed the prediction to be 77% accurate over the residues exhibiting strong sequence similarity.

The concurrent report of the crystal structure of the *v-src* SH2 module complexed with low affinity phosphopeptides (Waksman *et al.*, 1992) enabled a comparison to be made between the two structures, specifically with reference to the residues shown to be involved in phosphopeptide binding. The two structures were found to be globally very similar, and it was postulated only minor structural adjustments take place upon phosphopeptide binding to SH2s. This hypothesis was

confirmed by report of the conformational change associated with *Src*-peptide binding (Waksman *et al.*, 1993). Based on the protein-peptide interactions observed in the low affinity *v-src* complex and the comparison of this structure to the uncomplexed p85α-N SH2 structure, the mechanism of high affinity phosphopeptide binding was proposed to involve specific interaction of the peptide +3 residue with a hydrophobic binding pocket. This interaction was subsequently observed in the structures of two high affinity SH2-peptide complexes (Eck *et al.*, 1993; Waksman *et al.*, 1993).

The C-terminal SH2 module from the p85α subunit of PI3-kinase and the N-terminal GAP SH2 were modelled based on the p85α-N SH2 structure and were used to explain differential peptide affinities of these three modules (Fantl *et al.*, 1992; Panayotou *et al.*, 1993). Sequence specific interactions involving residues βE4 and βD5 with peptide positions +3 and +1/+3 respectively were postulated and later observed in high affinity complexes.

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Appendix I

Bruker NMR Spectrometer Pulse Sequences

All of the following pulse sequences were implemented on Bruker 500 MHz and 600 MHz NMR spectrometers by Dr. P. C. Driscoll.

D_2O EXPERIMENTS:

A. 1-dimensional experiment with solvent presaturation

```
1 ZE
2 D1 HG S3
  D2 DO
  P1 PH1
3 GO=2 PH2 DO
4 EXIT

PH1= 0 0 2 2 1 1 3 3
PH2= R0 R0 R2 R2 R1 R1 R3 R3
```

B. 2-dimensional DQF COSY experiment, phase sensitive with solvent presaturation

```
1 ZE
2 D1 HG S1
  D4 DO
3 P1 PH1
4 DO
5 P1 PH2
  D3
  P1 PH3
6 GO=2 PH4 DO
7 WR #1
8 IF #1
9 IP1
10 IN=1
   EXIT


PH1=A0 A0 A0 A0 A1 A1 A1 A1 A2 A2 A2 A2 A3 A3 A3 A3
PH2=A0 A0 A0 A0 A1 A1 A1 A1 A2 A2 A2 A2 A3 A3 A3 A3
PH3=A0 A1 A2 A3 A1 A2 A3 A0 A2 A3 A0 A1 A3 A0 A1 A2
PH4=R0 R3 R2 R1 R1 R0 R3 R2 R2 R1 R0 R3 R3 R2 R1 R0
```

C. 2-dimensional NOESY experiment with solvent presaturation

```
1 ZE
2 D1 HG S3
  D2 DO
3 P1 PH1
4 DO
5 P1 PH2
6 D4 HG S3
  D2 DO
7 P1 PH3
  D6
  D6 PH7
  D7
  D8 ADC
  RCYC=2 PH4
;8 GO=2 PH4 DO
9 WR #1
10 IF #1
   IP1
11 IN=1
12 EXIT

PH1=A0 A2
PH2=A0 A0 A0 A0 A0 A0 A0 A0
    A2 A2 A2 A2 A2 A2 A2 A2
PH3=A0 A0 A2 A2 A1 A1 A3 A3
PH4=R0 R2 R2 R0 R1 R3 R3 R1
    R2 R0 R0 R2 R3 R1 R1 R3
PH7=(360) 250
```

D. 2-dimensional WALTZ-17 HOHAHA experiment with solvent presaturation

```
1  ZE
   D10 S1
   D10 S2
2  D1
   (P20):C6
   (P8 PH1):D:C6:E
   (P21):C6
   (P20):C5
   (P5 PH5):D:C5:E
   (P21):C5
   DO
   (P20):C5
   (P15 PH2):D:C5:E
6  (P3 PH3 P4 PH4 P2 PH3 P3 PH4 P1 PH3 P2 PH4):D:C5:E
   (P4 PH3 P2 PH4 P3 PH3 P3 PH4 P4 PH3 P2 PH4):D:C5:E
   (P3 PH3 P1 PH4 P2 PH3 P4 PH4 P2 PH3 P3 PH4):D:C5:E
   (P3 PH4 P4 PH3 P2 PH4 P3 PH3 P1 PH4 P2 PH3):D:C5:E
```

```
    (P4 PH4 P2 PH3 P3 PH4 P3 PH3 P4 PH4 P2 PH3):D:C5:E
    (P3 PH4 P1 PH3 P2 PH4 P4 PH3 P2 PH4 P3 PH3):D:C5:E
    (P6 PH2):D:C5:E
    L2 TO 6 TIMES C
    (P15 PH2):D:C5:E
    (P21):C5
    D6
    D6:D PH7
    D7
    D8 ADC
    RCYC=2 PH8
    WR #1
    IF #1
    IP5
    IN=1
    EXIT

PH1=0
PH5=1 3 2 0 3 1 0 2
PH2=0 0 1 1 2 2 3 3
PH3=1 1 2 2 3 3 0 0
PH4=3 3 0 0 1 1 2 2
PH7=(360) 260

PH8=R1 R3 R2 R0 R3 R1 R0 R2
PH6 = A0
```

E. 2-dimensional PE-COSY experiment with solvent presaturation

```
1   ZE
2   D1 HG S3
    D2 D0
    P1 PH1
    D0
    P2 PH2
;    D4
    D6
    D6 PH3
    D7
    D8 ADC
    RCYC=2 PH4
    WR #1
    IF #1
 ;   LO TO 1 TIMES 8
    IP1
    IN=1
    D0
    EXIT

;PH1=0 2 1 3 2 0 3 1
PH1=0 2 2 0 1 3 3 1
;PH2=0 0 1 1 2 2 3 3
PH2=0 0 2 2 1 1 3 3
PH3=(360) 216
;PH4=R0 R2 R1 R3 R2 R0 R3 R1
PH4=R0 R2 R2 R0 R1 R3 R3 R1
```

H_2O EXPERIMENTS:

A. 2-dimensional COSY experiment with SCUBA for recovery of CαH resonances under H_2O

```
1   ZE
    D10 S2
    D10 S3
2   (P20):C6
    (P7 PH6):C6:D:E
    (P21):C6
    (P20):C5
    (P1 PH1):C5:D:E
    (P21):C5
    D0
    (P20):C5
    (P2 PH2):C5:D:E
    (P21):C5
    D6
    D6:D PH7
    D7
    D8 ADC
    RCYC=2 PH3
;    GO=2 PH3
    WR #1
    IF #1
    IP1
    IN=1
    DO
    EXIT

PH1 = 0 2 1 3 2 0 3 1
PH2 = 0 0 1 1 2 2 3 3
PH3 = R0 R2 R1 R3 R2 R0 R3 R1
PH7 = (360)200
PH8 = 0 0 0 0 0 0 0 0
      1 1 1 1 1 1 1 1
      2 2 2 2 2 2 2 2
      3 3 3 3 3 3 3 3
PH9 = 1 1 1 1 1 1 1 1
      2 2 2 2 2 2 2 2
      3 3 3 3 3 3 3 3
      0 0 0 0 0 0 0 0
PH6 = 0
```

B. 2-dimensional NOESY experiment with jump and return read pulse

```
1 ZE
2 D1; HG S3
    ; D2 DO
  P1 PH1
  DO
  P1 PH2
  D4
  P1 PH3
  D3
  P2 PH4
  D6
  D6 PH7
  D7
  D8 ADC
  RCYC=2 PH6
  IP1
  WR#1
  IF#1
  IN=1
  EXIT

PH1=0 0 2 2 0 0 2 2
    1 1 3 3 1 1 3 3
    2 2 0 0 2 2 0 0
    3 3 1 1 3 3 1 1
PH2=(8) 5 5 5 5 5 5 5 5
        7 7 7 7 7 7 7 7
        1 1 1 1 1 1 1 1
        3 3 3 3 3 3 3 3
PH3=0 1 0 1 2 3 2 3
    1 2 1 2 3 0 3 0
    2 3 2 3 0 1 0 1
    3 0 3 0 1 2 1 2
PH4=2 3 2 3 0 1 0 1
    3 0 3 0 1 2 1 2
    0 1 0 1 2 3 2 3
    1 2 1 2 3 0 3 0
PH7=(360)150


PH6=R0 R1 R2 R3 R2 R3 R0 R1
    R1 R2 R3 R0 R3 R0 R1 R2
    R2 R3 R0 R1 R0 R1 R2 R3
    R3 R0 R1 R2 R1 R2 R3 R0
```

C. 2-dimensional WALTZ-17 HOHAHA experiment with jump and return read pulse

```
1 ZE
  D3 S3 DO
2 (P15 PH2):D:E
  D15
  D1
  (P1 PH1):D:E
  DO
5 (P1 PH5):D:E
  (P5 PH2):D:E
6 (P3 PH3 P4 PH4 P2 PH3 P3 PH4 P1 PH3 P2 PH4):D:E
  (P4 PH3 P2 PH4 P3 PH3 P3 PH4 P4 PH3 P2 PH4):D:E
  (P3 PH3 P1 PH4 P2 PH3 P4 PH4 P2 PH3 P3 PH4):D:E
  (P3 PH4 P4 PH3 P2 PH4 P3 PH3 P1 PH4 P2 PH3):D:E
  (P4 PH4 P2 PH3 P3 PH4 P3 PH3 P4 PH4 P2 PH3):D:E
  (P3 PH4 P1 PH3 P2 PH4 P4 PH3 P2 PH4 P3 PH3):D:E
;  (P6 PH2):D:E
  L2 TO 6 TIMES C
  (P5 PH2):D:E
  D6
  (P1 PH9):D:E
  D11
  (P1 PH10 D2 P13 PH11):D:E
  D6
  D6:D PH6
  D7
  D8 ADC
  RCYC=2 PH7
  WR #1
  IF #1
  ID
  IP1
  IP1
  ;  IN=1
  LO TO 1 TIMES 2
  IP1
  IP1
  IP1
  IP1
  LO TO 1 TIMES 512

  DO
  EXIT

PH1=(8) 5 5 5 5 1 1 1 1
PH2=0
PH5=0 0 0 0 0 0 0 0 2 2 2 2 2 2 2 2
PH9=1 1 1 1 3 3 3 3
PH3=1
PH4=3
PH7=R0 R1 R2 R3
PH10=0 1 2 3
PH11=2 3 0 1
PH6=(360) 163
```

Appendix II

XPLOR Experimental Restraints Used in Structure Calculations

A. t-PA F1 Restraints

```
! INTRARESIDUE RESTRAINTS FOR TPA F1 MODULE
assign (resid    2 and   name HB#  )(resid    2 and   name HN   ) 0.0 0.6 4.1
assign (resid    2 and   name HB#  )(resid    2 and   name HA   ) 0.0 1.0 4.5
assign (resid    3 and   name HB1  )(resid    3 and   name HN   ) 0.0 0.0 5.0
assign (resid    3 and   name HB2  )(resid    3 and   name HN   ) 0.0 0.0 5.0
assign (resid    3 and   name HG#  )(resid    3 and   name HN   ) 0.0 1.0 4.5
assign (resid    3 and   name HB1  )(resid    3 and   name HA   ) 0.0 0.0 3.5
assign (resid    3 and   name HB2  )(resid    3 and   name HA   ) 0.0 0.0 3.5
assign (resid    3 and   name HG#  )(resid    3 and   name HA   ) 0.0 1.0 4.5
assign (resid    4 and   name HG1# )(resid    4 and   name HN   ) 0.0 1.0 4.5
assign (resid    4 and   name HG2# )(resid    4 and   name HN   ) 0.0 1.0 4.5
assign (resid    4 and   name HB   )(resid    4 and   name HN   ) 0.0 0.0 3.5
assign (resid    4 and   name HG1# )(resid    4 and   name HA   ) 0.0 1.0 4.5
assign (resid    4 and   name HG2# )(resid    4 and   name HA   ) 0.0 1.0 4.5
assign (resid    4 and   name HB   )(resid    4 and   name HA   ) 0.0 0.0 3.5
assign (resid    5 and   name HB   )(resid    5 and   name HN   ) 0.0 0.0 3.5
assign (resid    5 and   name HG11 )(resid    5 and   name HN   ) 0.0 0.0 2.5
assign (resid    5 and   name HG12 )(resid    5 and   name HN   ) 0.0 0.0 2.5
assign (resid    5 and   name HG2# )(resid    5 and   name HN   ) 0.0 1.0 3.5
assign (resid    5 and   name HD#  )(resid    5 and   name HN   ) 0.0 1.0 4.5
assign (resid    5 and   name HB   )(resid    5 and   name HA   ) 0.0 0.0 2.5
assign (resid    5 and   name HG11 )(resid    5 and   name HA   ) 0.0 0.0 3.5
assign (resid    5 and   name HG12 )(resid    5 and   name HA   ) 0.0 0.0 5.0
assign (resid    5 and   name HG2# )(resid    5 and   name HA   ) 0.0 1.0 3.5
assign (resid    5 and   name HD#  )(resid    5 and   name HA   ) 0.0 1.0 6.0
assign (resid    6 and   name HB1  )(resid    6 and   name HN   ) 0.0 0.0 3.5
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 HA   ) 0.0 0.0 3.5
assign (resid    6 and   name HB2  )(resid    6 and   name HA   ) 0.0 0.0 3.5
assign (resid    7 and   name HB1  )(resid    7 and   name HN   ) 0.0 0.0 2.5
assign (resid    7 and   name HB2  )(resid    7 and   name HN   ) 0.0 0.0 2.5
assign (resid    7 and   name HG1  )(resid    7 and   name HN   ) 0.0 0.0 5.0
assign (resid    7 and   name HG2  )(resid    7 and   name HN   ) 0.0 0.0 5.0
assign (resid    7 and   name HD#  )(resid    7 and   name HN   ) 0.0 1.0 6.0
assign (resid    7 and   name HB1  )(resid    7 and   name HA   ) 0.0 0.0 3.5
assign (resid    7 and   name HB2  )(resid    7 and   name HA   ) 0.0 0.0 3.5
assign (resid    8 and   name HB1  )(resid    8 and   name HN   ) 0.0 0.0 3.5
assign (resid    8 and   name HB2  )(resid    8 and   name HN   ) 0.0 0.0 3.5
assign (resid    8 and   name HB1  )(resid    8 and   name HA   ) 0.0 0.0 3.5
assign (resid    8 and   name HB2  )(resid    8 and   name HA   ) 0.0 0.0 2.5
assign (resid    9 and   name HB1  )(resid    9 and   name HN   ) 0.0 0.0 3.5
assign (resid    9 and   name HB2  )(resid    9 and   name HN   ) 0.0 0.0 3.5
assign (resid    9 and   name HG1  )(resid    9 and   name HN   ) 0.0 0.0 3.5
assign (resid    9 and   name HG2  )(resid    9 and   name HN   ) 0.0 0.0 3.5
assign (resid    9 and   name HB1  )(resid    9 and   name HA   ) 0.0 0.0 2.5
assign (resid    9 and   name HB2  )(resid    9 and   name HA   ) 0.0 0.0 2.5
assign (resid    9 and   name HG1  )(resid    9 and   name HA   ) 0.0 0.0 5.0
assign (resid    9 and   name HG2  )(resid    9 and   name HA   ) 0.0 0.0 5.0
assign (resid   10 and   name HB1  )(resid   10 and   name HN   ) 0.0 0.0 3.5
assign (resid   10 and   name HB2  )(resid   10 and   name HN   ) 0.0 0.0 3.5
assign (resid   10 and   name HG#  )(resid   10 and   name HN   ) 0.0 1.0 4.5
assign (resid   10 and   name HD#  )(resid   10 and   name HN   ) 0.0 1.0 3.5
assign (resid   10 and   name HB1  )(resid   10 and   name HA   ) 0.0 0.0 3.5
assign (resid   10 and   name HB2  )(resid   10 and   name HA   ) 0.0 0.0 3.5
```

```
assign (resid  10 and  name HG#  )(resid  10 and  name HA   ) 0.0 1.0 4.5
assign (resid  10 and  name HD#  )(resid  10 and  name HA   ) 0.0 1.0 4.5
assign (resid  10 and  name HE#  )(resid  10 and  name HA   ) 0.0 1.0 6.0
assign (resid  11 and  name HG2# )(resid  11 and  name HN   ) 0.0 1.0 3.5
assign (resid  11 and  name HG2# )(resid  11 and  name HA   ) 0.0 1.0 5.0
assign (resid  12 and  name HB1  )(resid  12 and  name HN   ) 0.0 0.0 5.0
assign (resid  12 and  name HB2  )(resid  12 and  name HN   ) 0.0 0.0 5.0
assign (resid  12 and  name HG#  )(resid  12 and  name HN   ) 0.0 1.0 4.5
assign (resid  12 and  name HB1  )(resid  12 and  name HA   ) 0.0 0.0 3.5
assign (resid  12 and  name HB2  )(resid  12 and  name HA   ) 0.0 0.0 3.5
assign (resid  12 and  name HG#  )(resid  12 and  name HA   ) 0.0 1.0 4.5
assign (resid  13 and  name HB1  )(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 3.5
assign (resid  13 and  name HG#  )(resid  13 and  name HN   ) 0.0 1.0 4.5
assign (resid  13 and  name HB1  )(resid  13 and  name HA   ) 0.0 0.0 3.5
assign (resid  13 and  name HB2  )(resid  13 and  name HA   ) 0.0 0.0 3.5
assign (resid  13 and  name HG#  )(resid  13 and  name HA   ) 0.0 1.0 3.5
assign (resid  14 and  name HB   )(resid  14 and  name HN   ) 0.0 0.0 2.5
assign (resid  14 and  name HG11 )(resid  14 and  name HN   ) 0.0 0.0 3.5
assign (resid  14 and  name HG12 )(resid  14 and  name HN   ) 0.0 0.0 3.5
assign (resid  14 and  name HG2# )(resid  14 and  name HN   ) 0.0 1.0 4.5
assign (resid  14 and  name HD#  )(resid  14 and  name HN   ) 0.0 1.0 4.5
assign (resid  14 and  name HB   )(resid  14 and  name HA   ) 0.0 0.0 3.5
assign (resid  14 and  name HG11 )(resid  14 and  name HA   ) 0.0 0.0 3.5
assign (resid  14 and  name HG12 )(resid  14 and  name HA   ) 0.0 0.0 3.5
assign (resid  14 and  name HG2# )(resid  14 and  name HA   ) 0.0 1.0 3.5
assign (resid  14 and  name HD#  )(resid  14 and  name HA   ) 0.0 1.0 4.5
assign (resid  15 and  name HB1  )(resid  15 and  name HN   ) 0.0 0.0 5.0
assign (resid  15 and  name HB2  )(resid  15 and  name HN   ) 0.0 0.0 3.5
assign (resid  15 and  name HB1  )(resid  15 and  name HA   ) 0.0 0.0 2.5
assign (resid  15 and  name HB2  )(resid  15 and  name HA   ) 0.0 0.0 3.5
assign (resid  16 and  name HB1  )(resid  16 and  name HN   ) 0.0 0.0 3.5
assign (resid  16 and  name HB2  )(resid  16 and  name HN   ) 0.0 0.0 3.5
assign (resid  16 and  name HG1  )(resid  16 and  name HN   ) 0.0 0.0 3.5
assign (resid  16 and  name HG2  )(resid  16 and  name HN   ) 0.0 0.0 3.5
assign (resid  16 and  name HB1  )(resid  16 and  name HA   ) 0.0 0.0 3.5
assign (resid  16 and  name HB2  )(resid  16 and  name HA   ) 0.0 0.0 3.5
assign (resid  16 and  name HG1  )(resid  16 and  name HA   ) 0.0 0.0 3.5
assign (resid  16 and  name HG2  )(resid  16 and  name HA   ) 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  17 and  name HB2  )(resid  17 and  name HN   ) 0.0 0.0 2.5
assign (resid  17 and  name HG#  )(resid  17 and  name HN   ) 0.0 1.0 4.5
assign (resid  17 and  name HB1  )(resid  17 and  name HA   ) 0.0 0.0 3.5
assign (resid  17 and  name HB2  )(resid  17 and  name HA   ) 0.0 0.0 3.5
assign (resid  17 and  name HG#  )(resid  17 and  name HA   ) 0.0 1.0 4.5
assign (resid  18 and  name HB#  )(resid  18 and  name HN   ) 0.0 0.6 4.1
assign (resid  18 and  name HB#  )(resid  18 and  name HA   ) 0.0 1.0 4.5
assign (resid  19 and  name HB#  )(resid  19 and  name HN   ) 0.0 0.6 3.1
assign (resid  19 and  name HG#  )(resid  19 and  name HN   ) 0.0 1.0 4.5
assign (resid  19 and  name HB#  )(resid  19 and  name HA   ) 0.0 1.0 4.5
assign (resid  19 and  name HG#  )(resid  19 and  name HA   ) 0.0 1.0 4.5
assign (resid  20 and  name HB#  )(resid  20 and  name HN   ) 0.0 0.6 3.1
assign (resid  20 and  name HB#  )(resid  20 and  name HA   ) 0.0 1.0 3.5
assign (resid  21 and  name HB1  )(resid  21 and  name HN   ) 0.0 0.0 5.0
assign (resid  21 and  name HB2  )(resid  21 and  name HN   ) 0.0 0.0 5.0
assign (resid  21 and  name HB1  )(resid  21 and  name HA   ) 0.0 0.0 3.5
assign (resid  21 and  name HB2  )(resid  21 and  name HA   ) 0.0 0.0 3.5
assign (resid  22 and  name HB1  )(resid  22 and  name HN   ) 0.0 0.0 3.5
assign (resid  22 and  name HB2  )(resid  22 and  name HN   ) 0.0 0.0 3.5
assign (resid  22 and  name HG   )(resid  22 and  name HN   ) 0.0 0.0 5.0
assign (resid  22 and  name HD1# )(resid  22 and  name HN   ) 0.0 1.0 6.0
assign (resid  22 and  name HD2# )(resid  22 and  name HN   ) 0.0 1.0 6.0
assign (resid  22 and  name HB1  )(resid  22 and  name HA   ) 0.0 0.0 3.5
assign (resid  22 and  name HB2  )(resid  22 and  name HA   ) 0.0 0.0 3.5
```

```
assign (resid  22 and  name HG   )(resid  22 and  name HA   ) 0.0 0.0 5.0
assign (resid  22 and  name HD1# )(resid  22 and  name HA   ) 0.0 1.0 4.5
assign (resid  22 and  name HD2# )(resid  22 and  name HA   ) 0.0 1.0 4.5
assign (resid  23 and  name HB#  )(resid  23 and  name HN   ) 0.0 0.6 3.1
assign (resid  23 and  name HG#  )(resid  23 and  name HN   ) 0.0 1.0 4.5
assign (resid  23 and  name HB#  )(resid  23 and  name HA   ) 0.0 1.0 4.5
assign (resid  23 and  name HG#  )(resid  23 and  name HA   ) 0.0 1.0 4.5
assign (resid  23 and  name HD1  )(resid  23 and  name HA   ) 0.0 0.0 5.0
assign (resid  23 and  name HD2  )(resid  23 and  name HA   ) 0.0 0.0 5.0
assign (resid  24 and  name HB1  )(resid  24 and  name HA   ) 0.0 0.0 3.5
assign (resid  24 and  name HB2  )(resid  24 and  name HA   ) 0.0 0.0 3.5
assign (resid  24 and  name HG1  )(resid  24 and  name HA   ) 0.0 0.0 5.0
assign (resid  24 and  name HG2  )(resid  24 and  name HA   ) 0.0 0.0 5.0
assign (resid  24 and  name HD1  )(resid  24 and  name HA   ) 0.0 0.0 5.0
assign (resid  24 and  name HD2  )(resid  24 and  name HA   ) 0.0 0.0 5.0
assign (resid  25 and  name HG1# )(resid  25 and  name HN   ) 0.0 1.0 4.5
assign (resid  25 and  name HG2# )(resid  25 and  name HN   ) 0.0 1.0 4.5
assign (resid  25 and  name HB   )(resid  25 and  name HN   ) 0.0 0.0 3.5
assign (resid  25 and  name HG1# )(resid  25 and  name HA   ) 0.0 1.0 4.5
assign (resid  25 and  name HG2# )(resid  25 and  name HA   ) 0.0 1.0 4.5
assign (resid  25 and  name HB   )(resid  25 and  name HA   ) 0.0 0.0 3.5
assign (resid  26 and  name HB#  )(resid  26 and  name HN   ) 0.0 0.6 3.1
assign (resid  26 and  name HG   )(resid  26 and  name HN   ) 0.0 0.0 2.5
assign (resid  26 and  name HD1# )(resid  26 and  name HN   ) 0.0 1.0 4.5
assign (resid  26 and  name HD2# )(resid  26 and  name HN   ) 0.0 1.0 4.5
assign (resid  26 and  name HB#  )(resid  26 and  name HA   ) 0.0 1.0 4.5
assign (resid  26 and  name HG   )(resid  26 and  name HA   ) 0.0 0.0 3.5
assign (resid  26 and  name HD1# )(resid  26 and  name HA   ) 0.0 1.0 6.0
assign (resid  26 and  name HD2# )(resid  26 and  name HA   ) 0.0 1.0 6.0
assign (resid  27 and  name HB1  )(resid  27 and  name HN   ) 0.0 0.0 5.0
assign (resid  27 and  name HB2  )(resid  27 and  name HN   ) 0.0 0.0 3.5
assign (resid  27 and  name HG#  )(resid  27 and  name HN   ) 0.0 1.0 3.5
assign (resid  27 and  name HD#  )(resid  27 and  name HN   ) 0.0 1.0 6.0
assign (resid  27 and  name HB1  )(resid  27 and  name HA   ) 0.0 0.0 3.5
assign (resid  27 and  name HB2  )(resid  27 and  name HA   ) 0.0 0.0 3.5
assign (resid  27 and  name HG#  )(resid  27 and  name HA   ) 0.0 1.0 3.5
assign (resid  27 and  name HD#  )(resid  27 and  name HA   ) 0.0 1.0 6.0
assign (resid  28 and  name HB1  )(resid  28 and  name HN   ) 0.0 0.0 3.5
assign (resid  28 and  name HB2  )(resid  28 and  name HN   ) 0.0 0.0 3.5
assign (resid  28 and  name HB1  )(resid  28 and  name HA   ) 0.0 0.0 3.5
assign (resid  28 and  name HB2  )(resid  28 and  name HA   ) 0.0 0.0 3.5
assign (resid  29 and  name HB#  )(resid  29 and  name HN   ) 0.0 0.6 3.1
assign (resid  29 and  name HB#  )(resid  29 and  name HA   ) 0.0 1.0 4.5
assign (resid  30 and  name HB1  )(resid  30 and  name HN   ) 0.0 0.0 3.5
assign (resid  30 and  name HB2  )(resid  30 and  name HN   ) 0.0 0.0 3.5
assign (resid  30 and  name HG#  )(resid  30 and  name HN   ) 0.0 1.0 3.5
assign (resid  30 and  name HD#  )(resid  30 and  name HN   ) 0.0 1.0 4.5
assign (resid  30 and  name HB1  )(resid  30 and  name HA   ) 0.0 0.0 3.5
assign (resid  30 and  name HB2  )(resid  30 and  name HA   ) 0.0 0.0 3.5
assign (resid  30 and  name HG#  )(resid  30 and  name HA   ) 0.0 1.0 3.5
assign (resid  30 and  name HD#  )(resid  30 and  name HA   ) 0.0 1.0 4.5
assign (resid  31 and  name HG#  )(resid  31 and  name HN   ) 0.0 1.7 4.2
assign (resid  31 and  name HB   )(resid  31 and  name HN   ) 0.0 0.0 5.0
assign (resid  31 and  name HG#  )(resid  31 and  name HA   ) 0.0 1.7 4.2
assign (resid  31 and  name HB   )(resid  31 and  name HA   ) 0.0 0.0 3.5
assign (resid  32 and  name HB1  )(resid  32 and  name HN   ) 0.0 0.0 3.5
assign (resid  32 and  name HB2  )(resid  32 and  name HN   ) 0.0 0.0 3.5
assign (resid  32 and  name HG1  )(resid  32 and  name HN   ) 0.0 0.0 5.0
assign (resid  32 and  name HG2  )(resid  32 and  name HN   ) 0.0 0.0 5.0
assign (resid  32 and  name HB1  )(resid  32 and  name HA   ) 0.0 0.0 3.5
assign (resid  32 and  name HB2  )(resid  32 and  name HA   ) 0.0 0.0 3.5
assign (resid  32 and  name HG1  )(resid  32 and  name HA   ) 0.0 0.0 3.5
assign (resid  32 and  name HG2  )(resid  32 and  name HA   ) 0.0 0.0 3.5
assign (resid  33 and  name HB#  )(resid  33 and  name HN   ) 0.0 0.6 3.1
```

```
assign (resid  33 and  name HB#  )(resid  33 and  name HA   ) 0.0 1.0 4.5
assign (resid  35 and  name HB#  )(resid  35 and  name HN   ) 0.0 0.6 4.1
assign (resid  35 and  name HB#  )(resid  35 and  name HA   ) 0.0 1.0 4.5
assign (resid  36 and  name HB1  )(resid  36 and  name HN   ) 0.0 0.0 3.5
assign (resid  36 and  name HB2  )(resid  36 and  name HN   ) 0.0 0.0 3.5
assign (resid  36 and  name HB1  )(resid  36 and  name HA   ) 0.0 0.0 3.5
assign (resid  36 and  name HB2  )(resid  36 and  name HA   ) 0.0 0.0 3.5
assign (resid  37 and  name HB#  )(resid  37 and  name HN   ) 0.0 0.6 3.1
assign (resid  37 and  name HB#  )(resid  37 and  name HA   ) 0.0 1.0 4.5
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  38 and  name HB1  )(resid  38 and  name HA   ) 0.0 0.0 3.5
assign (resid  38 and  name HB2  )(resid  38 and  name HA   ) 0.0 0.0 3.5
assign (resid  39 and  name HA2  )(resid  39 and  name HN   ) 0.0 0.0 3.5
assign (resid  40 and  name HB#  )(resid  40 and  name HN   ) 0.0 0.6 4.1
assign (resid  40 and  name HG1  )(resid  40 and  name HN   ) 0.0 0.0 3.5
assign (resid  40 and  name HG2  )(resid  40 and  name HN   ) 0.0 0.0 3.5
assign (resid  40 and  name HD#  )(resid  40 and  name HN   ) 0.0 1.0 6.0
assign (resid  40 and  name HB#  )(resid  40 and  name HA   ) 0.0 1.0 3.5
assign (resid  40 and  name HG1  )(resid  40 and  name HA   ) 0.0 0.0 5.0
assign (resid  40 and  name HG2  )(resid  40 and  name HA   ) 0.0 0.0 5.0
assign (resid  40 and  name HD#  )(resid  40 and  name HA   ) 0.0 1.0 6.0
assign (resid  41 and  name HB#  )(resid  41 and  name HN   ) 0.0 0.6 4.1
assign (resid  41 and  name HB#  )(resid  41 and  name HA   ) 0.0 1.0 3.5
assign (resid  42 and  name HB1  )(resid  42 and  name HN   ) 0.0 0.0 3.5
assign (resid  42 and  name HB2  )(resid  42 and  name HN   ) 0.0 0.0 3.5
assign (resid  42 and  name HG#  )(resid  42 and  name HN   ) 0.0 1.0 4.5
assign (resid  42 and  name HB1  )(resid  42 and  name HA   ) 0.0 0.0 3.5
assign (resid  42 and  name HB2  )(resid  42 and  name HA   ) 0.0 0.0 3.5
assign (resid  42 and  name HG#  )(resid  42 and  name HA   ) 0.0 1.0 3.5
assign (resid  43 and  name HB1  )(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 HA   ) 0.0 0.0 3.5
assign (resid  43 and  name HB2  )(resid  43 and  name HA   ) 0.0 0.0 3.5
assign (resid  44 and  name HB#  )(resid  44 and  name HN   ) 0.0 0.6 4.1
assign (resid  44 and  name HB1  )(resid  44 and  name HA   ) 0.0 0.0 2.5
assign (resid  44 and  name HB2  )(resid  44 and  name HA   ) 0.0 0.0 3.5
assign (resid  45 and  name HB#  )(resid  45 and  name HN   ) 0.0 0.6 3.1
assign (resid  45 and  name HB#  )(resid  45 and  name HA   ) 0.0 1.0 3.5
assign (resid  46 and  name HG1# )(resid  46 and  name HN   ) 0.0 1.0 4.5
assign (resid  46 and  name HG2# )(resid  46 and  name HN   ) 0.0 1.0 4.5
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 HA   ) 0.0 1.0 4.5
assign (resid  46 and  name HG2# )(resid  46 and  name HA   ) 0.0 1.0 4.5
assign (resid  46 and  name HB   )(resid  46 and  name HA   ) 0.0 0.0 3.5
assign (resid  47 and  name HB1  )(resid  47 and  name HA   ) 0.0 0.0 2.5
assign (resid  47 and  name HB2  )(resid  47 and  name HA   ) 0.0 0.0 3.5
assign (resid  47 and  name HG1  )(resid  47 and  name HA   ) 0.0 0.0 5.0
assign (resid  47 and  name HG2  )(resid  47 and  name HA   ) 0.0 0.0 5.0
assign (resid  47 and  name HD1  )(resid  47 and  name HA   ) 0.0 0.0 5.0
assign (resid  47 and  name HD2  )(resid  47 and  name HA   ) 0.0 0.0 5.0
assign (resid  48 and  name HG#  )(resid  48 and  name HN   ) 0.0 1.7 4.2
assign (resid  48 and  name HB   )(resid  48 and  name HN   ) 0.0 0.0 2.5
assign (resid  48 and  name HG#  )(resid  48 and  name HA   ) 0.0 1.7 4.2
assign (resid  48 and  name HB   )(resid  48 and  name HA   ) 0.0 0.0 2.5
assign (resid  49 and  name HB1  )(resid  49 and  name HN   ) 0.0 0.0 3.5
assign (resid  49 and  name HB2  )(resid  49 and  name HN   ) 0.0 0.0 3.5
assign (resid  49 and  name HB1  )(resid  49 and  name HA   ) 0.0 0.0 3.5
assign (resid  49 and  name HB2  )(resid  49 and  name HA   ) 0.0 0.0 3.5
assign (resid  49 and  name HG#  )(resid  49 and  name HA   ) 0.0 1.0 6.0
assign (resid  49 and  name HD#  )(resid  49 and  name HA   ) 0.0 1.0 6.0
assign (resid  49 and  name HE#  )(resid  49 and  name HA   ) 0.0 1.0 6.0
assign (resid  50 and  name HB#  )(resid  50 and  name HN   ) 0.0 0.6 3.1
assign (resid  50 and  name HB#  )(resid  50 and  name HA   ) 0.0 1.0 4.5
```

```
assign (resid   2 and   name HA   )(resid   2 and   name HD#  ) 0.0 2.0 4.5
assign (resid   2 and   name HB#  )(resid   2 and   name HD#  ) 0.0 3.0 5.5
assign (resid  15 and   name HA   )(resid  15 and   name HD#  ) 0.0 2.0 5.5
assign (resid  15 and   name HB2  )(resid  15 and   name HD#  ) 0.0 2.0 5.5
assign (resid  15 and   name HB1  )(resid  15 and   name HD#  ) 0.0 2.0 5.5
assign (resid  15 and   name HB1  )(resid  15 and   name HE#  ) 0.0 2.0 7.0
assign (resid  15 and   name HB2  )(resid  15 and   name HE#  ) 0.0 2.0 7.0
assign (resid  18 and   name HA   )(resid  18 and   name HD2  ) 0.0 0.0 2.5
assign (resid  18 and   name HB#  )(resid  18 and   name HD2  ) 0.0 1.0 4.5
assign (resid  21 and   name HA   )(resid  21 and   name HD1  ) 0.0 0.0 5.0
assign (resid  21 and   name HB1  )(resid  21 and   name HD1  ) 0.0 0.0 5.0
assign (resid  21 and   name HB2  )(resid  21 and   name HD1  ) 0.0 0.0 5.0
assign (resid  21 and   name HA   )(resid  21 and   name HE3  ) 0.0 0.0 5.0
assign (resid  33 and   name HA   )(resid  33 and   name HD#  ) 0.0 2.0 4.5
assign (resid  33 and   name HB#  )(resid  33 and   name HD#  ) 0.0 3.0 5.5
assign (resid  33 and   name HA   )(resid  33 and   name HE#  ) 0.0 2.0 5.5
assign (resid  33 and   name HB#  )(resid  33 and   name HE#  ) 0.0 3.0 6.5
assign (resid  35 and   name HA   )(resid  35 and   name HE3  ) 0.0 0.0 5.0
assign (resid  35 and   name HA   )(resid  35 and   name HD1  ) 0.0 0.0 5.0
assign (resid  35 and   name HB#  )(resid  35 and   name HE1  ) 0.0 1.0 6.0
assign (resid  44 and   name HA   )(resid  44 and   name HD2  ) 0.0 0.0 5.0
assign (resid  44 and   name HB#  )(resid  44 and   name HD2  ) 0.0 1.0 4.5
! SEQUENTIAL RESTRAINTS FOR TPA F1 MODULE
assign (resid   3 and   name HN   )(resid   2 and   name HA   ) 0.0 0.0 2.5
assign (resid   4 and   name HN   )(resid   3 and   name HA   ) 0.0 0.0 2.5
assign (resid   5 and   name HN   )(resid   4 and   name HA   ) 0.0 0.0 3.5
assign (resid   6 and   name HN   )(resid   5 and   name HA   ) 0.0 0.0 3.5
assign (resid   7 and   name HN   )(resid   6 and   name HA   ) 0.0 0.0 2.5
assign (resid   8 and   name HN   )(resid   7 and   name HA   ) 0.0 0.0 2.5
assign (resid   9 and   name HN   )(resid   8 and   name HA   ) 0.0 0.0 2.5
assign (resid  10 and   name HN   )(resid   9 and   name HA   ) 0.0 0.0 3.5
assign (resid  11 and   name HN   )(resid  12 and   name HA   ) 0.0 0.0 5.0
assign (resid  12 and   name HN   )(resid  11 and   name HA   ) 0.0 0.0 3.5
assign (resid  13 and   name HN   )(resid  12 and   name HA   ) 0.0 0.0 3.5
assign (resid  14 and   name HN   )(resid  13 and   name HA   ) 0.0 0.0 2.5
assign (resid  15 and   name HN   )(resid  14 and   name HA   ) 0.0 0.0 2.5
assign (resid  16 and   name HN   )(resid  15 and   name HA   ) 0.0 0.0 2.5
assign (resid  17 and   name HN   )(resid  16 and   name HA   ) 0.0 0.0 2.5
assign (resid  18 and   name HN   )(resid  17 and   name HA   ) 0.0 0.0 2.5
assign (resid  19 and   name HN   )(resid  18 and   name HA   ) 0.0 0.0 3.5
assign (resid  21 and   name HN   )(resid  20 and   name HA   ) 0.0 0.0 3.5
assign (resid  22 and   name HN   )(resid  21 and   name HA   ) 0.0 0.0 2.5
assign (resid  23 and   name HN   )(resid  22 and   name HA   ) 0.0 0.0 3.5
assign (resid  25 and   name HN   )(resid  24 and   name HA   ) 0.0 0.0 2.5
assign (resid  26 and   name HN   )(resid  25 and   name HA   ) 0.0 0.0 2.5
assign (resid  27 and   name HN   )(resid  26 and   name HA   ) 0.0 0.0 3.5
assign (resid  28 and   name HN   )(resid  27 and   name HA   ) 0.0 0.0 3.5
assign (resid  29 and   name HN   )(resid  28 and   name HA   ) 0.0 0.0 3.5
assign (resid  30 and   name HN   )(resid  29 and   name HA   ) 0.0 0.0 3.5
assign (resid  31 and   name HN   )(resid  30 and   name HA   ) 0.0 0.0 2.5
assign (resid  32 and   name HN   )(resid  31 and   name HA   ) 0.0 0.0 2.5
assign (resid  34 and   name HN   )(resid  33 and   name HA   ) 0.0 0.0 3.5
assign (resid  35 and   name HN   )(resid  34 and   name HA   ) 0.0 0.0 3.5
assign (resid  36 and   name HN   )(resid  35 and   name HA   ) 0.0 0.0 2.5
assign (resid  37 and   name HN   )(resid  36 and   name HA   ) 0.0 0.0 2.5
assign (resid  38 and   name HN   )(resid  37 and   name HA   ) 0.0 0.0 3.5
assign (resid  39 and   name HN   )(resid  38 and   name HA   ) 0.0 0.0 3.5
assign (resid  40 and   name HN   )(resid  39 and   name HA1  ) 0.0 0.0 3.5
assign (resid  42 and   name HN   )(resid  41 and   name HA   ) 0.0 0.0 2.5
assign (resid  43 and   name HN   )(resid  42 and   name HA   ) 0.0 0.0 2.5
assign (resid  44 and   name HN   )(resid  43 and   name HA   ) 0.0 0.0 2.5
assign (resid  45 and   name HN   )(resid  44 and   name HA   ) 0.0 0.0 2.5
assign (resid  46 and   name HN   )(resid  45 and   name HA   ) 0.0 0.0 2.5
assign (resid  48 and   name HN   )(resid  47 and   name HA   ) 0.0 0.0 2.5
```

```
assign (resid  49 and  name HN  )(resid  48 and  name HA  ) 0.0 0.0 3.5
assign (resid  50 and  name HN  )(resid  49 and  name HA  ) 0.0 0.0 2.5
assign (resid   5 and  name HN  )(resid   4 and  name HN  ) 0.0 0.0 3.5
assign (resid   6 and  name HN  )(resid   5 and  name HN  ) 0.0 0.0 5.0
assign (resid   7 and  name HN  )(resid   6 and  name HN  ) 0.0 0.0 5.0
assign (resid   9 and  name HN  )(resid   8 and  name HN  ) 0.0 0.0 5.0
assign (resid  10 and  name HN  )(resid   9 and  name HN  ) 0.0 0.0 2.5
assign (resid  11 and  name HN  )(resid  10 and  name HN  ) 0.0 0.0 2.5
assign (resid  12 and  name HN  )(resid  11 and  name HN  ) 0.0 0.0 2.5
assign (resid  13 and  name HN  )(resid  12 and  name HN  ) 0.0 0.0 2.5
assign (resid  15 and  name HN  )(resid  14 and  name HN  ) 0.0 0.0 5.0
assign (resid  17 and  name HN  )(resid  16 and  name HN  ) 0.0 0.0 5.0
assign (resid  18 and  name HN  )(resid  17 and  name HN  ) 0.0 0.0 5.0
assign (resid  19 and  name HN  )(resid  18 and  name HN  ) 0.0 0.0 3.5
assign (resid  21 and  name HN  )(resid  20 and  name HN  ) 0.0 0.0 5.0
assign (resid  22 and  name HN  )(resid  21 and  name HN  ) 0.0 0.0 5.0
assign (resid  26 and  name HN  )(resid  25 and  name HN  ) 0.0 0.0 5.0
assign (resid  27 and  name HN  )(resid  26 and  name HN  ) 0.0 0.0 3.5
assign (resid  28 and  name HN  )(resid  27 and  name HN  ) 0.0 0.0 2.5
assign (resid  29 and  name HN  )(resid  28 and  name HN  ) 0.0 0.0 3.5
assign (resid  30 and  name HN  )(resid  29 and  name HN  ) 0.0 0.0 3.5
assign (resid  31 and  name HN  )(resid  30 and  name HN  ) 0.0 0.0 5.0
assign (resid  32 and  name HN  )(resid  31 and  name HN  ) 0.0 0.0 5.0
assign (resid  35 and  name HN  )(resid  34 and  name HN  ) 0.0 0.0 5.0
assign (resid  36 and  name HN  )(resid  35 and  name HN  ) 0.0 0.0 5.0
assign (resid  37 and  name HN  )(resid  36 and  name HN  ) 0.0 0.0 5.0
assign (resid  38 and  name HN  )(resid  37 and  name HN  ) 0.0 0.0 3.5
assign (resid  39 and  name HN  )(resid  38 and  name HN  ) 0.0 0.0 3.5
assign (resid  40 and  name HN  )(resid  39 and  name HN  ) 0.0 0.0 2.5
assign (resid  41 and  name HN  )(resid  40 and  name HN  ) 0.0 0.0 5.0
assign (resid  43 and  name HN  )(resid  42 and  name HN  ) 0.0 0.0 5.0
assign (resid  44 and  name HN  )(resid  43 and  name HN  ) 0.0 0.0 5.0
assign (resid  45 and  name HN  )(resid  44 and  name HN  ) 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  49 and  name HN  )(resid  48 and  name HN  ) 0.0 0.0 3.5
assign (resid  50 and  name HN  )(resid  49 and  name HN  ) 0.0 0.0 5.0
assign (resid   2 and  name HB2 )(resid   3 and  name HN  ) 0.0 0.0 3.5
assign (resid   2 and  name HB1 )(resid   3 and  name HN  ) 0.0 0.0 3.5
assign (resid   3 and  name HG# )(resid   4 and  name HN  ) 0.0 1.0 4.5
assign (resid   3 and  name HB# )(resid   4 and  name HN  ) 0.0 1.0 4.5
assign (resid   5 and  name HG12)(resid   4 and  name HN  ) 0.0 0.0 5.0
assign (resid   5 and  name HG11)(resid   4 and  name HN  ) 0.0 0.0 5.0
assign (resid   4 and  name HB  )(resid   5 and  name HN  ) 0.0 0.0 5.0
assign (resid   4 and  name HG# )(resid   5 and  name HN  ) 0.0 2.4 5.9
assign (resid   5 and  name HB  )(resid   6 and  name HN  ) 0.0 0.0 2.5
assign (resid   5 and  name HG12)(resid   6 and  name HN  ) 0.0 0.0 5.0
assign (resid   5 and  name HG11)(resid   6 and  name HN  ) 0.0 0.0 5.0
assign (resid   5 and  name HG2#)(resid   6 and  name HN  ) 0.0 1.0 4.5
assign (resid   5 and  name HD# )(resid   6 and  name HN  ) 0.0 1.0 4.5
assign (resid   5 and  name HD# )(resid   6 and  name HA  ) 0.0 1.0 6.0
assign (resid   6 and  name HB1 )(resid   7 and  name HN  ) 0.0 0.0 5.0
assign (resid   6 and  name HB2 )(resid   7 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 HB1 )(resid   8 and  name HN  ) 0.0 0.0 5.0
assign (resid   7 and  name HG2 )(resid   8 and  name HN  ) 0.0 0.0 3.5
assign (resid   7 and  name HG1 )(resid   8 and  name HN  ) 0.0 0.0 3.5
assign (resid   8 and  name HB1 )(resid   9 and  name HN  ) 0.0 0.0 5.0
assign (resid   9 and  name HG# )(resid  10 and  name HN  ) 0.0 1.0 6.0
assign (resid   9 and  name HB# )(resid  10 and  name HN  ) 0.0 1.0 4.5
assign (resid  11 and  name HG2#)(resid  10 and  name HN  ) 0.0 1.0 4.5
assign (resid  10 and  name HB2 )(resid  11 and  name HN  ) 0.0 0.0 3.5
assign (resid  10 and  name HB1 )(resid  11 and  name HN  ) 0.0 0.0 3.5
assign (resid  10 and  name HD# )(resid  11 and  name HN  ) 0.0 1.0 6.0
assign (resid  10 and  name HG# )(resid  11 and  name HN  ) 0.0 1.0 6.0
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assign (resid  11 and  name HG2# )(resid  12 and  name HN  ) 0.0 1.0 3.5
assign (resid  13 and  name HA  )(resid  12 and  name HN  ) 0.0 0.0 5.0
assign (resid  14 and  name HG1# )(resid  13 and  name HA  ) 0.0 1.0 4.5
assign (resid  14 and  name HD#  )(resid  13 and  name HA  ) 0.0 1.0 6.0
assign (resid  13 and  name HG#  )(resid  14 and  name HN  ) 0.0 1.0 4.5
assign (resid  13 and  name HB#  )(resid  14 and  name HN  ) 0.0 1.0 6.0
assign (resid  14 and  name HB  )(resid  15 and  name HN  ) 0.0 0.0 5.0
assign (resid  14 and  name HG1# )(resid  15 and  name HN  ) 0.0 1.0 6.0
assign (resid  14 and  name HD#  )(resid  15 and  name HN  ) 0.0 1.0 6.0
assign (resid  14 and  name HG2# )(resid  15 and  name HN  ) 0.0 1.0 3.5
assign (resid  16 and  name HG#  )(resid  17 and  name HN  ) 0.0 1.0 4.5
assign (resid  17 and  name HG#  )(resid  18 and  name HN  ) 0.0 1.0 6.0
assign (resid  17 and  name HB#  )(resid  18 and  name HN  ) 0.0 1.0 4.5
assign (resid  18 and  name HB#  )(resid  19 and  name HN  ) 0.0 1.0 6.0
assign (resid  19 and  name HG#  )(resid  20 and  name HN  ) 0.0 1.0 3.5
assign (resid  19 and  name HB#  )(resid  20 and  name HN  ) 0.0 1.0 4.5
assign (resid  20 and  name HB#  )(resid  21 and  name HN  ) 0.0 1.0 3.5
assign (resid  21 and  name HB2  )(resid  22 and  name HN  ) 0.0 0.0 5.0
assign (resid  21 and  name HB1  )(resid  22 and  name HN  ) 0.0 0.0 5.0
assign (resid  22 and  name HB#  )(resid  23 and  name HN  ) 0.0 1.0 4.5
assign (resid  22 and  name HG  )(resid  23 and  name HN  ) 0.0 0.0 5.0
assign (resid  22 and  name HD#  )(resid  23 and  name HN  ) 0.0 2.4 5.9
assign (resid  24 and  name HD2  )(resid  23 and  name HA  ) 0.0 0.0 2.5
assign (resid  24 and  name HD1  )(resid  23 and  name HA  ) 0.0 0.0 2.5
assign (resid  24 and  name HG2  )(resid  23 and  name HA  ) 0.0 0.0 5.0
assign (resid  24 and  name HG1  )(resid  23 and  name HA  ) 0.0 0.0 5.0
assign (resid  23 and  name HB#  )(resid  24 and  name HD#  ) 0.0 2.0 5.5
assign (resid  23 and  name HG#  )(resid  24 and  name HD#  ) 0.0 2.0 7.0
assign (resid  24 and  name HB#  )(resid  25 and  name HN  ) 0.0 1.0 4.5
assign (resid  24 and  name HG2  )(resid  25 and  name HN  ) 0.0 0.0 5.0
assign (resid  24 and  name HG1  )(resid  25 and  name HN  ) 0.0 0.0 5.0
assign (resid  25 and  name HB  )(resid  26 and  name HN  ) 0.0 0.0 3.5
assign (resid  25 and  name HG#  )(resid  26 and  name HN  ) 0.0 2.4 4.9
assign (resid  26 and  name HB1  )(resid  27 and  name HN  ) 0.0 0.0 3.5
assign (resid  26 and  name HB2  )(resid  27 and  name HN  ) 0.0 0.0 3.5
assign (resid  26 and  name HG  )(resid  27 and  name HN  ) 0.0 0.0 3.5
assign (resid  26 and  name HD2# )(resid  27 and  name HN  ) 0.0 1.0 6.0
assign (resid  26 and  name HD1# )(resid  27 and  name HN  ) 0.0 1.0 6.0
assign (resid  27 and  name HB1  )(resid  28 and  name HN  ) 0.0 0.0 5.0
assign (resid  27 and  name HB2  )(resid  28 and  name HN  ) 0.0 0.0 3.5
assign (resid  27 and  name HG#  )(resid  28 and  name HN  ) 0.0 1.0 4.5
assign (resid  28 and  name HB2  )(resid  29 and  name HN  ) 0.0 0.0 3.5
assign (resid  28 and  name HB1  )(resid  29 and  name HN  ) 0.0 0.0 3.5
assign (resid  29 and  name HB#  )(resid  30 and  name HN  ) 0.0 1.0 4.5
assign (resid  30 and  name HB#  )(resid  31 and  name HN  ) 0.0 1.0 4.5
assign (resid  30 and  name HG#  )(resid  31 and  name HN  ) 0.0 1.0 4.5
assign (resid  30 and  name HD#  )(resid  31 and  name HN  ) 0.0 1.0 4.5
assign (resid  31 and  name HB  )(resid  32 and  name HN  ) 0.0 0.0 3.5
assign (resid  31 and  name HG#  )(resid  32 and  name HN  ) 0.0 2.4 4.9
assign (resid  32 and  name HB#  )(resid  33 and  name HN  ) 0.0 1.0 4.5
assign (resid  32 and  name HG#  )(resid  33 and  name HN  ) 0.0 1.0 4.5
assign (resid  34 and  name HA  )(resid  33 and  name HN  ) 0.0 0.0 5.0
assign (resid  33 and  name HB#  )(resid  34 and  name HN  ) 0.0 1.0 4.5
assign (resid  35 and  name HA  )(resid  34 and  name HN  ) 0.0 0.0 5.0
assign (resid  35 and  name HB#  )(resid  36 and  name HN  ) 0.0 1.0 4.5
assign (resid  36 and  name HB1  )(resid  37 and  name HN  ) 0.0 0.0 5.0
assign (resid  36 and  name HB2  )(resid  37 and  name HN  ) 0.0 0.0 5.0
assign (resid  37 and  name HB#  )(resid  38 and  name HN  ) 0.0 1.0 6.0
assign (resid  40 and  name HB#  )(resid  41 and  name HN  ) 0.0 1.0 4.5
assign (resid  40 and  name HD#  )(resid  41 and  name HN  ) 0.0 1.0 6.0
assign (resid  40 and  name HG2  )(resid  41 and  name HN  ) 0.0 0.0 3.5
assign (resid  40 and  name HG1  )(resid  41 and  name HN  ) 0.0 0.0 3.5
assign (resid  41 and  name HB#  )(resid  42 and  name HN  ) 0.0 1.0 4.5
assign (resid  42 and  name HG#  )(resid  43 and  name HN  ) 0.0 1.0 4.5
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assign (resid   42 and   name HB#  )(resid   43 and   name HN   ) 0.0 1.0 6.0
assign (resid   43 and   name HB1  )(resid   44 and   name HN   ) 0.0 0.0 3.5
assign (resid   43 and   name HB2  )(resid   44 and   name HN   ) 0.0 0.0 3.5
assign (resid   44 and   name HB#  )(resid   45 and   name HN   ) 0.0 1.0 4.5
assign (resid   45 and   name HB#  )(resid   46 and   name HN   ) 0.0 1.0 4.5
assign (resid   47 and   name HD2  )(resid   46 and   name HA   ) 0.0 0.0 2.5
assign (resid   47 and   name HD1  )(resid   46 and   name HA   ) 0.0 0.0 2.5
assign (resid   47 and   name HD2  )(resid   46 and   name HN   ) 0.0 0.0 5.0
assign (resid   47 and   name HD1  )(resid   46 and   name HN   ) 0.0 0.0 5.0
assign (resid   47 and   name HB1  )(resid   48 and   name HN   ) 0.0 0.0 3.5
assign (resid   47 and   name HB2  )(resid   48 and   name HN   ) 0.0 0.0 2.5
assign (resid   47 and   name HG#  )(resid   48 and   name HN   ) 0.0 1.0 6.0
assign (resid   48 and   name HB   )(resid   49 and   name HN   ) 0.0 0.0 5.0
assign (resid   48 and   name HG#  )(resid   49 and   name HN   ) 0.0 2.4 5.9
assign (resid   49 and   name HB#  )(resid   50 and   name HN   ) 0.0 1.0 4.5
assign (resid   49 and   name HG#  )(resid   50 and   name HN   ) 0.0 1.0 6.0
assign (resid   16 and   name HG#  )(resid   15 and   name HD#  ) 0.0 3.0 8.0
assign (resid   16 and   name HA   )(resid   15 and   name HD#  ) 0.0 2.0 7.0
assign (resid   20 and   name HA   )(resid   21 and   name HZ3  ) 0.0 0.0 5.0
assign (resid   20 and   name HB#  )(resid   21 and   name HE3  ) 0.0 1.0 6.0
assign (resid   20 and   name HA   )(resid   21 and   name HE3  ) 0.0 0.0 3.5
assign (resid   22 and   name HN   )(resid   21 and   name HD1  ) 0.0 0.0 5.0
assign (resid   20 and   name HN   )(resid   21 and   name HE3  ) 0.0 0.0 5.0
assign (resid   34 and   name HN   )(resid   33 and   name HE#  ) 0.0 2.0 7.0
assign (resid   34 and   name HN   )(resid   33 and   name HD#  ) 0.0 2.0 5.5
assign (resid   34 and   name HA   )(resid   35 and   name HE3  ) 0.0 0.0 5.0
assign (resid   36 and   name HN   )(resid   35 and   name HD1  ) 0.0 0.0 3.5
assign (resid   45 and   name HN   )(resid   44 and   name HD2  ) 0.0 0.0 3.5
! SHORT RANGE RESTRAINTS FOR TPA F1 MODULE
assign (resid    3 and   name HA   )(resid    5 and   name HN   ) 0.0 0.0 3.5
assign (resid    5 and   name HD#  )(resid    7 and   name HN   ) 0.0 1.0 6.0
assign (resid   11 and   name HN   )(resid    9 and   name HN   ) 0.0 0.0 5.0
assign (resid   12 and   name HA   )(resid    9 and   name HA   ) 0.0 0.0 3.5
assign (resid   12 and   name HN   )(resid   10 and   name HN   ) 0.0 0.0 3.5
assign (resid   12 and   name HA   )(resid   10 and   name HN   ) 0.0 0.0 5.0
assign (resid    8 and   name HA   )(resid   11 and   name HN   ) 0.0 0.0 5.0
assign (resid    8 and   name HB1  )(resid   11 and   name HN   ) 0.0 0.0 3.5
assign (resid    9 and   name HA   )(resid   11 and   name HN   ) 0.0 0.0 3.5
assign (resid    9 and   name HG2  )(resid   11 and   name HN   ) 0.0 0.0 5.0
assign (resid    9 and   name HG1  )(resid   11 and   name HN   ) 0.0 0.0 5.0
assign (resid    9 and   name HB#  )(resid   11 and   name HN   ) 0.0 1.0 6.0
assign (resid   13 and   name HN   )(resid   11 and   name HN   ) 0.0 0.0 3.5
assign (resid   10 and   name HB2  )(resid   12 and   name HN   ) 0.0 0.0 5.0
assign (resid   10 and   name HB1  )(resid   12 and   name HN   ) 0.0 0.0 5.0
assign (resid   11 and   name HG2# )(resid   13 and   name HN   ) 0.0 1.0 6.0
assign (resid   11 and   name HB   )(resid   13 and   name HN   ) 0.0 0.0 5.0
assign (resid   19 and   name HB#  )(resid   17 and   name HN   ) 0.0 1.0 4.5
assign (resid   17 and   name HA   )(resid   19 and   name HN   ) 0.0 0.0 3.5
assign (resid   17 and   name HN   )(resid   19 and   name HN   ) 0.0 0.0 5.0
assign (resid   26 and   name HA   )(resid   28 and   name HN   ) 0.0 0.0 5.0
assign (resid   28 and   name HA   )(resid   30 and   name HN   ) 0.0 0.0 5.0
assign (resid   28 and   name HB2  )(resid   30 and   name HN   ) 0.0 0.0 3.5
assign (resid   28 and   name HB1  )(resid   30 and   name HN   ) 0.0 0.0 3.5
assign (resid   28 and   name HN   )(resid   30 and   name HN   ) 0.0 0.0 5.0
assign (resid   27 and   name HN   )(resid   30 and   name HN   ) 0.0 0.0 5.0
assign (resid   40 and   name HN   )(resid   38 and   name HN   ) 0.0 0.0 5.0
assign (resid   38 and   name HB2  )(resid   40 and   name HN   ) 0.0 0.0 5.0
assign (resid   38 and   name HA   )(resid   40 and   name HN   ) 0.0 0.0 5.0
assign (resid   38 and   name HB1  )(resid   40 and   name HN   ) 0.0 0.0 5.0
assign (resid   44 and   name HB#  )(resid   46 and   name HN   ) 0.0 1.0 6.0
assign (resid   47 and   name HA   )(resid   49 and   name HN   ) 0.0 0.0 5.0
assign (resid   48 and   name HG#  )(resid   50 and   name HN   ) 0.0 2.4 5.9
assign (resid   12 and   name HN   )(resid    8 and   name HN   ) 0.0 0.0 5.0
assign (resid    8 and   name HB1  )(resid   12 and   name HN   ) 0.0 0.0 3.5
```

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assign (resid   8 and   name HA   )(resid  12 and   name HN   ) 0.0 0.0 5.0
assign (resid   9 and   name HA   )(resid  12 and   name HN   ) 0.0 0.0 3.5
assign (resid  30 and   name HB#  )(resid  26 and   name HN   ) 0.0 1.0 6.0
assign (resid  41 and   name HB#  )(resid  37 and   name HN   ) 0.0 1.0 6.0
assign (resid  40 and   name HN   )(resid  37 and   name HN   ) 0.0 0.0 3.5
assign (resid  36 and   name HA   )(resid  40 and   name HN   ) 0.0 0.0 5.0
assign (resid  37 and   name HN   )(resid  41 and   name HN   ) 0.0 0.0 5.0
assign (resid  13 and   name HB2  )(resid  15 and   name HE#  ) 0.0 2.0 7.0
assign (resid  13 and   name HB1  )(resid  15 and   name HE#  ) 0.0 2.0 7.0
assign (resid  13 and   name HN   )(resid  15 and   name HE#  ) 0.0 2.0 7.0
assign (resid  23 and   name HG#  )(resid  21 and   name HD1  ) 0.0 1.0 4.5
assign (resid  23 and   name HD2  )(resid  21 and   name HD1  ) 0.0 0.0 3.5
assign (resid  23 and   name HD1  )(resid  21 and   name HD1  ) 0.0 0.0 3.5
assign (resid  23 and   name HA   )(resid  21 and   name HD1  ) 0.0 0.0 5.0
assign (resid  23 and   name HD2  )(resid  21 and   name HZ2  ) 0.0 0.0 5.0
assign (resid  23 and   name HD1  )(resid  21 and   name HZ2  ) 0.0 0.0 5.0
assign (resid  23 and   name HG#  )(resid  21 and   name HE1  ) 0.0 1.0 4.5
assign (resid  23 and   name HD2  )(resid  21 and   name HE1  ) 0.0 0.0 3.5
assign (resid  23 and   name HD1  )(resid  21 and   name HE1  ) 0.0 0.0 3.5
assign (resid  23 and   name HN   )(resid  21 and   name HD1  ) 0.0 0.0 5.0
assign (resid  19 and   name HN   )(resid  21 and   name HZ3  ) 0.0 0.0 5.0
assign (resid  35 and   name HB#  )(resid  33 and   name HE#  ) 0.0 3.0 6.5
assign (resid  35 and   name HA   )(resid  33 and   name HE#  ) 0.0 2.0 7.0
assign (resid  35 and   name HN   )(resid  33 and   name HE#  ) 0.0 2.0 7.0
assign (resid  35 and   name HN   )(resid  33 and   name HD#  ) 0.0 2.0 7.0
assign (resid  37 and   name HB#  )(resid  35 and   name HE1  ) 0.0 1.0 6.0
assign (resid  37 and   name HA   )(resid  35 and   name HE1  ) 0.0 0.0 5.0
assign (resid  37 and   name HN   )(resid  35 and   name HE1  ) 0.0 0.0 5.0
assign (resid  46 and   name HA   )(resid  44 and   name HD2  ) 0.0 0.0 5.0
assign (resid  46 and   name HG2# )(resid  44 and   name HD2  ) 0.0 1.0 6.0
assign (resid  46 and   name HG1# )(resid  44 and   name HD2  ) 0.0 1.0 6.0
! LONG RANGE RESTRAINTS FOR TPA F1 MODULE
assign (resid  16 and   name HA   )(resid   5 and   name HA   ) 0.0 0.0 2.5
assign (resid  17 and   name HA   )(resid   5 and   name HA   ) 0.0 0.0 5.0
assign (resid  16 and   name HA   )(resid   5 and   name HN   ) 0.0 0.0 5.0
assign (resid  39 and   name HA2  )(resid   6 and   name HA   ) 0.0 0.0 5.0
assign (resid  39 and   name HA1  )(resid   6 and   name HA   ) 0.0 0.0 5.0
assign (resid  41 and   name HB#  )(resid   6 and   name HB1  ) 0.0 1.0 6.0
assign (resid  41 and   name HB#  )(resid   6 and   name HB2  ) 0.0 1.0 6.0
assign (resid  16 and   name HA   )(resid   6 and   name HN   ) 0.0 0.0 5.0
assign (resid  15 and   name HN   )(resid   6 and   name HN   ) 0.0 0.0 3.5
assign (resid  14 and   name HB   )(resid   6 and   name HN   ) 0.0 0.0 5.0
assign (resid  14 and   name HD#  )(resid   6 and   name HN   ) 0.0 1.0 6.0
assign (resid  14 and   name HA   )(resid   7 and   name HA   ) 0.0 0.0 2.5
assign (resid  14 and   name HG1# )(resid   7 and   name HA   ) 0.0 1.0 4.5
assign (resid  14 and   name HG2# )(resid   7 and   name HA   ) 0.0 1.0 4.5
assign (resid  14 and   name HD#  )(resid   7 and   name HA   ) 0.0 1.0 4.5
assign (resid  14 and   name HD#  )(resid   8 and   name HN   ) 0.0 1.0 6.0
assign (resid  14 and   name HG2# )(resid   8 and   name HN   ) 0.0 1.0 6.0
assign (resid  14 and   name HA   )(resid   8 and   name HN   ) 0.0 0.0 3.5
assign (resid  13 and   name HG#  )(resid   8 and   name HN   ) 0.0 1.0 6.0
assign (resid  13 and   name HA   )(resid   8 and   name HN   ) 0.0 0.0 5.0
assign (resid  13 and   name HN   )(resid   8 and   name HN   ) 0.0 0.0 3.5
assign (resid  41 and   name HB#  )(resid   8 and   name HA   ) 0.0 1.0 6.0
assign (resid   7 and   name HG#  )(resid  12 and   name HN   ) 0.0 1.0 6.0
assign (resid   8 and   name HB1  )(resid  13 and   name HN   ) 0.0 0.0 3.5
assign (resid   8 and   name HN   )(resid  14 and   name HN   ) 0.0 0.0 5.0
assign (resid   6 and   name HB#  )(resid  15 and   name HN   ) 0.0 1.0 6.0
assign (resid   4 and   name HB   )(resid  16 and   name HA   ) 0.0 0.0 5.0
assign (resid   5 and   name HB   )(resid  16 and   name HA   ) 0.0 0.0 5.0
assign (resid   5 and   name HG2# )(resid  16 and   name HA   ) 0.0 1.0 4.5
assign (resid  36 and   name HB1  )(resid  17 and   name HA   ) 0.0 0.0 2.5
assign (resid  36 and   name HB2  )(resid  17 and   name HA   ) 0.0 0.0 3.5
assign (resid  36 and   name HB1  )(resid  18 and   name HN   ) 0.0 0.0 2.5
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assign (resid  36 and  name HB2  )(resid  18 and  name HN   ) 0.0 0.0 3.5
assign (resid  36 and  name HA   )(resid  18 and  name HN   ) 0.0 0.0 5.0
assign (resid  36 and  name HN   )(resid  18 and  name HN   ) 0.0 0.0 5.0
assign (resid  36 and  name HB1  )(resid  19 and  name HN   ) 0.0 0.0 3.5
assign (resid  36 and  name HB2  )(resid  19 and  name HN   ) 0.0 0.0 5.0
assign (resid  36 and  name HA   )(resid  19 and  name HN   ) 0.0 0.0 5.0
assign (resid  35 and  name HA   )(resid  20 and  name HN   ) 0.0 0.0 5.0
assign (resid  35 and  name HB#  )(resid  20 and  name HN   ) 0.0 1.0 4.5
assign (resid  35 and  name HA   )(resid  20 and  name HA   ) 0.0 0.0 2.5
assign (resid  35 and  name HB#  )(resid  20 and  name HA   ) 0.0 1.0 4.5
assign (resid  35 and  name HA   )(resid  21 and  name HN   ) 0.0 0.0 3.5
assign (resid  36 and  name HN   )(resid  21 and  name HN   ) 0.0 0.0 5.0
assign (resid  33 and  name HA   )(resid  22 and  name HA   ) 0.0 0.0 3.5
assign (resid  33 and  name HA   )(resid  22 and  name HN   ) 0.0 0.0 5.0
assign (resid  33 and  name HB#  )(resid  22 and  name HA   ) 0.0 1.0 6.0
assign (resid  33 and  name HA   )(resid  23 and  name HN   ) 0.0 0.0 5.0
assign (resid  31 and  name HA   )(resid  23 and  name HN   ) 0.0 0.0 5.0
assign (resid  32 and  name HN   )(resid  23 and  name HN   ) 0.0 0.0 3.5
assign (resid  11 and  name HG2# )(resid  23 and  name HD2  ) 0.0 1.0 6.0
assign (resid  11 and  name HG2# )(resid  23 and  name HD1  ) 0.0 1.0 6.0
assign (resid  11 and  name HG2# )(resid  23 and  name HA   ) 0.0 1.0 6.0
assign (resid  11 and  name HG2# )(resid  24 and  name HD2  ) 0.0 1.0 6.0
assign (resid  11 and  name HG2# )(resid  24 and  name HD1  ) 0.0 1.0 6.0
assign (resid  31 and  name HA   )(resid  25 and  name HA   ) 0.0 0.0 3.5
assign (resid  32 and  name HN   )(resid  25 and  name HN   ) 0.0 0.0 5.0
assign (resid  25 and  name HB   )(resid  30 and  name HN   ) 0.0 0.0 3.5
assign (resid  25 and  name HG2# )(resid  30 and  name HN   ) 0.0 1.0 4.5
assign (resid  25 and  name HG1# )(resid  30 and  name HN   ) 0.0 1.0 4.5
assign (resid  25 and  name HA   )(resid  30 and  name HN   ) 0.0 0.0 5.0
assign (resid  47 and  name HA   )(resid  30 and  name HA   ) 0.0 0.0 2.5
assign (resid  47 and  name HB#  )(resid  30 and  name HA   ) 0.0 1.0 6.0
assign (resid  47 and  name HG#  )(resid  30 and  name HA   ) 0.0 1.0 6.0
assign (resid  48 and  name HB   )(resid  30 and  name HA   ) 0.0 0.0 5.0
assign (resid  48 and  name HN   )(resid  31 and  name HN   ) 0.0 0.0 5.0
assign (resid  47 and  name HA   )(resid  31 and  name HN   ) 0.0 0.0 3.5
assign (resid  45 and  name HB#  )(resid  32 and  name HA   ) 0.0 1.0 4.5
assign (resid  23 and  name HA   )(resid  32 and  name HN   ) 0.0 0.0 5.0
assign (resid  22 and  name HA   )(resid  32 and  name HN   ) 0.0 0.0 5.0
assign (resid  22 and  name HA   )(resid  33 and  name HN   ) 0.0 0.0 5.0
assign (resid  22 and  name HD#  )(resid  33 and  name HN   ) 0.0 2.4 5.9
assign (resid  43 and  name HB#  )(resid  33 and  name HN   ) 0.0 1.0 6.0
assign (resid  44 and  name HB#  )(resid  33 and  name HN   ) 0.0 1.0 6.0
assign (resid  45 and  name HB#  )(resid  33 and  name HN   ) 0.0 1.0 6.0
assign (resid  45 and  name HA   )(resid  33 and  name HN   ) 0.0 0.0 3.5
assign (resid  22 and  name HA   )(resid  34 and  name HN   ) 0.0 0.0 3.5
assign (resid  22 and  name HD2# )(resid  34 and  name HN   ) 0.0 1.0 4.5
assign (resid  22 and  name HD1# )(resid  34 and  name HN   ) 0.0 1.0 4.5
assign (resid  22 and  name HN   )(resid  34 and  name HN   ) 0.0 0.0 5.0
assign (resid  23 and  name HB#  )(resid  34 and  name HN   ) 0.0 1.0 6.0
assign (resid  43 and  name HA   )(resid  34 and  name HN   ) 0.0 0.0 5.0
assign (resid  43 and  name HB1  )(resid  34 and  name HA   ) 0.0 0.0 5.0
assign (resid  43 and  name HB2  )(resid  34 and  name HA   ) 0.0 0.0 5.0
assign (resid  43 and  name HA   )(resid  35 and  name HN   ) 0.0 0.0 3.5
assign (resid  41 and  name HA   )(resid  35 and  name HN   ) 0.0 0.0 5.0
assign (resid  41 and  name HB#  )(resid  35 and  name HN   ) 0.0 1.0 6.0
assign (resid  20 and  name HB#  )(resid  35 and  name HA   ) 0.0 1.0 4.5
assign (resid  15 and  name HB#  )(resid  36 and  name HN   ) 0.0 1.0 6.0
assign (resid  20 and  name HA   )(resid  36 and  name HN   ) 0.0 0.0 5.0
assign (resid  20 and  name HB#  )(resid  36 and  name HN   ) 0.0 1.0 6.0
assign (resid  18 and  name HA   )(resid  36 and  name HN   ) 0.0 0.0 5.0
assign (resid  19 and  name HN   )(resid  36 and  name HN   ) 0.0 0.0 3.5
assign (resid  41 and  name HA   )(resid  36 and  name HN   ) 0.0 0.0 5.0
assign (resid  41 and  name HA   )(resid  36 and  name HA   ) 0.0 0.0 2.5
assign (resid  41 and  name HB#  )(resid  36 and  name HA   ) 0.0 1.0 6.0
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assign (resid   6 and   name HB#  )(resid   36 and   name HB#  ) 0.0 2.0 7.0
assign (resid   6 and   name HB1  )(resid   36 and   name HA   ) 0.0 0.0 5.0
assign (resid   6 and   name HB2  )(resid   36 and   name HA   ) 0.0 0.0 5.0
assign (resid  42 and   name HG#  )(resid   37 and   name HN   ) 0.0 1.0 4.5
assign (resid  18 and   name HA   )(resid   37 and   name HA   ) 0.0 0.0 5.0
assign (resid  17 and   name HA   )(resid   38 and   name HN   ) 0.0 0.0 5.0
assign (resid  36 and   name HB1  )(resid   41 and   name HA   ) 0.0 0.0 5.0
assign (resid  36 and   name HB2  )(resid   41 and   name HA   ) 0.0 0.0 5.0
assign (resid   6 and   name HB1  )(resid   41 and   name HN   ) 0.0 0.0 5.0
assign (resid   6 and   name HB2  )(resid   41 and   name HN   ) 0.0 0.0 5.0
assign (resid  34 and   name HA   )(resid   42 and   name HN   ) 0.0 0.0 5.0
assign (resid  36 and   name HA   )(resid   42 and   name HN   ) 0.0 0.0 3.5
assign (resid  35 and   name HA   )(resid   42 and   name HN   ) 0.0 0.0 5.0
assign (resid  35 and   name HB#  )(resid   42 and   name HN   ) 0.0 1.0 6.0
assign (resid  37 and   name HN   )(resid   42 and   name HN   ) 0.0 0.0 3.5
assign (resid  35 and   name HN   )(resid   42 and   name HN   ) 0.0 0.0 5.0
assign (resid  34 and   name HA   )(resid   43 and   name HA   ) 0.0 0.0 2.5
assign (resid  34 and   name HA   )(resid   43 and   name HN   ) 0.0 0.0 5.0
assign (resid  32 and   name HB#  )(resid   44 and   name HN   ) 0.0 1.0 6.0
assign (resid  34 and   name HA   )(resid   44 and   name HN   ) 0.0 0.0 3.5
assign (resid  33 and   name HN   )(resid   44 and   name HN   ) 0.0 0.0 3.5
assign (resid  34 and   name HN   )(resid   44 and   name HN   ) 0.0 0.0 5.0
assign (resid  35 and   name HN   )(resid   44 and   name HN   ) 0.0 0.0 5.0
assign (resid  33 and   name HA   )(resid   44 and   name HN   ) 0.0 0.0 5.0
assign (resid  33 and   name HB#  )(resid   44 and   name HN   ) 0.0 1.0 6.0
assign (resid  32 and   name HA   )(resid   44 and   name HN   ) 0.0 0.0 5.0
assign (resid  30 and   name HD#  )(resid   45 and   name HB#  ) 0.0 2.0 5.5
assign (resid  33 and   name HB#  )(resid   45 and   name HA   ) 0.0 1.0 6.0
assign (resid  30 and   name HD#  )(resid   47 and   name HA   ) 0.0 1.0 4.5
assign (resid  30 and   name HB#  )(resid   47 and   name HA   ) 0.0 1.0 4.5
assign (resid  30 and   name HG#  )(resid   47 and   name HA   ) 0.0 1.0 6.0
assign (resid  30 and   name HA   )(resid   48 and   name HN   ) 0.0 0.0 3.5
assign (resid  30 and   name HD#  )(resid   48 and   name HN   ) 0.0 1.0 6.0
assign (resid  31 and   name HG#  )(resid   48 and   name HN   ) 0.0 1.0 6.0
assign (resid  29 and   name HB#  )(resid   48 and   name HN   ) 0.0 1.0 6.0
assign (resid   8 and   name HB2  )(resid   15 and   name HE#  ) 0.0 2.0 5.5
assign (resid   8 and   name HB1  )(resid   15 and   name HE#  ) 0.0 2.0 5.5
assign (resid  21 and   name HB2  )(resid   15 and   name HD#  ) 0.0 2.0 7.0
assign (resid  21 and   name HB1  )(resid   15 and   name HD#  ) 0.0 2.0 7.0
assign (resid   8 and   name HN   )(resid   15 and   name HE#  ) 0.0 2.0 5.5
assign (resid   6 and   name HN   )(resid   15 and   name HD#  ) 0.0 2.0 7.0
assign (resid   8 and   name HN   )(resid   15 and   name HD#  ) 0.0 2.0 5.5
assign (resid  37 and   name HA   )(resid   18 and   name HD2  ) 0.0 0.0 2.5
assign (resid  38 and   name HN   )(resid   18 and   name HD2  ) 0.0 0.0 5.0
assign (resid  11 and   name HG2# )(resid   21 and   name HD1  ) 0.0 1.0 4.5
assign (resid   8 and   name HB2  )(resid   21 and   name HZ2  ) 0.0 0.0 5.0
assign (resid   6 and   name HB1  )(resid   21 and   name HZ2  ) 0.0 0.0 5.0
assign (resid   6 and   name HB2  )(resid   21 and   name HZ2  ) 0.0 0.0 5.0
assign (resid  41 and   name HB#  )(resid   21 and   name HZ2  ) 0.0 1.0 3.5
assign (resid  41 and   name HA   )(resid   21 and   name HZ2  ) 0.0 0.0 5.0
assign (resid  41 and   name HB#  )(resid   21 and   name HH2  ) 0.0 1.0 3.5
assign (resid  41 and   name HA   )(resid   21 and   name HH2  ) 0.0 0.0 3.5
assign (resid   6 and   name HB1  )(resid   21 and   name HH2  ) 0.0 0.0 2.5
assign (resid   6 and   name HB2  )(resid   21 and   name HH2  ) 0.0 0.0 3.5
assign (resid  36 and   name HB1  )(resid   21 and   name HH2  ) 0.0 0.0 5.0
assign (resid  36 and   name HB2  )(resid   21 and   name HH2  ) 0.0 0.0 3.5
assign (resid  36 and   name HA   )(resid   21 and   name HH2  ) 0.0 0.0 3.5
assign (resid  41 and   name HB#  )(resid   21 and   name HZ3  ) 0.0 1.0 6.0
assign (resid  41 and   name HA   )(resid   21 and   name HZ3  ) 0.0 0.0 5.0
assign (resid   6 and   name HB1  )(resid   21 and   name HZ3  ) 0.0 0.0 5.0
assign (resid   6 and   name HB2  )(resid   21 and   name HZ3  ) 0.0 0.0 5.0
assign (resid  36 and   name HB1  )(resid   21 and   name HZ3  ) 0.0 0.0 5.0
assign (resid  36 and   name HB2  )(resid   21 and   name HZ3  ) 0.0 0.0 2.5
assign (resid  36 and   name HA   )(resid   21 and   name HZ3  ) 0.0 0.0 5.0
```

```
assign (resid   35 and   name HA   )(resid   21 and   name HZ3  ) 0.0 0.0 3.5
assign (resid   42 and   name HN   )(resid   21 and   name HZ3  ) 0.0 0.0 5.0
assign (resid    6 and   name HB#  )(resid   21 and   name HE3  ) 0.0 1.0 6.0
assign (resid   35 and   name HA   )(resid   21 and   name HE3  ) 0.0 0.0 3.5
assign (resid   11 and   name HG2# )(resid   21 and   name HE1  ) 0.0 1.0 6.0
assign (resid   41 and   name HB#  )(resid   21 and   name HE1  ) 0.0 1.0 6.0
assign (resid    8 and   name HB2  )(resid   21 and   name HE1  ) 0.0 0.0 3.5
assign (resid    8 and   name HB1  )(resid   21 and   name HE1  ) 0.0 0.0 3.5
assign (resid    8 and   name HA   )(resid   21 and   name HE1  ) 0.0 0.0 5.0
assign (resid    8 and   name HN   )(resid   21 and   name HZ2  ) 0.0 0.0 5.0
assign (resid   36 and   name HN   )(resid   21 and   name HH2  ) 0.0 0.0 5.0
assign (resid   41 and   name HN   )(resid   21 and   name HH2  ) 0.0 0.0 5.0
assign (resid   36 and   name HN   )(resid   21 and   name HZ3  ) 0.0 0.0 3.5
assign (resid   36 and   name HN   )(resid   21 and   name HE3  ) 0.0 0.0 5.0
assign (resid   22 and   name HD1# )(resid   33 and   name HE#  ) 0.0 3.0 5.5
assign (resid   22 and   name HD2# )(resid   33 and   name HE#  ) 0.0 3.0 5.5
assign (resid   22 and   name HB#  )(resid   33 and   name HE#  ) 0.0 3.0 8.0
assign (resid   46 and   name HG#  )(resid   33 and   name HE#  ) 0.0 4.4 7.9
assign (resid   44 and   name HB#  )(resid   33 and   name HE#  ) 0.0 3.0 8.0
assign (resid   20 and   name HB#  )(resid   33 and   name HE#  ) 0.0 3.0 5.5
assign (resid   20 and   name HB#  )(resid   33 and   name HD#  ) 0.0 3.0 6.5
assign (resid   22 and   name HD1# )(resid   33 and   name HD#  ) 0.0 3.0 5.5
assign (resid   22 and   name HD2# )(resid   33 and   name HD#  ) 0.0 3.0 5.5
assign (resid   22 and   name HB#  )(resid   33 and   name HD#  ) 0.0 3.0 6.5
assign (resid   46 and   name HB   )(resid   33 and   name HD#  ) 0.0 2.0 7.0
assign (resid   46 and   name HG#  )(resid   33 and   name HD#  ) 0.0 4.4 7.9
assign (resid   22 and   name HA   )(resid   33 and   name HD#  ) 0.0 2.0 7.0
assign (resid   44 and   name HN   )(resid   33 and   name HE#  ) 0.0 2.0 7.0
assign (resid   44 and   name HN   )(resid   33 and   name HD#  ) 0.0 2.0 5.5
assign (resid   42 and   name HB1  )(resid   35 and   name HD1  ) 0.0 0.0 5.0
assign (resid   20 and   name HB#  )(resid   35 and   name HD1  ) 0.0 1.0 6.0
assign (resid   20 and   name HA   )(resid   35 and   name HD1  ) 0.0 0.0 5.0
assign (resid   18 and   name HA   )(resid   35 and   name HD1  ) 0.0 0.0 3.5
assign (resid   18 and   name HB#  )(resid   35 and   name HD1  ) 0.0 1.0 6.0
assign (resid   18 and   name HA   )(resid   35 and   name HD1  ) 0.0 0.0 3.5
assign (resid   18 and   name HB#  )(resid   35 and   name HD1  ) 0.0 1.0 6.0
assign (resid   42 and   name HB2  )(resid   35 and   name HH2  ) 0.0 0.0 5.0
assign (resid   42 and   name HG#  )(resid   35 and   name HH2  ) 0.0 1.0 6.0
assign (resid   42 and   name HG#  )(resid   35 and   name HE3  ) 0.0 1.0 6.0
assign (resid   20 and   name HB#  )(resid   35 and   name HE3  ) 0.0 1.0 6.0
assign (resid   42 and   name HB1  )(resid   35 and   name HE1  ) 0.0 0.0 5.0
assign (resid   42 and   name HB2  )(resid   35 and   name HE1  ) 0.0 0.0 5.0
assign (resid   42 and   name HG#  )(resid   35 and   name HE1  ) 0.0 1.0 6.0
assign (resid   18 and   name HB#  )(resid   35 and   name HE1  ) 0.0 1.0 6.0
assign (resid   18 and   name HA   )(resid   35 and   name HE1  ) 0.0 0.0 5.0
assign (resid   19 and   name HN   )(resid   35 and   name HD1  ) 0.0 0.0 5.0
assign (resid   42 and   name HN   )(resid   35 and   name HD1  ) 0.0 0.0 5.0
assign (resid   42 and   name HN   )(resid   35 and   name HE3  ) 0.0 0.0 5.0
assign (resid   18 and   name HN   )(resid   35 and   name HE1  ) 0.0 0.0 5.0
assign (resid   42 and   name HN   )(resid   35 and   name HE1  ) 0.0 0.0 5.0
assign (resid   33 and   name HB#  )(resid   44 and   name HD2  ) 0.0 1.0 6.0
! RESTRAINTS DEFINING DISULPHIDE BONDS IN TPA F1 MODULE
assign (resid    6 and   name SG   )(resid   36 and   name SG   ) 0.0 0.0 2.2
assign (resid   34 and   name SG   )(resid   43 and   name SG   ) 0.0 0.0 2.2
!HYDROGEN BOND RESTRAINTS LIST FOR TPA F1 MODULE
!class H-bonds
! 6-15
assign (resid    6 and   name O    )(resid   15 and   name N    ) 0.0 1.2 3.3
assign (resid    6 and   name O    )(resid   15 and   name HN   ) 0.0 0.2 2.3
assign (resid   15 and   name O    )(resid    6 and   name N    ) 0.0 1.2 3.3
assign (resid   15 and   name O    )(resid    6 and   name HN   ) 0.0 0.2 2.3
! 8-13
assign (resid    8 and   name O    )(resid   13 and   name N    ) 0.0 1.2 3.3
assign (resid    8 and   name O    )(resid   13 and   name HN   ) 0.0 0.2 2.3
assign (resid   13 and   name O    )(resid    8 and   name N    ) 0.0 1.2 3.3
assign (resid   13 and   name O    )(resid    8 and   name HN   ) 0.0 0.2 2.3
```

```
! 23-32
assign (resid   23 and   name O    )(resid   32 and   name N    ) 0.0 1.2 3.3
assign (resid   23 and   name O    )(resid   32 and   name HN   ) 0.0 0.2 2.3
assign (resid   32 and   name O    )(resid   23 and   name N    ) 0.0 1.2 3.3
assign (resid   32 and   name O    )(resid   23 and   name HN   ) 0.0 0.2 2.3
! 35-42
assign (resid   35 and   name O    )(resid   42 and   name N    ) 0.0 1.2 3.3
assign (resid   35 and   name O    )(resid   42 and   name HN   ) 0.0 0.2 2.3
assign (resid   42 and   name O    )(resid   35 and   name N    ) 0.0 1.2 3.3
assign (resid   42 and   name O    )(resid   35 and   name HN   ) 0.0 0.2 2.3
! DIHEDRAL ANGLES RESTRAINTS LIST FOR TPA F1 MODULE
constraints dihedral reset
  !!   13
 assign (resid  12 and name c ) (resid  13 and name n )
        (resid  13 and name ca) (resid  13 and name c )  1  -120.0  30.0  2
  !!   14
 assign (resid  13 and name c ) (resid  14 and name n )
        (resid  14 and name ca) (resid  14 and name c )  1  -120.0  30.0  2
  !!   15
 assign (resid  14 and name c ) (resid  15 and name n )
        (resid  15 and name ca) (resid  15 and name c )  1  -120.0  30.0  2
  !!   31
 assign (resid  30 and name c ) (resid  31 and name n )
        (resid  31 and name ca) (resid  31 and name c )  1  -120.0  30.0  2
  !!   32
 assign (resid  31 and name c ) (resid  32 and name n )
        (resid  32 and name ca) (resid  32 and name c )  1  -120.0  30.0  2
  !!   33
 assign (resid  32 and name c ) (resid  33 and name n )
        (resid  33 and name ca) (resid  33 and name c )  1  -120.0  30.0  2
  !!   34
 assign (resid  33 and name c ) (resid  34 and name n )
        (resid  34 and name ca) (resid  34 and name c )  1  -120.0  30.0  2
  !! cys 6
! Phi
 assign (resid  5 and name c ) (resid 6 and name n )
        (resid  6 and name ca) (resid 6 and name c )     1  -120.0   30.0  2
! Psi
 assign (resid  6 and name n ) (resid 6 and name ca )
        (resid  6 and name c ) (resid  7 and name n )    1   110.0  65.0  2
  !! asp 8
! Psi
 assign (resid  8 and name n ) (resid 8 and name ca )
        (resid  8 and name c ) (resid  9 and name n )    1   115.0  70.0  2
! Chi1
 assign (resid 8 and name n) (resid 8 and name ca)
        (resid 8 and name cb) (resid 8 and name cg)      1   -175.0  20.0  2
  !! met 13
! Psi
 assign (resid  13 and name n ) (resid 13 and name ca )
        (resid  13 and name c ) (resid  14 and name n )    1   115.0  70.0  2
! Chi1
 assign (resid  13 and name n) (resid 13 and name ca)
        (resid  13 and name cb) (resid 13 and name cg)     1   -170.0  25.0  2
  !! cys 36
! Psi
 assign (resid  36 and name n ) (resid 36 and name ca )
        (resid  36 and name c ) (resid  37 and name n )    1   115.0  70.0  2
! Chi1
 assign (resid  36 and name n) (resid 36 and name ca)
        (resid  36 and name cb) (resid 36 and name sg)     1   -165.0  30.0  2
  !! cys 43
! Psi
 assign (resid  43 and name n ) (resid 43 and name ca )
```

```
        (resid  43 and name c ) (resid  44 and name n )     1   165.0  50.0  2
! Chi1
 assign (resid  43 and name n) (resid 43 and name ca)
        (resid  43 and name cb) (resid 43 and name sg)      1   -55.0  20.0  2
  !! his 44
! Phi
 assign (resid  43 and name c ) (resid 44 and name n )
        (resid  44 and name ca) (resid 44 and name c )      1  -120.0   35.0  2
! Psi
 assign (resid  44 and name n ) (resid 44 and name ca )
        (resid  44 and name c ) (resid  45 and name n )     1   155.0  50.0  2
! Chi1
 assign (resid  44 and name n) (resid 44 and name ca)
        (resid  44 and name cb) (resid 44 and name cg)      1    60.0  20.0  2
  !! pro 47
! Psi
 assign (resid  47 and name n ) (resid 47 and name ca )
        (resid  47 and name c ) (resid  48 and name n )     1   165.0  50.0  2
! Chi1
 assign (resid  47 and name n) (resid 47 and name ca)
        (resid  47 and name cb) (resid 47 and name cg)      1   -20.0  20.0  2
  !! ser 20
! Phi
 assign (resid  19 and name c ) (resid  20 and name n )
        (resid  20 and name ca) (resid  20 and name c )   1  -115.0  30.0  2
  !! trp 21
! Phi
 assign (resid  20 and name c ) (resid  21 and name n )
        (resid  21 and name ca) (resid  21 and name c )   1  -120.0  35.0  2
  !! arg 23
! Phi
 assign (resid  22 and name c ) (resid  23 and name n )
        (resid  23 and name ca) (resid  23 and name c )   1  -115.0  30.0  2
  !! arg 27
! Phi
 assign (resid  26 and name c ) (resid  27 and name n )
        (resid  27 and name ca) (resid  27 and name c )   1  -120.0  35.0  2
  !! asn 37
! Phi
 assign (resid  36 and name c ) (resid  37 and name n )
        (resid  37 and name ca) (resid  37 and name c )   1  -120.0  35.0  2
  !! ser 38
! Phi
 assign (resid  37 and name c ) (resid  38 and name n )
        (resid  38 and name ca) (resid  38 and name c )   1  -120.0  55.0  2
  !! arg 40
! Phi
 assign (resid  39 and name c ) (resid  40 and name n )
        (resid  40 and name ca) (resid  40 and name c )   1  -120.0  35.0  2
  !! ala 41
! Phi
 assign (resid  40 and name c ) (resid  41 and name n )
        (resid  41 and name ca) (resid  41 and name c )   1   60.0  30.0  2
  !! asp 8
! Phi
 assign (resid  7 and name c ) (resid  8 and name n )
        (resid  8 and name ca) (resid  8 and name c )   1  -120.0  35.0  2
  !! ile 14
! Psi
 assign (resid  14 and name n ) (resid 14 and name ca )
        (resid  14 and name c ) (resid  15 and name n )     1   125.0  60.0  2
  !! val 25
! Phi
 assign (resid  24 and name c ) (resid  25 and name n )
```

```
        (resid  25 and name ca) (resid  25 and name c )  1  -120.0  35.0  2
! Psi
 assign (resid  25 and name n ) (resid 25 and name ca )
        (resid  25 and name c ) (resid  26 and name n )    1   125.0  60.0  2
  !! arg 30
! Psi
 assign (resid  30 and name n ) (resid 30 and name ca )
        (resid  30 and name c ) (resid  31 and name n )    1   125.0  60.0  2
  !! val 31
! Psi
 assign (resid  31 and name n ) (resid 31 and name ca )
        (resid  31 and name c ) (resid  32 and name n )    1   125.0  60.0  2
  !! trp 35
! Phi
 assign (resid  34 and name c ) (resid  35 and name n )
        (resid  35 and name ca) (resid  35 and name c )  1  -120.0  35.0  2
! Psi
 assign (resid  35 and name n ) (resid 35 and name ca )
        (resid  35 and name c ) (resid  36 and name n )    1   125.0  60.0  2
  !! ser 45
! Psi
 assign (resid  45 and name n ) (resid 45 and name ca )
        (resid  45 and name c ) (resid  46 and name n )    1   125.0  60.0  2
end end
```

B. p85α-N SH2 Restraints

```
! INTRARESIDUE RESTRAINTS FOR SH2 MODULE
assign (resid   17 and   name  HT# )(resid   17 and   name  HB# ) 0.0 0.6 3.9
assign (resid   17 and   name  HT# )(resid   17 and   name   HG ) 0.0 0.0 5.0
assign (resid   17 and   name   HA )(resid   17 and   name  HB# ) 0.0 1.0 3.8
assign (resid   17 and   name   HA )(resid   17 and   name HD1# ) 0.0 1.5 4.3
assign (resid   17 and   name  HB# )(resid   17 and   name HD1# ) 0.0 2.5 5.3
assign (resid   17 and   name   HG )(resid   17 and   name HD1# ) 0.0 1.5 4.3
assign (resid   17 and   name  HT# )(resid   17 and   name HD1# ) 0.0 1.5 6.5
assign (resid   17 and   name   HA )(resid   17 and   name HD2# ) 0.0 1.5 4.3
assign (resid   17 and   name  HB# )(resid   17 and   name HD2# ) 0.0 2.5 5.3
assign (resid   17 and   name HD1# )(resid   17 and   name HD2# ) 0.0 3.0 5.8
assign (resid   17 and   name   HG )(resid   17 and   name HD2# ) 0.0 1.5 4.3
assign (resid   17 and   name  HT# )(resid   17 and   name HD2# ) 0.0 1.5 6.5
assign (resid   17 and   name   HA )(resid   17 and   name   HG ) 0.0 0.0 3.3
assign (resid   17 and   name  HB# )(resid   17 and   name   HG ) 0.0 1.0 3.8
assign (resid   18 and   name   HN )(resid   18 and   name  HB# ) 0.0 0.6 3.4
assign (resid   18 and   name   HN )(resid   18 and   name  HG# ) 0.0 1.0 4.3
assign (resid   19 and   name   HA )(resid   19 and   name  HB1 ) 0.0 0.0 2.8
assign (resid   19 and   name   HN )(resid   19 and   name  HB1 ) 0.0 0.0 3.3
assign (resid   19 and   name   HA )(resid   19 and   name  HB2 ) 0.0 0.0 2.8
assign (resid   19 and   name   HN )(resid   19 and   name  HB2 ) 0.0 0.0 3.3
assign (resid   20 and   name   HA )(resid   20 and   name  HB# ) 0.0 1.5 4.3
assign (resid   20 and   name   HN )(resid   20 and   name  HB# ) 0.0 1.1 3.9
assign (resid   21 and   name   HN )(resid   21 and   name  HB# ) 0.0 0.6 3.9
assign (resid   21 and   name   HN )(resid   21 and   name  HG# ) 0.0 1.0 4.3
assign (resid   22 and   name   HA )(resid   22 and   name  HB# ) 0.0 1.0 3.8
assign (resid   22 and   name   HA )(resid   22 and   name  HB1 ) 0.0 0.0 2.8
assign (resid   22 and   name   HN )(resid   22 and   name  HB1 ) 0.0 0.0 5.0
assign (resid   22 and   name   HA )(resid   22 and   name  HB2 ) 0.0 0.0 2.8
assign (resid   22 and   name   HN )(resid   22 and   name  HB2 ) 0.0 0.0 5.0
assign (resid   23 and   name   HN )(resid   23 and   name  HB1 ) 0.0 0.0 5.0
assign (resid   23 and   name   HN )(resid   23 and   name  HB2 ) 0.0 0.0 5.0
assign (resid   23 and   name   HA )(resid   23 and   name  HB# ) 0.0 1.0 4.3
assign (resid   23 and   name   HA )(resid   23 and   name  HB1 ) 0.0 0.0 3.3
assign (resid   23 and   name   HA )(resid   23 and   name  HB2 ) 0.0 0.0 3.3
assign (resid   24 and   name   HN )(resid   24 and   name  HB1 ) 0.0 0.0 5.0
assign (resid   24 and   name   HN )(resid   24 and   name  HB2 ) 0.0 0.0 5.0
assign (resid   24 and   name   HA )(resid   24 and   name  HB1 ) 0.0 0.0 3.3
assign (resid   24 and   name   HA )(resid   24 and   name  HB2 ) 0.0 0.0 3.3
assign (resid   26 and   name   HA )(resid   26 and   name  HB# ) 0.0 1.0 3.8
assign (resid   26 and   name   HN )(resid   26 and   name  HB# ) 0.0 0.6 3.4
assign (resid   28 and   name   HA )(resid   28 and   name  HB# ) 0.0 1.0 3.8
assign (resid   28 and   name   HN )(resid   28 and   name  HB# ) 0.0 0.6 3.9
assign (resid   29 and   name   HA )(resid   29 and   name  HB1 ) 0.0 0.0 2.8
assign (resid   29 and   name   HN )(resid   29 and   name  HB1 ) 0.0 0.0 5.0
assign (resid   29 and   name   HA )(resid   29 and   name  HB2 ) 0.0 0.0 2.8
assign (resid   29 and   name  HB1 )(resid   29 and   name  HB2 ) 0.0 0.0 2.8
assign (resid   29 and   name   HN )(resid   29 and   name  HB2 ) 0.0 0.0 5.0
assign (resid   29 and   name   HA )(resid   29 and   name  HD# ) 0.0 1.0 4.3
assign (resid   29 and   name  HB# )(resid   29 and   name  HD# ) 0.0 2.0 4.8
assign (resid   29 and   name  HB1 )(resid   29 and   name  HD# ) 0.0 1.0 3.8
assign (resid   29 and   name  HB2 )(resid   29 and   name  HD# ) 0.0 1.0 3.8
assign (resid   29 and   name  HG1 )(resid   29 and   name  HD# ) 0.0 1.0 3.8
assign (resid   29 and   name  HG2 )(resid   29 and   name  HD# ) 0.0 1.0 3.8
assign (resid   29 and   name   HN )(resid   29 and   name  HD# ) 0.0 1.0 3.8
assign (resid   29 and   name   HA )(resid   29 and   name  HG# ) 0.0 1.0 4.3
assign (resid   29 and   name   HA )(resid   29 and   name  HG1 ) 0.0 0.0 5.0
assign (resid   29 and   name  HB# )(resid   29 and   name  HG1 ) 0.0 1.0 3.8
assign (resid   29 and   name   HN )(resid   29 and   name  HG1 ) 0.0 0.0 3.3
assign (resid   29 and   name   HA )(resid   29 and   name  HG2 ) 0.0 0.0 5.0
assign (resid   29 and   name  HB# )(resid   29 and   name  HG2 ) 0.0 1.0 3.8
assign (resid   29 and   name  HG1 )(resid   29 and   name  HG2 ) 0.0 0.0 2.8
assign (resid   29 and   name   HN )(resid   29 and   name  HG2 ) 0.0 0.0 3.3
assign (resid   30 and   name   HA )(resid   30 and   name  HB1 ) 0.0 0.0 3.3
```

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assign (resid  30 and  name   HN )(resid  30 and  name  HB# ) 0.0 1.0 4.3
assign (resid  30 and  name   HA )(resid  30 and  name  HB2 ) 0.0 0.0 3.3
assign (resid  30 and  name   HA )(resid  30 and  name  HG1 ) 0.0 0.0 3.3
assign (resid  30 and  name   HN )(resid  30 and  name  HG1 ) 0.0 0.0 2.8
assign (resid  30 and  name   HA )(resid  30 and  name  HG2 ) 0.0 0.0 3.3
assign (resid  30 and  name   HN )(resid  30 and  name  HG2 ) 0.0 0.0 2.8
assign (resid  31 and  name   HA )(resid  31 and  name  HB# ) 0.0 1.0 3.8
assign (resid  31 and  name   HN )(resid  31 and  name  HB# ) 0.0 0.6 3.4
assign (resid  31 and  name   HA )(resid  31 and  name  HG# ) 0.0 1.0 3.8
assign (resid  31 and  name  HB# )(resid  31 and  name  HG# ) 0.0 2.0 4.8
assign (resid  31 and  name   HN )(resid  31 and  name  HG# ) 0.0 1.0 4.3
assign (resid  32 and  name   HA )(resid  32 and  name   HB ) 0.0 0.0 3.3
assign (resid  32 and  name   HN )(resid  32 and  name   HB ) 0.0 0.0 5.0
assign (resid  32 and  name   HA )(resid  32 and  name HG1# ) 0.0 1.5 4.3
assign (resid  32 and  name   HB )(resid  32 and  name HG1# ) 0.0 1.5 4.3
assign (resid  32 and  name   HN )(resid  32 and  name HG1# ) 0.0 1.5 4.8
assign (resid  32 and  name   HA )(resid  32 and  name HG2# ) 0.0 1.5 4.3
assign (resid  32 and  name   HB )(resid  32 and  name HG2# ) 0.0 1.5 4.3
assign (resid  32 and  name   HN )(resid  32 and  name HG2# ) 0.0 1.5 4.8
assign (resid  33 and  name   HA )(resid  33 and  name  HB1 ) 0.0 0.0 3.3
assign (resid  33 and  name   HN )(resid  33 and  name  HB1 ) 0.0 0.0 2.8
assign (resid  33 and  name   HA )(resid  33 and  name  HB2 ) 0.0 0.0 3.3
assign (resid  33 and  name   HN )(resid  33 and  name  HB2 ) 0.0 0.0 2.8
assign (resid  34 and  name   HN )(resid  34 and  name  HB# ) 0.0 0.6 3.4
assign (resid  34 and  name   HN )(resid  34 and  name  HG# ) 0.0 1.0 3.8
assign (resid  34 and  name   HA )(resid  34 and  name  HG# ) 0.0 1.0 3.8
assign (resid  35 and  name   HN )(resid  35 and  name  HB1 ) 0.0 0.0 3.3
assign (resid  35 and  name   HN )(resid  35 and  name  HB2 ) 0.0 0.0 3.3
assign (resid  35 and  name   HN )(resid  35 and  name  HD# ) 0.0 1.0 6.0
assign (resid  35 and  name   HN )(resid  35 and  name  HG1 ) 0.0 0.0 5.0
assign (resid  35 and  name   HN )(resid  35 and  name  HG2 ) 0.0 0.0 5.0
assign (resid  35 and  name   HA )(resid  35 and  name  HB1 ) 0.0 0.0 2.8
assign (resid  35 and  name   HA )(resid  35 and  name  HB2 ) 0.0 0.0 2.8
assign (resid  35 and  name   HA )(resid  35 and  name  HD# ) 0.0 1.0 3.8
assign (resid  35 and  name  HB1 )(resid  35 and  name  HD# ) 0.0 1.0 3.8
assign (resid  35 and  name  HB2 )(resid  35 and  name  HD# ) 0.0 1.0 3.8
assign (resid  35 and  name  HB1 )(resid  35 and  name  HG# ) 0.0 1.0 4.3
assign (resid  35 and  name  HB2 )(resid  35 and  name  HG# ) 0.0 1.0 4.3
assign (resid  35 and  name   HA )(resid  35 and  name  HG1 ) 0.0 0.0 3.3
assign (resid  35 and  name   HA )(resid  35 and  name  HG2 ) 0.0 0.0 3.3
assign (resid  36 and  name   HA )(resid  36 and  name   HG ) 0.0 0.0 2.8
assign (resid  37 and  name   HA )(resid  37 and  name  HB1 ) 0.0 0.0 2.8
assign (resid  37 and  name   HN )(resid  37 and  name  HB1 ) 0.0 0.0 5.0
assign (resid  37 and  name   HA )(resid  37 and  name  HB2 ) 0.0 0.0 2.8
assign (resid  37 and  name   HN )(resid  37 and  name  HB2 ) 0.0 0.0 5.0
assign (resid  37 and  name   HA )(resid  37 and  name  HD# ) 0.0 1.0 4.3
assign (resid  37 and  name   HN )(resid  37 and  name  HD# ) 0.0 1.0 6.0
assign (resid  37 and  name   HA )(resid  37 and  name  HG# ) 0.0 1.0 3.8
assign (resid  37 and  name  HB# )(resid  37 and  name  HG# ) 0.0 2.0 4.8
assign (resid  37 and  name   HN )(resid  37 and  name  HG1 ) 0.0 0.0 3.3
assign (resid  37 and  name   HN )(resid  37 and  name  HG2 ) 0.0 0.0 3.3
assign (resid  38 and  name   HA )(resid  38 and  name  HB1 ) 0.0 0.0 3.3
assign (resid  38 and  name   HN )(resid  38 and  name  HB1 ) 0.0 0.0 3.3
assign (resid  38 and  name   HA )(resid  38 and  name  HB2 ) 0.0 0.0 3.3
assign (resid  38 and  name   HN )(resid  38 and  name  HB2 ) 0.0 0.0 3.3
assign (resid  39 and  name   HA )(resid  39 and  name HG2# ) 0.0 1.5 4.3
assign (resid  39 and  name   HN )(resid  39 and  name HG2# ) 0.0 1.5 4.8
assign (resid  40 and  name   HN )(resid  40 and  name  HB# ) 0.0 1.1 3.9
assign (resid  41 and  name   HA )(resid  41 and  name  HB1 ) 0.0 0.0 3.3
assign (resid  41 and  name   HN )(resid  41 and  name  HB1 ) 0.0 0.0 2.8
assign (resid  41 and  name   HA )(resid  41 and  name  HB2 ) 0.0 0.0 3.3
assign (resid  41 and  name   HN )(resid  41 and  name  HB2 ) 0.0 0.0 2.8
assign (resid  43 and  name   HA )(resid  43 and  name   HB ) 0.0 0.0 3.3
assign (resid  43 and  name   HN )(resid  43 and  name   HB ) 0.0 0.0 2.8
```

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assign (resid  43 and  name   HA )(resid  43 and  name HG2# ) 0.0 1.5 4.3
assign (resid  43 and  name   HN )(resid  43 and  name HG2# ) 0.0 1.5 4.3
assign (resid  44 and  name   HN )(resid  44 and  name  HB1 ) 0.0 0.0 5.0
assign (resid  44 and  name   HN )(resid  44 and  name  HB2 ) 0.0 0.0 5.0
assign (resid  44 and  name   HA )(resid  44 and  name  HB1 ) 0.0 0.0 2.8
assign (resid  44 and  name   HA )(resid  44 and  name  HB2 ) 0.0 0.0 2.8
assign (resid  45 and  name   HN )(resid  45 and  name  HB# ) 0.0 0.6 3.9
assign (resid  45 and  name   HN )(resid  45 and  name HD1# ) 0.0 1.5 4.8
assign (resid  45 and  name   HN )(resid  45 and  name HD2# ) 0.0 1.5 4.8
assign (resid  45 and  name   HN )(resid  45 and  name   HG ) 0.0 0.0 3.3
assign (resid  45 and  name   HA )(resid  45 and  name  HB# ) 0.0 1.0 3.8
assign (resid  45 and  name   HA )(resid  45 and  name HD1# ) 0.0 1.5 4.8
assign (resid  45 and  name  HB# )(resid  45 and  name HD1# ) 0.0 2.5 5.3
assign (resid  45 and  name   HG )(resid  45 and  name HD1# ) 0.0 1.5 4.3
assign (resid  45 and  name   HA )(resid  45 and  name HD2# ) 0.0 1.5 4.8
assign (resid  45 and  name  HB# )(resid  45 and  name HD2# ) 0.0 2.5 5.3
assign (resid  45 and  name HD1# )(resid  45 and  name HD2# ) 0.0 3.0 5.8
assign (resid  45 and  name   HG )(resid  45 and  name HD2# ) 0.0 1.5 4.3
assign (resid  45 and  name   HA )(resid  45 and  name   HG ) 0.0 0.0 5.0
assign (resid  45 and  name  HB# )(resid  45 and  name   HG ) 0.0 1.0 3.8
assign (resid  46 and  name   HB )(resid  46 and  name   HN ) 0.0 0.0 3.3
assign (resid  46 and  name HG1# )(resid  46 and  name   HN ) 0.0 1.5 6.5
assign (resid  46 and  name HG2# )(resid  46 and  name   HN ) 0.0 1.5 6.5
assign (resid  46 and  name   HA )(resid  46 and  name   HB ) 0.0 0.0 3.3
assign (resid  46 and  name   HA )(resid  46 and  name HG1# ) 0.0 1.5 4.3
assign (resid  46 and  name   HB )(resid  46 and  name HG1# ) 0.0 1.5 4.3
assign (resid  46 and  name   HA )(resid  46 and  name HG2# ) 0.0 1.5 4.3
assign (resid  46 and  name   HB )(resid  46 and  name HG2# ) 0.0 1.5 4.3
assign (resid  47 and  name  HB1 )(resid  47 and  name   HN ) 0.0 0.0 3.3
assign (resid  47 and  name  HB2 )(resid  47 and  name   HN ) 0.0 0.0 3.3
assign (resid  47 and  name  HG# )(resid  47 and  name   HN ) 0.0 1.0 6.0
assign (resid  47 and  name   HA )(resid  47 and  name  HB1 ) 0.0 0.0 3.3
assign (resid  47 and  name   HA )(resid  47 and  name  HB2 ) 0.0 0.0 3.3
assign (resid  47 and  name  HB1 )(resid  47 and  name  HD# ) 0.0 1.0 6.0
assign (resid  47 and  name  HB2 )(resid  47 and  name  HD# ) 0.0 1.0 6.0
assign (resid  47 and  name  HG1 )(resid  47 and  name  HD# ) 0.0 1.0 4.3
assign (resid  47 and  name  HG2 )(resid  47 and  name  HD# ) 0.0 1.0 4.3
assign (resid  47 and  name   HA )(resid  47 and  name  HD1 ) 0.0 0.0 3.3
assign (resid  47 and  name   HA )(resid  47 and  name  HD2 ) 0.0 0.0 3.3
assign (resid  47 and  name   HA )(resid  47 and  name  HG1 ) 0.0 0.0 5.0
assign (resid  47 and  name  HB1 )(resid  47 and  name  HG1 ) 0.0 0.0 3.3
assign (resid  47 and  name  HB2 )(resid  47 and  name  HG1 ) 0.0 0.0 3.3
assign (resid  47 and  name   HA )(resid  47 and  name  HG2 ) 0.0 0.0 5.0
assign (resid  47 and  name  HB1 )(resid  47 and  name  HG2 ) 0.0 0.0 3.3
assign (resid  47 and  name  HB2 )(resid  47 and  name  HG2 ) 0.0 0.0 3.3
assign (resid  48 and  name   HA )(resid  48 and  name  HB1 ) 0.0 0.0 3.3
assign (resid  48 and  name   HN )(resid  48 and  name  HB1 ) 0.0 0.0 2.8
assign (resid  48 and  name   HA )(resid  48 and  name  HB2 ) 0.0 0.0 3.3
assign (resid  48 and  name   HN )(resid  48 and  name  HB2 ) 0.0 0.0 2.8
assign (resid  51 and  name   HN )(resid  51 and  name HG2# ) 0.0 1.5 4.8
assign (resid  53 and  name   HN )(resid  53 and  name  HB# ) 0.0 0.6 3.9
assign (resid  53 and  name   HN )(resid  53 and  name  HG# ) 0.0 1.0 6.0
assign (resid  54 and  name   HN )(resid  54 and  name  HB# ) 0.0 0.6 5.6
assign (resid  54 and  name   HA )(resid  54 and  name  HB# ) 0.0 1.0 4.3
assign (resid  56 and  name   HA )(resid  56 and  name  HB1 ) 0.0 0.0 2.8
assign (resid  56 and  name   HN )(resid  56 and  name  HB1 ) 0.0 0.0 3.3
assign (resid  56 and  name   HA )(resid  56 and  name  HB2 ) 0.0 0.0 2.8
assign (resid  56 and  name   HN )(resid  56 and  name  HB2 ) 0.0 0.0 3.3
assign (resid  57 and  name   HA )(resid  57 and  name  HB# ) 0.0 1.0 4.3
assign (resid  57 and  name   HA )(resid  57 and  name  HB1 ) 0.0 0.0 3.3
assign (resid  57 and  name   HN )(resid  57 and  name  HB1 ) 0.0 0.0 5.0
assign (resid  57 and  name   HA )(resid  57 and  name  HB2 ) 0.0 0.0 3.3
assign (resid  57 and  name   HN )(resid  57 and  name  HB2 ) 0.0 0.0 5.0
assign (resid  58 and  name   HA )(resid  58 and  name   HB ) 0.0 0.0 2.8
```

```
assign (resid  58 and  name  HN )(resid  58 and  name  HB ) 0.0 0.0 2.8
assign (resid  58 and  name  HA )(resid  58 and  name HG2# ) 0.0 1.5 4.3
assign (resid  58 and  name  HB )(resid  58 and  name HG2# ) 0.0 1.5 4.3
assign (resid  58 and  name  HN )(resid  58 and  name HG2# ) 0.0 1.5 4.3
assign (resid  59 and  name  HA )(resid  59 and  name  HD# ) 0.0 1.7 4.5
assign (resid  59 and  name HB# )(resid  59 and  name  HD# ) 0.0 2.7 6.0
assign (resid  59 and  name  HA )(resid  59 and  name  HG ) 0.0 0.0 2.8
assign (resid  60 and  name  HA )(resid  60 and  name  HB ) 0.0 0.0 2.8
assign (resid  60 and  name  HN )(resid  60 and  name  HB ) 0.0 0.0 3.3
assign (resid  60 and  name  HA )(resid  60 and  name HG2# ) 0.0 1.5 4.3
assign (resid  60 and  name  HB )(resid  60 and  name HG2# ) 0.0 1.5 4.3
assign (resid  60 and  name  HN )(resid  60 and  name HG2# ) 0.0 1.5 4.3
assign (resid  61 and  name  HN )(resid  61 and  name  HB1 ) 0.0 0.0 5.0
assign (resid  61 and  name  HN )(resid  61 and  name  HB2 ) 0.0 0.0 5.0
assign (resid  61 and  name  HN )(resid  61 and  name HD1# ) 0.0 1.5 6.5
assign (resid  61 and  name  HN )(resid  61 and  name HD2# ) 0.0 1.5 6.5
assign (resid  61 and  name  HN )(resid  61 and  name  HG ) 0.0 0.0 3.3
assign (resid  61 and  name  HA )(resid  61 and  name  HB1 ) 0.0 0.0 2.8
assign (resid  61 and  name  HA )(resid  61 and  name  HB2 ) 0.0 0.0 2.8
assign (resid  61 and  name  HA )(resid  61 and  name HD1# ) 0.0 1.5 4.8
assign (resid  61 and  name HB1 )(resid  61 and  name HD1# ) 0.0 1.5 4.3
assign (resid  61 and  name HB2 )(resid  61 and  name HD1# ) 0.0 1.5 4.3
assign (resid  61 and  name  HG )(resid  61 and  name HD1# ) 0.0 1.5 4.3
assign (resid  61 and  name  HA )(resid  61 and  name HD2# ) 0.0 1.5 4.8
assign (resid  61 and  name HB1 )(resid  61 and  name HD2# ) 0.0 1.5 4.3
assign (resid  61 and  name HB2 )(resid  61 and  name HD2# ) 0.0 1.5 4.3
assign (resid  61 and  name  HG )(resid  61 and  name HD2# ) 0.0 1.5 4.3
assign (resid  61 and  name  HA )(resid  61 and  name  HG ) 0.0 0.0 5.0
assign (resid  61 and  name HB1 )(resid  61 and  name  HG ) 0.0 0.0 2.8
assign (resid  61 and  name HB2 )(resid  61 and  name  HG ) 0.0 0.0 2.8
assign (resid  62 and  name  HN )(resid  62 and  name HB1 ) 0.0 0.0 3.3
assign (resid  61 and  name HB2 )(resid  61 and  name  HG ) 0.0 0.0 2.8
assign (resid  62 and  name  HN )(resid  62 and  name HB1 ) 0.0 0.0 3.3
assign (resid  62 and  name  HN )(resid  62 and  name HB2 ) 0.0 0.0 3.3
assign (resid  62 and  name  HN )(resid  62 and  name HG# ) 0.0 1.0 4.3
assign (resid  62 and  name  HA )(resid  62 and  name HB1 ) 0.0 0.0 3.3
assign (resid  62 and  name  HA )(resid  62 and  name HB2 ) 0.0 0.0 3.3
assign (resid  62 and  name  HA )(resid  62 and  name HD1 ) 0.0 0.0 3.3
assign (resid  62 and  name HB1 )(resid  62 and  name HD1 ) 0.0 0.0 5.0
assign (resid  62 and  name HB2 )(resid  62 and  name HD1 ) 0.0 0.0 5.0
assign (resid  62 and  name  HA )(resid  62 and  name HD2 ) 0.0 0.0 3.3
assign (resid  62 and  name HB1 )(resid  62 and  name HD2 ) 0.0 0.0 5.0
assign (resid  62 and  name HB2 )(resid  62 and  name HD2 ) 0.0 0.0 5.0
assign (resid  62 and  name  HA )(resid  62 and  name HG# ) 0.0 1.0 3.8
assign (resid  62 and  name HB1 )(resid  62 and  name HG# ) 0.0 1.0 3.8
assign (resid  62 and  name HB2 )(resid  62 and  name HG# ) 0.0 1.0 3.8
assign (resid  63 and  name  HA )(resid  63 and  name HB1 ) 0.0 0.0 2.8
assign (resid  63 and  name  HA )(resid  63 and  name HB2 ) 0.0 0.0 2.8
assign (resid  63 and  name  HA )(resid  63 and  name HG1 ) 0.0 0.0 3.3
assign (resid  63 and  name  HA )(resid  63 and  name HG2 ) 0.0 0.0 3.3
assign (resid  66 and  name  HA )(resid  66 and  name HB1 ) 0.0 0.0 3.3
assign (resid  66 and  name  HN )(resid  66 and  name HB1 ) 0.0 0.0 2.8
assign (resid  66 and  name  HA )(resid  66 and  name HB2 ) 0.0 0.0 3.3
assign (resid  66 and  name  HN )(resid  66 and  name HB2 ) 0.0 0.0 2.8
assign (resid  67 and  name  HA )(resid  67 and  name HB1 ) 0.0 0.0 3.3
assign (resid  67 and  name  HN )(resid  67 and  name HB1 ) 0.0 0.0 3.3
assign (resid  67 and  name  HA )(resid  67 and  name HB2 ) 0.0 0.0 3.3
assign (resid  67 and  name  HN )(resid  67 and  name HB2 ) 0.0 0.0 3.3
assign (resid  68 and  name  HN )(resid  68 and  name HB# ) 0.0 0.6 3.9
assign (resid  68 and  name  HN )(resid  68 and  name HD1 ) 0.0 0.0 5.0
assign (resid  68 and  name  HN )(resid  68 and  name HD2 ) 0.0 0.0 5.0
assign (resid  69 and  name  HN )(resid  69 and  name  HD# ) 0.0 1.7 5.0
assign (resid  69 and  name  HA )(resid  69 and  name HB# ) 0.0 1.0 3.8
assign (resid  69 and  name  HA )(resid  69 and  name  HD# ) 0.0 1.7 4.5
assign (resid  69 and  name  HA )(resid  69 and  name  HG ) 0.0 0.0 2.8
assign (resid  70 and  name  HN )(resid  70 and  name  HD# ) 0.0 1.5 6.5
```

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assign (resid  70 and  name  HN ) (resid  70 and  name HG11 ) 0.0 0.0 5.0
assign (resid  70 and  name  HN ) (resid  70 and  name HG12 ) 0.0 0.0 5.0
assign (resid  70 and  name  HN ) (resid  70 and  name HG2# ) 0.0 1.5 6.5
assign (resid  70 and  name  HA ) (resid  70 and  name   HB ) 0.0 0.0 3.3
assign (resid  70 and  name  HA ) (resid  70 and  name  HD# ) 0.0 1.5 4.3
assign (resid  70 and  name  HB ) (resid  70 and  name  HD# ) 0.0 1.5 4.3
assign (resid  70 and  name  HA ) (resid  70 and  name HG1# ) 0.0 1.0 3.8
assign (resid  70 and  name  HA ) (resid  70 and  name HG2# ) 0.0 1.5 4.3
assign (resid  71 and  name  HN ) (resid  71 and  name  HB# ) 0.0 0.6 3.4
assign (resid  72 and  name  HN ) (resid  72 and  name   HB ) 0.0 0.0 3.3
assign (resid  72 and  name  HN ) (resid  72 and  name  HD# ) 0.0 1.5 6.5
assign (resid  72 and  name  HN ) (resid  72 and  name HG11 ) 0.0 0.0 5.0
assign (resid  72 and  name  HN ) (resid  72 and  name HG12 ) 0.0 0.0 5.0
assign (resid  72 and  name  HN ) (resid  72 and  name HG2# ) 0.0 1.5 4.8
assign (resid  72 and  name  HA ) (resid  72 and  name  HD# ) 0.0 1.5 4.3
assign (resid  72 and  name  HA ) (resid  72 and  name HG2# ) 0.0 1.5 4.3
assign (resid  73 and  name  HN ) (resid  73 and  name  HB1 ) 0.0 0.0 5.0
assign (resid  73 and  name  HN ) (resid  73 and  name  HB2 ) 0.0 0.0 5.0
assign (resid  73 and  name  HA ) (resid  73 and  name  HB# ) 0.0 1.0 3.8
assign (resid  73 and  name  HA ) (resid  73 and  name  HB1 ) 0.0 0.0 2.8
assign (resid  73 and  name  HA ) (resid  73 and  name  HB2 ) 0.0 0.0 2.8
assign (resid  74 and  name  HN ) (resid  74 and  name  HB1 ) 0.0 0.0 3.3
assign (resid  74 and  name  HN ) (resid  74 and  name  HB2 ) 0.0 0.0 3.3
assign (resid  74 and  name  HA ) (resid  74 and  name  HB# ) 0.0 1.0 3.8
assign (resid  74 and  name  HA ) (resid  74 and  name  HD2 ) 0.0 0.0 3.3
assign (resid  75 and  name  HN ) (resid  75 and  name  HB# ) 0.0 0.6 3.9
assign (resid  75 and  name  HN ) (resid  75 and  name  HG1 ) 0.0 0.0 5.0
assign (resid  75 and  name  HN ) (resid  75 and  name  HG2 ) 0.0 0.0 5.0
assign (resid  75 and  name  HA ) (resid  75 and  name  HB# ) 0.0 1.0 3.8
assign (resid  75 and  name  HA ) (resid  75 and  name  HD# ) 0.0 1.0 4.3
assign (resid  75 and  name HB# ) (resid  75 and  name  HD# ) 0.0 2.0 5.3
assign (resid  75 and  name HG# ) (resid  75 and  name  HD# ) 0.0 2.0 5.3
assign (resid  75 and  name  HA ) (resid  75 and  name  HG1 ) 0.0 0.0 3.3
assign (resid  75 and  name  HA ) (resid  75 and  name  HG2 ) 0.0 0.0 3.3
assign (resid  76 and  name  HN ) (resid  76 and  name  HB1 ) 0.0 0.0 5.0
assign (resid  76 and  name  HN ) (resid  76 and  name  HB2 ) 0.0 0.0 5.0
assign (resid  76 and  name  HA ) (resid  76 and  name  HB1 ) 0.0 0.0 2.8
assign (resid  76 and  name  HA ) (resid  76 and  name  HB2 ) 0.0 0.0 2.8
assign (resid  78 and  name  HN ) (resid  78 and  name  HB1 ) 0.0 0.0 3.3
assign (resid  78 and  name  HN ) (resid  78 and  name  HB2 ) 0.0 0.0 3.3
assign (resid  78 and  name  HN ) (resid  78 and  name  HG# ) 0.0 1.0 4.3
assign (resid  78 and  name  HA ) (resid  78 and  name  HB1 ) 0.0 0.0 2.8
assign (resid  78 and  name  HA ) (resid  78 and  name  HB2 ) 0.0 0.0 2.8
assign (resid  78 and  name  HA ) (resid  78 and  name  HG1 ) 0.0 0.0 5.0
assign (resid  78 and  name  HA ) (resid  78 and  name  HG2 ) 0.0 0.0 5.0
assign (resid  79 and  name  HN ) (resid  79 and  name  HB1 ) 0.0 0.0 5.0
assign (resid  79 and  name  HN ) (resid  79 and  name  HB2 ) 0.0 0.0 5.0
assign (resid  79 and  name  HA ) (resid  79 and  name  HB# ) 0.0 1.0 4.3
assign (resid  79 and  name  HA ) (resid  79 and  name  HB1 ) 0.0 0.0 3.3
assign (resid  79 and  name  HA ) (resid  79 and  name  HB2 ) 0.0 0.0 3.3
assign (resid  81 and  name  HN ) (resid  81 and  name  HB1 ) 0.0 0.0 3.3
assign (resid  81 and  name  HN ) (resid  81 and  name  HB2 ) 0.0 0.0 3.3
assign (resid  81 and  name  HA ) (resid  81 and  name  HB# ) 0.0 1.0 4.3
assign (resid  81 and  name  HA ) (resid  81 and  name  HB1 ) 0.0 0.0 3.3
assign (resid  81 and  name  HA ) (resid  81 and  name  HB2 ) 0.0 0.0 3.3
assign (resid  82 and  name  HN ) (resid  82 and  name  HB# ) 0.0 0.6 3.9
assign (resid  82 and  name  HA ) (resid  82 and  name  HB1 ) 0.0 0.0 2.8
assign (resid  82 and  name  HA ) (resid  82 and  name  HB2 ) 0.0 0.0 2.8
assign (resid  83 and  name  HN ) (resid  83 and  name  HB1 ) 0.0 0.0 3.3
assign (resid  83 and  name  HN ) (resid  83 and  name  HB2 ) 0.0 0.0 3.3
assign (resid  83 and  name  HA ) (resid  83 and  name  HB1 ) 0.0 0.0 3.3
assign (resid  83 and  name  HA ) (resid  83 and  name  HB2 ) 0.0 0.0 3.3
assign (resid  85 and  name  HN ) (resid  85 and  name  HB# ) 0.0 0.6 3.9
assign (resid  85 and  name  HN ) (resid  85 and  name HD1# ) 0.0 1.5 6.5
```

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assign (resid  85 and  name   HN ) (resid  85 and  name HD2# ) 0.0 1.5 6.5
assign (resid  85 and  name   HN ) (resid  85 and  name   HG ) 0.0 0.0 3.3
assign (resid  85 and  name   HA ) (resid  85 and  name  HB# ) 0.0 1.0 3.8
assign (resid  85 and  name   HA ) (resid  85 and  name HD1# ) 0.0 1.5 4.3
assign (resid  85 and  name   HG ) (resid  85 and  name HD1# ) 0.0 1.5 4.3
assign (resid  85 and  name   HA ) (resid  85 and  name HD2# ) 0.0 1.5 4.3
assign (resid  85 and  name   HG ) (resid  85 and  name HD2# ) 0.0 1.5 4.3
assign (resid  86 and  name   HN ) (resid  86 and  name HG2# ) 0.0 1.5 6.5
assign (resid  86 and  name   HA ) (resid  86 and  name HG2# ) 0.0 1.5 4.3
assign (resid  87 and  name   HN ) (resid  87 and  name  HB# ) 0.0 0.6 3.9
assign (resid  87 and  name   HA ) (resid  87 and  name  HB1 ) 0.0 0.0 3.3
assign (resid  87 and  name   HA ) (resid  87 and  name  HB2 ) 0.0 0.0 3.3
assign (resid  88 and  name   HN ) (resid  88 and  name  HB# ) 0.0 1.0 4.3
assign (resid  88 and  name   HA ) (resid  88 and  name  HB# ) 0.0 1.0 3.8
assign (resid  88 and  name  HB# ) (resid  88 and  name  HD# ) 0.0 2.0 4.8
assign (resid  88 and  name   HN ) (resid  88 and  name  HD# ) 0.0 1.0 4.3
assign (resid  89 and  name   HN ) (resid  89 and  name  HB1 ) 0.0 0.0 5.0
assign (resid  89 and  name   HN ) (resid  89 and  name  HB2 ) 0.0 0.0 5.0
assign (resid  89 and  name   HA ) (resid  89 and  name  HB1 ) 0.0 0.0 2.8
assign (resid  89 and  name   HA ) (resid  89 and  name  HB2 ) 0.0 0.0 2.8
assign (resid  90 and  name   HB ) (resid  90 and  name   HN ) 0.0 0.0 2.8
assign (resid  90 and  name HG1# ) (resid  90 and  name   HN ) 0.0 1.5 4.8
assign (resid  90 and  name HG2# ) (resid  90 and  name   HN ) 0.0 1.5 4.8
assign (resid  90 and  name   HA ) (resid  90 and  name   HB ) 0.0 0.0 3.3
assign (resid  90 and  name   HA ) (resid  90 and  name HG1# ) 0.0 1.5 4.3
assign (resid  90 and  name   HB ) (resid  90 and  name HG1# ) 0.0 1.5 4.3
assign (resid  90 and  name   HA ) (resid  90 and  name HG2# ) 0.0 1.5 4.3
assign (resid  90 and  name   HB ) (resid  90 and  name HG2# ) 0.0 1.5 4.3
assign (resid  91 and  name   HN ) (resid  91 and  name   HB ) 0.0 0.0 2.8
assign (resid  91 and  name   HN ) (resid  91 and  name HG1# ) 0.0 1.5 4.8
assign (resid  91 and  name   HN ) (resid  91 and  name HG2# ) 0.0 1.5 4.8
assign (resid  91 and  name   HA ) (resid  91 and  name HG1# ) 0.0 1.5 4.3
assign (resid  91 and  name   HB ) (resid  91 and  name HG1# ) 0.0 1.5 4.3
assign (resid  91 and  name   HA ) (resid  91 and  name HG2# ) 0.0 1.5 4.3
assign (resid  91 and  name   HB ) (resid  91 and  name HG2# ) 0.0 1.5 4.3
assign (resid  92 and  name   HN ) (resid  92 and  name  HB# ) 0.0 0.6 3.9
assign (resid  92 and  name   HN ) (resid  92 and  name  HG# ) 0.0 1.0 4.3
assign (resid  92 and  name   HA ) (resid  92 and  name  HG# ) 0.0 1.0 3.8
assign (resid  93 and  name   HN ) (resid  93 and  name  HB# ) 0.0 0.6 3.4
assign (resid  93 and  name   HN ) (resid  93 and  name HD1# ) 0.0 1.5 6.5
assign (resid  93 and  name   HN ) (resid  93 and  name HD2# ) 0.0 1.5 6.5
assign (resid  93 and  name   HN ) (resid  93 and  name   HG ) 0.0 0.0 5.0
assign (resid  93 and  name   HA ) (resid  93 and  name  HB# ) 0.0 1.0 4.3
assign (resid  93 and  name   HA ) (resid  93 and  name HD1# ) 0.0 1.5 4.8
assign (resid  93 and  name  HB# ) (resid  93 and  name HD1# ) 0.0 2.5 5.8
assign (resid  93 and  name   HG ) (resid  93 and  name HD1# ) 0.0 1.5 4.3
assign (resid  93 and  name   HA ) (resid  93 and  name HD2# ) 0.0 1.5 4.8
assign (resid  93 and  name  HB# ) (resid  93 and  name HD2# ) 0.0 2.5 5.8
assign (resid  93 and  name   HG ) (resid  93 and  name HD2# ) 0.0 1.5 4.3
assign (resid  93 and  name   HA ) (resid  93 and  name   HG ) 0.0 0.0 3.3
assign (resid  93 and  name  HB# ) (resid  93 and  name   HG ) 0.0 1.0 4.3
assign (resid  94 and  name   HN ) (resid  94 and  name  HD# ) 0.0 1.5 4.8
assign (resid  94 and  name   HN ) (resid  94 and  name HG2# ) 0.0 1.5 4.8
assign (resid  95 and  name   HN ) (resid  95 and  name  HB1 ) 0.0 0.0 2.8
assign (resid  95 and  name   HN ) (resid  95 and  name  HB2 ) 0.0 0.0 2.8
assign (resid  95 and  name   HA ) (resid  95 and  name  HB1 ) 0.0 0.0 3.3
assign (resid  95 and  name   HA ) (resid  95 and  name  HB2 ) 0.0 0.0 3.3
assign (resid  96 and  name   HN ) (resid  96 and  name  HB# ) 0.0 0.6 3.4
assign (resid  96 and  name   HA ) (resid  96 and  name  HB# ) 0.0 1.0 3.8
assign (resid  97 and  name   HN ) (resid  97 and  name  HB1 ) 0.0 0.0 5.0
assign (resid  97 and  name   HN ) (resid  97 and  name  HB2 ) 0.0 0.0 5.0
assign (resid  97 and  name   HA ) (resid  97 and  name  HB# ) 0.0 1.0 4.3
assign (resid  97 and  name   HA ) (resid  97 and  name  HB1 ) 0.0 0.0 3.3
assign (resid  97 and  name   HA ) (resid  97 and  name  HB2 ) 0.0 0.0 3.3
```

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assign (resid   98 and   name   HN )(resid   98 and   name   HB1 ) 0.0 0.0 3.3
assign (resid   98 and   name   HN )(resid   98 and   name   HB2 ) 0.0 0.0 3.3
assign (resid   98 and   name   HN )(resid   98 and   name   HG# ) 0.0 1.0 4.3
assign (resid   98 and   name   HA )(resid   98 and   name   HB# ) 0.0 1.0 3.8
assign (resid   98 and   name  HB# )(resid   98 and   name   HD# ) 0.0 2.0 4.8
assign (resid   98 and   name   HA )(resid   98 and   name   HD1 ) 0.0 0.0 5.0
assign (resid   98 and   name  HB# )(resid   98 and   name   HD1 ) 0.0 1.0 4.3
assign (resid   98 and   name  HG# )(resid   98 and   name   HD1 ) 0.0 1.0 4.3
assign (resid   98 and   name   HA )(resid   98 and   name   HD2 ) 0.0 0.0 5.0
assign (resid   98 and   name  HB# )(resid   98 and   name   HD2 ) 0.0 1.0 4.3
assign (resid   98 and   name  HG# )(resid   98 and   name   HD2 ) 0.0 1.0 4.3
assign (resid   98 and   name   HA )(resid   98 and   name   HG# ) 0.0 1.0 3.8
assign (resid   98 and   name  HB# )(resid   98 and   name   HG# ) 0.0 2.0 4.8
assign (resid   99 and   name   HN )(resid   99 and   name   HB1 ) 0.0 0.0 5.0
assign (resid   99 and   name   HN )(resid   99 and   name   HB2 ) 0.0 0.0 5.0
assign (resid   99 and   name   HA )(resid   99 and   name   HB1 ) 0.0 0.0 2.8
assign (resid   99 and   name   HA )(resid   99 and   name   HB2 ) 0.0 0.0 2.8
assign (resid  100 and   name   HN )(resid  100 and   name   HB# ) 0.0 0.6 3.9
assign (resid  100 and   name   HN )(resid  100 and   name   HG1 ) 0.0 0.0 3.3
assign (resid  100 and   name   HN )(resid  100 and   name   HG2 ) 0.0 0.0 3.3
assign (resid  100 and   name   HA )(resid  100 and   name   HB# ) 0.0 1.0 3.8
assign (resid  100 and   name   HA )(resid  100 and   name   HG1 ) 0.0 0.0 3.3
assign (resid  100 and   name  HB# )(resid  100 and   name   HG1 ) 0.0 1.0 4.3
assign (resid  100 and   name   HA )(resid  100 and   name   HG2 ) 0.0 0.0 3.3
assign (resid  100 and   name  HB# )(resid  100 and   name   HG2 ) 0.0 1.0 4.3
assign (resid  101 and   name   HN )(resid  101 and   name   HB1 ) 0.0 0.0 5.0
assign (resid  101 and   name   HN )(resid  101 and   name   HB2 ) 0.0 0.0 5.0
assign (resid  101 and   name   HA )(resid  101 and   name   HB1 ) 0.0 0.0 3.3
assign (resid  101 and   name   HA )(resid  101 and   name   HB2 ) 0.0 0.0 3.3
assign (resid  102 and   name   HN )(resid  102 and   name   HB1 ) 0.0 0.0 5.0
assign (resid  102 and   name   HN )(resid  102 and   name   HB2 ) 0.0 0.0 5.0
assign (resid  102 and   name   HN )(resid  102 and   name  HD1# ) 0.0 1.5 6.5
assign (resid  102 and   name   HN )(resid  102 and   name  HD2# ) 0.0 1.5 6.5
assign (resid  102 and   name   HN )(resid  102 and   name    HG ) 0.0 0.0 3.3
assign (resid  102 and   name   HA )(resid  102 and   name   HB1 ) 0.0 0.0 3.3
assign (resid  102 and   name   HG )(resid  102 and   name   HB1 ) 0.0 0.0 3.3
assign (resid  102 and   name   HA )(resid  102 and   name   HB2 ) 0.0 0.0 3.3
assign (resid  102 and   name   HG )(resid  102 and   name   HB2 ) 0.0 0.0 3.3
assign (resid  102 and   name  HB# )(resid  102 and   name  HD1# ) 0.0 2.5 5.3
assign (resid  102 and   name  HB1 )(resid  102 and   name  HD1# ) 0.0 1.5 4.3
assign (resid  102 and   name  HB2 )(resid  102 and   name  HD1# ) 0.0 1.5 4.3
assign (resid  102 and   name   HG )(resid  102 and   name  HD1# ) 0.0 1.5 4.3
assign (resid  102 and   name  HB# )(resid  102 and   name  HD2# ) 0.0 2.5 5.3
assign (resid  102 and   name  HB1 )(resid  102 and   name  HD2# ) 0.0 1.5 4.3
assign (resid  102 and   name  HB2 )(resid  102 and   name  HD2# ) 0.0 1.5 4.3
assign (resid  102 and   name   HG )(resid  102 and   name  HD2# ) 0.0 1.5 4.3
assign (resid  102 and   name   HA )(resid  102 and   name    HG ) 0.0 0.0 3.3
assign (resid  103 and   name   HN )(resid  103 and   name   HB# ) 0.0 1.1 3.9
assign (resid  103 and   name   HA )(resid  103 and   name   HB# ) 0.0 1.5 4.3
assign (resid  104 and   name   HN )(resid  104 and   name   HB# ) 0.0 0.6 3.4
assign (resid  104 and   name   HN )(resid  104 and   name   HG# ) 0.0 1.0 4.3
assign (resid  104 and   name   HA )(resid  104 and   name   HB# ) 0.0 1.0 3.8
assign (resid  104 and   name   HA )(resid  104 and   name   HG# ) 0.0 1.0 4.3
assign (resid  104 and   name  HB# )(resid  104 and   name   HG# ) 0.0 2.0 4.8
assign (resid  104 and   name HE21 )(resid  104 and   name    HN ) 0.0 0.0 5.0
assign (resid  104 and   name HE22 )(resid  104 and   name    HN ) 0.0 0.0 5.0
assign (resid  105 and   name   HN )(resid  105 and   name   HB# ) 0.0 0.6 3.9
assign (resid  105 and   name   HA )(resid  105 and   name   HB# ) 0.0 1.0 3.8
assign (resid  106 and   name   HN )(resid  106 and   name   HB1 ) 0.0 0.0 3.3
assign (resid  106 and   name   HN )(resid  106 and   name   HB2 ) 0.0 0.0 3.3
assign (resid  106 and   name   HA )(resid  106 and   name   HB1 ) 0.0 0.0 3.3
assign (resid  106 and   name   HA )(resid  106 and   name   HB2 ) 0.0 0.0 3.3
assign (resid  107 and   name   HA )(resid  107 and   name   HB1 ) 0.0 0.0 2.8
assign (resid  107 and   name   HA )(resid  107 and   name   HB2 ) 0.0 0.0 2.8
```

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assign (resid 107 and name  HA )(resid 107 and name  HD1 ) 0.0 0.0 5.0
assign (resid 107 and name HB1 )(resid 107 and name  HD1 ) 0.0 0.0 5.0
assign (resid 107 and name HB2 )(resid 107 and name  HD1 ) 0.0 0.0 5.0
assign (resid 107 and name HG# )(resid 107 and name  HD1 ) 0.0 1.0 3.8
assign (resid 107 and name  HA )(resid 107 and name  HD2 ) 0.0 0.0 5.0
assign (resid 107 and name HB1 )(resid 107 and name  HD2 ) 0.0 0.0 5.0
assign (resid 107 and name HB2 )(resid 107 and name  HD2 ) 0.0 0.0 5.0
assign (resid 107 and name HG# )(resid 107 and name  HD2 ) 0.0 1.0 3.8
assign (resid 107 and name  HA )(resid 107 and name  HG# ) 0.0 1.0 3.8
assign (resid 108 and name  HN )(resid 108 and name  HB# ) 0.0 0.6 3.4
assign (resid 108 and name  HN )(resid 108 and name  HG# ) 0.0 1.0 4.3
assign (resid 109 and name  HN )(resid 109 and name HD1# ) 0.0 1.5 6.5
assign (resid 109 and name  HN )(resid 109 and name HD2# ) 0.0 1.5 6.5
assign (resid 110 and name  HN )(resid 110 and name  HB1 ) 0.0 0.0 5.0
assign (resid 110 and name  HN )(resid 110 and name  HB2 ) 0.0 0.0 5.0
assign (resid 111 and name  HN )(resid 111 and name   HB ) 0.0 0.0 3.3
assign (resid 111 and name  HN )(resid 111 and name  HG# ) 0.0 1.7 5.0
assign (resid 113 and name  HN )(resid 113 and name HD1# ) 0.0 1.5 4.8
assign (resid 113 and name  HN )(resid 113 and name HD2# ) 0.0 1.5 4.8
assign (resid 114 and name  HN )(resid 114 and name HD1# ) 0.0 1.5 4.8
assign (resid 114 and name  HN )(resid 114 and name HD2# ) 0.0 1.5 4.8
assign (resid 115 and name  HN )(resid 115 and name  HB# ) 0.0 0.6 3.9
assign (resid 115 and name  HA )(resid 115 and name  HB# ) 0.0 1.0 3.8
assign (resid 116 and name  HA )(resid 116 and name  HB1 ) 0.0 0.0 2.8
assign (resid 116 and name  HA )(resid 116 and name  HB2 ) 0.0 0.0 2.8
assign (resid 116 and name  HA )(resid 116 and name  HD1 ) 0.0 0.0 5.0
assign (resid 116 and name HB1 )(resid 116 and name  HD1 ) 0.0 0.0 5.0
assign (resid 116 and name HB2 )(resid 116 and name  HD1 ) 0.0 0.0 5.0
assign (resid 116 and name HG1 )(resid 116 and name  HD1 ) 0.0 0.0 3.3
assign (resid 116 and name HG2 )(resid 116 and name  HD1 ) 0.0 0.0 3.3
assign (resid 116 and name  HA )(resid 116 and name  HD2 ) 0.0 0.0 5.0
assign (resid 116 and name HB1 )(resid 116 and name  HD2 ) 0.0 0.0 5.0
assign (resid 116 and name HB2 )(resid 116 and name  HD2 ) 0.0 0.0 5.0
assign (resid 116 and name HG1 )(resid 116 and name  HD2 ) 0.0 0.0 3.3
assign (resid 116 and name HG2 )(resid 116 and name  HD2 ) 0.0 0.0 3.3
assign (resid 116 and name  HA )(resid 116 and name  HG1 ) 0.0 0.0 5.0
assign (resid 116 and name HB1 )(resid 116 and name  HG1 ) 0.0 0.0 3.3
assign (resid 116 and name HB2 )(resid 116 and name  HG1 ) 0.0 0.0 3.3
assign (resid 116 and name  HA )(resid 116 and name  HG2 ) 0.0 0.0 5.0
assign (resid 116 and name HB1 )(resid 116 and name  HG2 ) 0.0 0.0 3.3
assign (resid 116 and name HB2 )(resid 116 and name  HG2 ) 0.0 0.0 3.3
assign (resid 117 and name  HN )(resid 117 and name   HB ) 0.0 0.0 5.0
assign (resid 117 and name  HN )(resid 117 and name  HG# ) 0.0 1.7 4.5
assign (resid 117 and name  HA )(resid 117 and name   HB ) 0.0 0.0 3.3
assign (resid 117 and name  HA )(resid 117 and name  HG# ) 0.0 1.7 4.5
assign (resid 117 and name  HA )(resid 117 and name HG1# ) 0.0 1.5 4.3
assign (resid 117 and name  HB )(resid 117 and name HG1# ) 0.0 1.5 4.3
assign (resid 117 and name  HA )(resid 117 and name HG2# ) 0.0 1.5 4.3
assign (resid 117 and name  HB )(resid 117 and name HG2# ) 0.0 1.5 4.3
assign (resid 118 and name  HN )(resid 118 and name  HB1 ) 0.0 0.0 3.3
assign (resid 118 and name  HN )(resid 118 and name  HB2 ) 0.0 0.0 3.3
assign (resid 118 and name  HA )(resid 118 and name  HB1 ) 0.0 0.0 2.8
assign (resid 118 and name  HA )(resid 118 and name  HB2 ) 0.0 0.0 2.8
assign (resid 119 and name  HN )(resid 119 and name  HB1 ) 0.0 0.0 5.0
assign (resid 119 and name  HN )(resid 119 and name  HB2 ) 0.0 0.0 5.0
assign (resid 119 and name  HN )(resid 119 and name  HD# ) 0.0 1.0 4.3
assign (resid 119 and name  HN )(resid 119 and name  HG# ) 0.0 1.0 4.3
assign (resid 119 and name  HA )(resid 119 and name  HB# ) 0.0 1.0 3.8
assign (resid 119 and name  HA )(resid 119 and name  HD1 ) 0.0 0.0 3.3
assign (resid 119 and name  HA )(resid 119 and name  HD2 ) 0.0 0.0 3.3
assign (resid 119 and name  HA )(resid 119 and name  HG# ) 0.0 1.0 3.8
assign (resid 119 and name HB# )(resid 119 and name  HG# ) 0.0 2.0 4.8
assign (resid 120 and name  HN )(resid 120 and name  HB1 ) 0.0 0.0 3.3
assign (resid 120 and name  HN )(resid 120 and name  HB2 ) 0.0 0.0 3.3
```

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assign (resid  120 and  name  HA ) (resid  120 and  name  HB1 ) 0.0 0.0 2.8
assign (resid  120 and  name  HA ) (resid  120 and  name  HB2 ) 0.0 0.0 2.8
assign (resid   22 and  name HD1 ) (resid   22 and  name   HA ) 0.0 0.0 3.3
assign (resid   23 and  name HE# ) (resid   23 and  name   HN ) 0.0 2.0 7.0
assign (resid   24 and  name  HA ) (resid   24 and  name HD1 ) 0.0 0.0 3.3
assign (resid   24 and  name HB# ) (resid   24 and  name HD1 ) 0.0 1.0 4.3
assign (resid   24 and  name  HA ) (resid   24 and  name HE3 ) 0.0 0.0 5.0
assign (resid   24 and  name HD1 ) (resid   24 and  name HZ2 ) 0.0 0.0 5.0
assign (resid   24 and  name HE3 ) (resid   24 and  name HZ2 ) 0.0 0.0 5.0
assign (resid   44 and  name HD1 ) (resid   44 and  name   HN ) 0.0 0.0 5.0
assign (resid   44 and  name HD2 ) (resid   44 and  name   HN ) 0.0 0.0 5.0
assign (resid   44 and  name  HA ) (resid   44 and  name HD# ) 0.0 2.0 5.3
assign (resid   44 and  name HB# ) (resid   44 and  name HD# ) 0.0 3.0 6.3
assign (resid   44 and  name HE# ) (resid   44 and  name HD# ) 0.0 4.0 7.3
assign (resid   44 and  name HD1 ) (resid   44 and  name HD2 ) 0.0 0.0 5.0
assign (resid   44 and  name HE1 ) (resid   44 and  name HE2 ) 0.0 0.0 5.0
assign (resid   44 and  name HD# ) (resid   44 and  name   HZ ) 0.0 2.0 5.3
assign (resid   57 and  name  HA ) (resid   57 and  name HD# ) 0.0 2.0 4.8
assign (resid   57 and  name HB# ) (resid   57 and  name HD# ) 0.0 3.0 5.8
assign (resid   57 and  name  HN ) (resid   57 and  name HD# ) 0.0 2.0 4.8
assign (resid   57 and  name  HA ) (resid   57 and  name HE# ) 0.0 2.0 5.3
assign (resid   57 and  name HB# ) (resid   57 and  name HE# ) 0.0 3.0 6.3
assign (resid   57 and  name  HN ) (resid   57 and  name HE# ) 0.0 2.0 5.3
assign (resid   73 and  name HD# ) (resid   73 and  name  HN ) 0.0 2.0 5.3
assign (resid   73 and  name  HA ) (resid   73 and  name HD# ) 0.0 2.0 4.8
assign (resid   73 and  name HB# ) (resid   73 and  name HD# ) 0.0 3.0 5.8
assign (resid   73 and  name  HZ ) (resid   73 and  name HD# ) 0.0 2.0 5.3
assign (resid   73 and  name  HA ) (resid   73 and  name HE# ) 0.0 2.0 7.0
assign (resid   73 and  name  HN ) (resid   73 and  name HE# ) 0.0 2.0 5.3
assign (resid   79 and  name  HA ) (resid   79 and  name HD# ) 0.0 2.0 4.8
assign (resid   79 and  name HB# ) (resid   79 and  name HD# ) 0.0 3.0 5.8
assign (resid   79 and  name  HA ) (resid   79 and  name HE# ) 0.0 2.0 5.3
assign (resid   79 and  name HB# ) (resid   79 and  name HE# ) 0.0 3.0 6.3
assign (resid   81 and  name  HA ) (resid   81 and  name HD# ) 0.0 2.0 4.8
assign (resid   81 and  name HB# ) (resid   81 and  name HD# ) 0.0 3.0 5.8
assign (resid   81 and  name  HZ ) (resid   81 and  name HD# ) 0.0 2.0 4.8
assign (resid   81 and  name  HA ) (resid   81 and  name HE# ) 0.0 2.0 5.3
assign (resid   81 and  name HB# ) (resid   81 and  name HE# ) 0.0 3.0 6.3
assign (resid   87 and  name HD# ) (resid   87 and  name  HN ) 0.0 2.0 5.3
assign (resid   87 and  name  HA ) (resid   87 and  name HD# ) 0.0 2.0 4.8
assign (resid   87 and  name HB# ) (resid   87 and  name HD# ) 0.0 3.0 5.8
assign (resid   87 and  name  HZ ) (resid   87 and  name HD# ) 0.0 2.0 4.8
assign (resid   87 and  name  HA ) (resid   87 and  name HE# ) 0.0 2.0 7.0
assign (resid   97 and  name HD# ) (resid   97 and  name  HN ) 0.0 2.0 7.0
assign (resid   97 and  name HE# ) (resid   97 and  name  HN ) 0.0 2.0 7.0
assign (resid   97 and  name  HA ) (resid   97 and  name HD# ) 0.0 2.0 4.8
assign (resid   97 and  name HB# ) (resid   97 and  name HD# ) 0.0 3.0 5.8
assign (resid   97 and  name  HA ) (resid   97 and  name HE# ) 0.0 2.0 4.8
assign (resid  105 and  name HD# ) (resid  105 and  name  HN ) 0.0 2.0 7.0
assign (resid  105 and  name  HA ) (resid  105 and  name HD# ) 0.0 2.0 4.8
assign (resid  105 and  name HB# ) (resid  105 and  name HD# ) 0.0 3.0 5.8
assign (resid  105 and  name  HA ) (resid  105 and  name HE# ) 0.0 2.0 5.3
assign (resid  105 and  name HB# ) (resid  105 and  name HE# ) 0.0 3.0 6.3
assign (resid  115 and  name HD# ) (resid  115 and  name  HN ) 0.0 2.0 5.3
assign (resid  115 and  name HE# ) (resid  115 and  name  HN ) 0.0 2.0 7.0
assign (resid  115 and  name  HA ) (resid  115 and  name HD# ) 0.0 2.0 4.8
assign (resid  115 and  name HB# ) (resid  115 and  name HD# ) 0.0 3.0 5.8
assign (resid  115 and  name  HA ) (resid  115 and  name HE# ) 0.0 2.0 5.3
assign (resid  115 and  name HB# ) (resid  115 and  name HE# ) 0.0 3.0 6.3
assign (resid  120 and  name HD# ) (resid  120 and  name  HN ) 0.0 2.0 5.3
assign (resid  120 and  name HE# ) (resid  120 and  name  HN ) 0.0 2.0 7.0
assign (resid  120 and  name  HA ) (resid  120 and  name HD# ) 0.0 2.0 4.8
assign (resid  120 and  name HB1 ) (resid  120 and  name HD# ) 0.0 2.0 4.8
assign (resid  120 and  name HB2 ) (resid  120 and  name HD# ) 0.0 2.0 4.8
```

```
assign (resid  120 and   name   HA )(resid  120 and   name  HE# ) 0.0 2.0 5.3
assign (resid  120 and   name  HB1 )(resid  120 and   name  HE# ) 0.0 2.0 7.0
assign (resid  120 and   name  HB2 )(resid  120 and   name  HE# ) 0.0 2.0 7.0
assign (resid   23 and   name   HN )(resid   23 and   name  HD# ) 0.0 2.0 7.0
assign (resid   68 and   name   HN )(resid   68 and   name  HG2 ) 0.0 0.0 5.0
assign (resid   68 and   name   HN )(resid   68 and   name  HG1 ) 0.0 0.0 5.0
assign (resid   79 and   name   HN )(resid   79 and   name  HD# ) 0.0 2.0 5.3
assign (resid   81 and   name   HN )(resid   81 and   name  HD# ) 0.0 2.0 7.0
! SEQUENTIAL RESTRAINTS FOR SH2 MODULE
assign (resid   18 and   name   HN )(resid   17 and   name   HG ) 0.0 0.0 5.0
assign (resid   18 and   name   HN )(resid   17 and   name   HA ) 0.0 0.0 3.3
assign (resid   18 and   name   HN )(resid   17 and   name  HB# ) 0.0 1.0 4.3
assign (resid   18 and   name   HN )(resid   17 and   name HD1# ) 0.0 1.5 6.5
assign (resid   18 and   name   HN )(resid   17 and   name  HT# ) 0.0 0.0 3.3
assign (resid   19 and   name   HN )(resid   18 and   name   HA ) 0.0 0.0 3.3
assign (resid   19 and   name   HN )(resid   18 and   name  HB# ) 0.0 1.0 4.3
assign (resid   19 and   name   HN )(resid   18 and   name  HG# ) 0.0 1.0 4.3
assign (resid   19 and   name   HN )(resid   18 and   name   HN ) 0.0 0.0 3.3
assign (resid   20 and   name   HN )(resid   19 and   name   HA ) 0.0 0.0 3.3
assign (resid   20 and   name   HN )(resid   19 and   name  HB1 ) 0.0 0.0 3.3
assign (resid   20 and   name   HN )(resid   19 and   name  HB2 ) 0.0 0.0 3.3
assign (resid   20 and   name  HB# )(resid   19 and   name   HN ) 0.0 1.5 4.8
assign (resid   20 and   name   HN )(resid   19 and   name   HN ) 0.0 0.0 2.8
assign (resid   21 and   name   HN )(resid   20 and   name   HA ) 0.0 0.0 2.8
assign (resid   21 and   name   HN )(resid   20 and   name  HB# ) 0.0 1.5 4.8
assign (resid   21 and   name   HN )(resid   20 and   name   HN ) 0.0 0.0 5.0
assign (resid   22 and   name   HN )(resid   21 and   name  HB# ) 0.0 1.0 6.0
assign (resid   22 and   name   HN )(resid   21 and   name   HA ) 0.0 0.0 5.0
assign (resid   22 and   name   HN )(resid   21 and   name  HG# ) 0.0 1.0 4.3
assign (resid   22 and   name   HN )(resid   21 and   name   HN ) 0.0 0.0 3.3
assign (resid   23 and   name   HN )(resid   22 and   name  HB1 ) 0.0 0.0 5.0
assign (resid   23 and   name   HN )(resid   22 and   name  HB2 ) 0.0 0.0 5.0
assign (resid   23 and   name   HN )(resid   22 and   name   HA ) 0.0 0.0 3.3
assign (resid   24 and   name   HN )(resid   23 and   name   HA ) 0.0 0.0 3.3
assign (resid   24 and   name   HN )(resid   23 and   name  HB1 ) 0.0 0.0 5.0
assign (resid   24 and   name   HN )(resid   23 and   name  HB2 ) 0.0 0.0 5.0
assign (resid   24 and   name   HN )(resid   23 and   name   HN ) 0.0 0.0 5.0
assign (resid   25 and   name   HN )(resid   24 and   name   HA ) 0.0 0.0 3.3
assign (resid   25 and   name   HN )(resid   24 and   name  HB# ) 0.0 1.0 4.3
assign (resid   25 and   name   HN )(resid   24 and   name   HN ) 0.0 0.0 3.3
assign (resid   26 and   name   HN )(resid   25 and   name  HA1 ) 0.0 0.0 3.3
assign (resid   26 and   name   HN )(resid   25 and   name  HA2 ) 0.0 0.0 3.3
assign (resid   26 and   name   HN )(resid   25 and   name   HN ) 0.0 0.0 3.3
assign (resid   27 and   name   HN )(resid   26 and   name   HA ) 0.0 0.0 2.8
assign (resid   27 and   name   HN )(resid   26 and   name  HB# ) 0.0 1.0 4.3
assign (resid   27 and   name   HN )(resid   26 and   name   HN ) 0.0 0.0 5.0
assign (resid   28 and   name   HN )(resid   27 and   name   HA ) 0.0 0.0 3.3
assign (resid   28 and   name   HN )(resid   27 and   name   HB ) 0.0 0.0 3.3
assign (resid   28 and   name   HN )(resid   27 and   name  HD# ) 0.0 1.5 4.8
assign (resid   28 and   name   HN )(resid   27 and   name HG11 ) 0.0 0.0 3.3
assign (resid   28 and   name   HN )(resid   27 and   name HG12 ) 0.0 0.0 3.3
assign (resid   28 and   name   HN )(resid   27 and   name HG2# ) 0.0 1.5 4.8
assign (resid   28 and   name   HN )(resid   27 and   name   HN ) 0.0 0.0 5.0
assign (resid   29 and   name   HN )(resid   28 and   name   HA ) 0.0 0.0 2.8
assign (resid   29 and   name   HA )(resid   28 and   name  HB# ) 0.0 1.0 6.0
assign (resid   29 and   name   HN )(resid   28 and   name  HB# ) 0.0 1.0 4.3
assign (resid   29 and   name   HN )(resid   28 and   name   HN ) 0.0 0.0 5.0
assign (resid   30 and   name   HN )(resid   29 and   name  HD# ) 0.0 1.0 6.0
assign (resid   30 and   name   HN )(resid   29 and   name   HA ) 0.0 0.0 3.3
assign (resid   30 and   name   HN )(resid   29 and   name  HB# ) 0.0 1.0 4.3
assign (resid   30 and   name   HN )(resid   29 and   name  HG# ) 0.0 1.0 4.3
assign (resid   30 and   name   HN )(resid   29 and   name   HN ) 0.0 0.0 3.3
assign (resid   31 and   name   HN )(resid   30 and   name  HG# ) 0.0 1.0 6.0
assign (resid   31 and   name   HN )(resid   30 and   name   HA ) 0.0 0.0 5.0
```

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assign (resid  31 and  name  HN )(resid  30 and  name   HN ) 0.0 0.0 3.3
assign (resid  32 and  name  HN )(resid  31 and  name  HB# ) 0.0 1.0 4.3
assign (resid  32 and  name  HN )(resid  31 and  name   HA ) 0.0 0.0 5.0
assign (resid  32 and  name  HN )(resid  31 and  name  HG# ) 0.0 1.0 4.3
assign (resid  32 and  name  HN )(resid  31 and  name   HN ) 0.0 0.0 2.8
assign (resid  33 and  name  HN )(resid  32 and  name   HA ) 0.0 0.0 5.0
assign (resid  33 and  name  HN )(resid  32 and  name   HB ) 0.0 0.0 2.8
assign (resid  33 and  name  HN )(resid  32 and  name  HG# ) 0.0 2.4 5.7
assign (resid  33 and  name  HN )(resid  32 and  name   HN ) 0.0 0.0 3.3
assign (resid  34 and  name  HN )(resid  33 and  name   HA ) 0.0 0.0 5.0
assign (resid  34 and  name  HN )(resid  33 and  name  HB1 ) 0.0 0.0 3.3
assign (resid  34 and  name  HN )(resid  33 and  name  HB2 ) 0.0 0.0 3.3
assign (resid  34 and  name  HN )(resid  33 and  name   HN ) 0.0 0.0 3.3
assign (resid  35 and  name  HN )(resid  34 and  name  HG# ) 0.0 1.0 6.0
assign (resid  35 and  name  HN )(resid  34 and  name   HA ) 0.0 0.0 3.3
assign (resid  35 and  name  HN )(resid  34 and  name  HB# ) 0.0 1.0 4.3
assign (resid  35 and  name  HN )(resid  34 and  name   HN ) 0.0 0.0 3.3
assign (resid  36 and  name  HN )(resid  35 and  name  HD# ) 0.0 1.0 6.0
assign (resid  36 and  name  HN )(resid  35 and  name  HG1 ) 0.0 0.0 5.0
assign (resid  36 and  name  HN )(resid  35 and  name  HG2 ) 0.0 0.0 5.0
assign (resid  36 and  name  HN )(resid  35 and  name   HA ) 0.0 0.0 3.3
assign (resid  36 and  name  HN )(resid  35 and  name   HN ) 0.0 0.0 3.3
assign (resid  37 and  name  HN )(resid  36 and  name  HD# ) 0.0 2.4 5.7
assign (resid  37 and  name  HN )(resid  36 and  name   HA ) 0.0 0.0 3.3
assign (resid  37 and  name  HN )(resid  36 and  name  HB# ) 0.0 1.0 4.3
assign (resid  37 and  name  HN )(resid  36 and  name   HN ) 0.0 0.0 2.8
assign (resid  38 and  name  HN )(resid  37 and  name  HD# ) 0.0 1.0 4.3
assign (resid  38 and  name  HN )(resid  37 and  name   HA ) 0.0 0.0 3.3
assign (resid  38 and  name  HN )(resid  37 and  name   HN ) 0.0 0.0 3.3
assign (resid  39 and  name  HN )(resid  38 and  name  HB1 ) 0.0 0.0 5.0
assign (resid  38 and  name  HN )(resid  37 and  name   HN ) 0.0 0.0 3.3
assign (resid  39 and  name  HN )(resid  38 and  name  HB1 ) 0.0 0.0 5.0
assign (resid  39 and  name  HN )(resid  38 and  name  HB2 ) 0.0 0.0 5.0
assign (resid  39 and  name  HN )(resid  38 and  name   HA ) 0.0 0.0 3.3
assign (resid  39 and  name  HN )(resid  38 and  name   HN ) 0.0 0.0 3.3
assign (resid  40 and  name  HN )(resid  39 and  name   HA ) 0.0 0.0 2.8
assign (resid  40 and  name  HN )(resid  39 and  name HG2# ) 0.0 1.5 4.8
assign (resid  40 and  name  HN )(resid  39 and  name   HN ) 0.0 0.0 5.0
assign (resid  41 and  name  HN )(resid  40 and  name   HA ) 0.0 0.0 2.8
assign (resid  41 and  name  HN )(resid  40 and  name  HB# ) 0.0 1.5 4.3
assign (resid  41 and  name  HN )(resid  40 and  name   HN ) 0.0 0.0 5.0
assign (resid  42 and  name  HN )(resid  41 and  name   HA ) 0.0 0.0 3.3
assign (resid  42 and  name  HN )(resid  41 and  name  HB1 ) 0.0 0.0 5.0
assign (resid  42 and  name  HN )(resid  41 and  name  HB2 ) 0.0 0.0 5.0
assign (resid  42 and  name  HN )(resid  41 and  name   HN ) 0.0 0.0 3.3
assign (resid  43 and  name  HN )(resid  42 and  name  HA# ) 0.0 1.0 6.0
assign (resid  43 and  name  HN )(resid  42 and  name   HN ) 0.0 0.0 3.3
assign (resid  44 and  name  HN )(resid  43 and  name   HA ) 0.0 0.0 3.3
assign (resid  44 and  name  HN )(resid  43 and  name HG2# ) 0.0 1.5 4.8
assign (resid  44 and  name  HN )(resid  43 and  name   HN ) 0.0 0.0 5.0
assign (resid  45 and  name  HN )(resid  44 and  name  HB1 ) 0.0 0.0 5.0
assign (resid  45 and  name  HN )(resid  44 and  name  HB2 ) 0.0 0.0 5.0
assign (resid  45 and  name  HN )(resid  44 and  name   HA ) 0.0 0.0 2.8
assign (resid  45 and  name  HN )(resid  44 and  name   HN ) 0.0 0.0 5.0
assign (resid  46 and  name  HN )(resid  45 and  name HD1# ) 0.0 1.5 6.5
assign (resid  46 and  name  HN )(resid  45 and  name HD2# ) 0.0 1.5 6.5
assign (resid  46 and  name  HN )(resid  45 and  name   HG ) 0.0 0.0 5.0
assign (resid  46 and  name  HN )(resid  45 and  name   HA ) 0.0 0.0 3.3
assign (resid  46 and  name  HN )(resid  45 and  name  HB# ) 0.0 1.0 4.3
assign (resid  47 and  name  HN )(resid  46 and  name   HA ) 0.0 0.0 3.3
assign (resid  47 and  name  HN )(resid  46 and  name   HB ) 0.0 0.0 5.0
assign (resid  47 and  name  HN )(resid  46 and  name HG1# ) 0.0 1.5 4.8
assign (resid  47 and  name  HN )(resid  46 and  name HG2# ) 0.0 1.5 4.8
assign (resid  47 and  name  HN )(resid  46 and  name   HN ) 0.0 0.0 5.0
assign (resid  49 and  name  HN )(resid  48 and  name   HA ) 0.0 0.0 3.3
assign (resid  49 and  name  HN )(resid  48 and  name  HB1 ) 0.0 0.0 5.0
```

```
assign (resid  49 and  name  HN )(resid  48 and  name  HB2 ) 0.0 0.0 5.0
assign (resid  49 and  name  HN )(resid  48 and  name   HN ) 0.0 0.0 5.0
assign (resid  52 and  name  HN )(resid  51 and  name   HA ) 0.0 0.0 3.3
assign (resid  52 and  name  HN )(resid  51 and  name HG2# ) 0.0 1.5 6.5
assign (resid  52 and  name  HN )(resid  51 and  name   HN ) 0.0 0.0 3.3
assign (resid  53 and  name  HN )(resid  52 and  name   HA ) 0.0 0.0 5.0
assign (resid  53 and  name  HN )(resid  52 and  name   HN ) 0.0 0.0 3.3
assign (resid  54 and  name  HN )(resid  53 and  name   HA ) 0.0 0.0 3.3
assign (resid  54 and  name  HN )(resid  53 and  name   HN ) 0.0 0.0 3.3
assign (resid  55 and  name  HN )(resid  54 and  name   HA ) 0.0 0.0 3.3
assign (resid  55 and  name  HN )(resid  54 and  name  HB1 ) 0.0 0.0 5.0
assign (resid  55 and  name  HN )(resid  54 and  name  HB2 ) 0.0 0.0 5.0
assign (resid  55 and  name  HN )(resid  54 and  name   HN ) 0.0 0.0 3.3
assign (resid  56 and  name  HN )(resid  55 and  name  HA# ) 0.0 1.0 4.3
assign (resid  56 and  name  HN )(resid  55 and  name  HA1 ) 0.0 0.0 3.3
assign (resid  56 and  name  HN )(resid  55 and  name  HA2 ) 0.0 0.0 3.3
assign (resid  56 and  name  HN )(resid  55 and  name   HN ) 0.0 0.0 5.0
assign (resid  57 and  name  HN )(resid  56 and  name   HA ) 0.0 0.0 5.0
assign (resid  57 and  name  HN )(resid  56 and  name  HB# ) 0.0 1.0 6.0
assign (resid  57 and  name  HN )(resid  56 and  name  HB1 ) 0.0 0.0 5.0
assign (resid  57 and  name  HN )(resid  56 and  name  HB2 ) 0.0 0.0 5.0
assign (resid  57 and  name  HN )(resid  56 and  name   HN ) 0.0 0.0 3.3
assign (resid  58 and  name  HN )(resid  57 and  name   HA ) 0.0 0.0 2.8
assign (resid  58 and  name  HN )(resid  57 and  name  HB1 ) 0.0 0.0 3.3
assign (resid  58 and  name  HN )(resid  57 and  name  HB2 ) 0.0 0.0 3.3
assign (resid  59 and  name  HN )(resid  58 and  name   HB ) 0.0 0.0 5.0
assign (resid  59 and  name  HN )(resid  58 and  name   HA ) 0.0 0.0 3.3
assign (resid  59 and  name  HN )(resid  58 and  name HG2# ) 0.0 1.5 4.8
assign (resid  60 and  name  HN )(resid  59 and  name HD1# ) 0.0 1.5 6.5
assign (resid  60 and  name  HN )(resid  59 and  name HD2# ) 0.0 1.5 6.5
assign (resid  60 and  name  HN )(resid  59 and  name   HA ) 0.0 0.0 2.8
assign (resid  60 and  name  HN )(resid  59 and  name  HB# ) 0.0 1.0 4.3
assign (resid  60 and  name  HN )(resid  59 and  name   HG ) 0.0 0.0 3.3
assign (resid  60 and  name  HN )(resid  59 and  name   HN ) 0.0 0.0 5.0
assign (resid  61 and  name  HN )(resid  60 and  name   HA ) 0.0 0.0 2.8
assign (resid  62 and  name  HN )(resid  61 and  name   HA ) 0.0 0.0 3.3
assign (resid  62 and  name  HN )(resid  61 and  name  HB1 ) 0.0 0.0 3.3
assign (resid  62 and  name  HN )(resid  61 and  name  HB2 ) 0.0 0.0 3.3
assign (resid  62 and  name  HN )(resid  61 and  name   HN ) 0.0 0.0 5.0
assign (resid  63 and  name  HN )(resid  62 and  name  HB1 ) 0.0 0.0 5.0
assign (resid  63 and  name  HN )(resid  62 and  name  HB2 ) 0.0 0.0 5.0
assign (resid  63 and  name  HN )(resid  62 and  name   HA ) 0.0 0.0 2.8
assign (resid  63 and  name  HA )(resid  62 and  name  HB# ) 0.0 1.0 6.0
assign (resid  63 and  name  HN )(resid  62 and  name   HN ) 0.0 0.0 5.0
assign (resid  64 and  name  HN )(resid  63 and  name  HB# ) 0.0 1.0 4.3
assign (resid  64 and  name  HN )(resid  63 and  name  HG# ) 0.0 1.0 6.0
assign (resid  64 and  name  HN )(resid  63 and  name   HA ) 0.0 0.0 2.8
assign (resid  64 and  name  HN )(resid  63 and  name   HN ) 0.0 0.0 5.0
assign (resid  65 and  name  HN )(resid  64 and  name  HA# ) 0.0 1.0 4.3
assign (resid  65 and  name  HN )(resid  64 and  name  HA1 ) 0.0 0.0 3.3
assign (resid  65 and  name  HN )(resid  64 and  name  HA2 ) 0.0 0.0 3.3
assign (resid  65 and  name  HN )(resid  64 and  name   HN ) 0.0 0.0 3.3
assign (resid  66 and  name  HN )(resid  65 and  name  HA# ) 0.0 1.0 4.3
assign (resid  66 and  name  HN )(resid  65 and  name  HA1 ) 0.0 0.0 3.3
assign (resid  66 and  name  HN )(resid  65 and  name  HA2 ) 0.0 0.0 3.3
assign (resid  66 and  name  HN )(resid  65 and  name   HN ) 0.0 0.0 3.3
assign (resid  67 and  name  HN )(resid  66 and  name   HA ) 0.0 0.0 2.8
assign (resid  67 and  name  HN )(resid  66 and  name  HB# ) 0.0 1.0 4.3
assign (resid  67 and  name  HN )(resid  66 and  name   HN ) 0.0 0.0 5.0
assign (resid  68 and  name  HN )(resid  67 and  name   HA ) 0.0 0.0 2.8
assign (resid  68 and  name  HN )(resid  67 and  name  HB1 ) 0.0 0.0 5.0
assign (resid  68 and  name  HN )(resid  67 and  name  HB2 ) 0.0 0.0 5.0
assign (resid  68 and  name  HN )(resid  67 and  name   HN ) 0.0 0.0 5.0
assign (resid  69 and  name  HN )(resid  68 and  name  HB# ) 0.0 1.0 4.3
```

```
assign (resid  69 and  name  HN )(resid  68 and  name  HA ) 0.0 0.0 2.8
assign (resid  69 and  name  HN )(resid  68 and  name  HN ) 0.0 0.0 5.0
assign (resid  70 and  name  HN )(resid  69 and  name  HD# ) 0.0 2.4 5.7
assign (resid  70 and  name  HN )(resid  69 and  name  HA ) 0.0 0.0 3.3
assign (resid  70 and  name  HN )(resid  69 and  name  HB# ) 0.0 1.0 4.3
assign (resid  70 and  name  HN )(resid  69 and  name  HN ) 0.0 0.0 5.0
assign (resid  71 and  name  HN )(resid  70 and  name  HB ) 0.0 0.0 5.0
assign (resid  71 and  name  HN )(resid  70 and  name HG11 ) 0.0 0.0 5.0
assign (resid  71 and  name  HN )(resid  70 and  name HG12 ) 0.0 0.0 5.0
assign (resid  71 and  name  HN )(resid  70 and  name  HA ) 0.0 0.0 2.8
assign (resid  71 and  name  HN )(resid  70 and  name HG2# ) 0.0 1.5 4.8
assign (resid  71 and  name  HN )(resid  70 and  name  HN ) 0.0 0.0 5.0
assign (resid  72 and  name  HN )(resid  71 and  name  HB# ) 0.0 1.0 6.0
assign (resid  73 and  name  HN )(resid  72 and  name  HD# ) 0.0 1.5 4.8
assign (resid  73 and  name  HN )(resid  72 and  name  HA ) 0.0 0.0 3.3
assign (resid  73 and  name  HN )(resid  72 and  name  HB ) 0.0 0.0 5.0
assign (resid  73 and  name  HN )(resid  72 and  name HG2# ) 0.0 1.5 6.5
assign (resid  73 and  name  HN )(resid  72 and  name  HN ) 0.0 0.0 5.0
assign (resid  74 and  name  HN )(resid  73 and  name  HB1 ) 0.0 0.0 5.0
assign (resid  74 and  name  HN )(resid  73 and  name  HB2 ) 0.0 0.0 5.0
assign (resid  74 and  name  HN )(resid  73 and  name  HA ) 0.0 0.0 3.3
assign (resid  74 and  name  HN )(resid  73 and  name  HN ) 0.0 0.0 5.0
assign (resid  75 and  name  HN )(resid  74 and  name  HA ) 0.0 0.0 2.8
assign (resid  75 and  name  HN )(resid  74 and  name  HB1 ) 0.0 0.0 5.0
assign (resid  75 and  name  HN )(resid  74 and  name  HB2 ) 0.0 0.0 5.0
assign (resid  75 and  name  HN )(resid  74 and  name  HN ) 0.0 0.0 5.0
assign (resid  76 and  name  HN )(resid  75 and  name  HG# ) 0.0 1.0 6.0
assign (resid  76 and  name  HN )(resid  75 and  name  HA ) 0.0 0.0 5.0
assign (resid  76 and  name  HN )(resid  75 and  name  HB# ) 0.0 1.0 6.0
assign (resid  76 and  name  HN )(resid  75 and  name  HN ) 0.0 0.0 5.0
assign (resid  77 and  name  HN )(resid  76 and  name  HA ) 0.0 0.0 5.0
assign (resid  77 and  name  HN )(resid  76 and  name  HB# ) 0.0 1.0 6.0
assign (resid  77 and  name  HN )(resid  76 and  name  HN ) 0.0 0.0 3.3
assign (resid  78 and  name  HN )(resid  77 and  name  HA# ) 0.0 1.0 4.3
assign (resid  78 and  name  HN )(resid  77 and  name  HA1 ) 0.0 0.0 3.3
assign (resid  78 and  name  HN )(resid  77 and  name  HA2 ) 0.0 0.0 3.3
assign (resid  78 and  name  HN )(resid  77 and  name  HN ) 0.0 0.0 3.3
assign (resid  79 and  name  HN )(resid  78 and  name  HG# ) 0.0 1.0 4.3
assign (resid  79 and  name  HN )(resid  78 and  name  HA ) 0.0 0.0 3.3
assign (resid  79 and  name  HN )(resid  78 and  name  HB1 ) 0.0 0.0 3.3
assign (resid  79 and  name  HN )(resid  78 and  name  HB2 ) 0.0 0.0 3.3
assign (resid  79 and  name  HN )(resid  78 and  name  HN ) 0.0 0.0 5.0
assign (resid  80 and  name  HN )(resid  79 and  name  HA ) 0.0 0.0 3.3
assign (resid  80 and  name  HN )(resid  79 and  name  HB1 ) 0.0 0.0 5.0
assign (resid  80 and  name  HN )(resid  79 and  name  HB2 ) 0.0 0.0 5.0
assign (resid  80 and  name  HN )(resid  79 and  name  HN ) 0.0 0.0 5.0
assign (resid  81 and  name  HN )(resid  80 and  name  HA# ) 0.0 1.0 4.3
assign (resid  81 and  name  HN )(resid  80 and  name  HA1 ) 0.0 0.0 3.3
assign (resid  81 and  name  HN )(resid  80 and  name  HA2 ) 0.0 0.0 3.3
assign (resid  81 and  name  HN )(resid  80 and  name  HN ) 0.0 0.0 5.0
assign (resid  82 and  name  HN )(resid  81 and  name  HA ) 0.0 0.0 3.3
assign (resid  82 and  name  HN )(resid  81 and  name  HB1 ) 0.0 0.0 3.3
assign (resid  82 and  name  HN )(resid  81 and  name  HB2 ) 0.0 0.0 3.3
assign (resid  82 and  name  HN )(resid  81 and  name  HN ) 0.0 0.0 2.8
assign (resid  83 and  name  HN )(resid  82 and  name  HA ) 0.0 0.0 3.3
assign (resid  83 and  name  HN )(resid  82 and  name  HB# ) 0.0 1.0 4.3
assign (resid  83 and  name  HN )(resid  82 and  name  HN ) 0.0 0.0 5.0
assign (resid  85 and  name  HN )(resid  84 and  name  HA ) 0.0 0.0 3.3
assign (resid  85 and  name  HN )(resid  84 and  name  HB1 ) 0.0 0.0 5.0
assign (resid  85 and  name  HN )(resid  84 and  name  HB2 ) 0.0 0.0 5.0
assign (resid  86 and  name  HN )(resid  85 and  name  HD# ) 0.0 2.4 7.4
assign (resid  86 and  name  HN )(resid  85 and  name  HA ) 0.0 0.0 3.3
assign (resid  86 and  name  HN )(resid  85 and  name  HB# ) 0.0 1.0 6.0
assign (resid  86 and  name  HN )(resid  85 and  name  HN ) 0.0 0.0 3.3
```

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assign (resid  87 and  name  HN )(resid  86 and  name  HA ) 0.0 0.0 5.0
assign (resid  87 and  name  HN )(resid  86 and  name HG2# ) 0.0 1.5 6.5
assign (resid  87 and  name  HN )(resid  86 and  name  HN ) 0.0 0.0 3.3
assign (resid  88 and  name  HN )(resid  87 and  name  HA ) 0.0 0.0 3.3
assign (resid  88 and  name  HN )(resid  87 and  name HB# ) 0.0 1.0 4.3
assign (resid  88 and  name  HN )(resid  87 and  name  HN ) 0.0 0.0 5.0
assign (resid  89 and  name  HN )(resid  88 and  name  HA ) 0.0 0.0 3.3
assign (resid  89 and  name  HN )(resid  88 and  name HB# ) 0.0 1.0 4.3
assign (resid  89 and  name  HN )(resid  88 and  name  HN ) 0.0 0.0 3.3
assign (resid  90 and  name  HN )(resid  89 and  name  HA ) 0.0 0.0 3.3
assign (resid  90 and  name  HN )(resid  89 and  name HB1 ) 0.0 0.0 3.3
assign (resid  90 and  name  HN )(resid  89 and  name HB2 ) 0.0 0.0 3.3
assign (resid  90 and  name  HN )(resid  89 and  name  HN ) 0.0 0.0 5.0
assign (resid  91 and  name  HA )(resid  90 and  name  HN ) 0.0 0.0 5.0
assign (resid  91 and  name  HB )(resid  90 and  name  HN ) 0.0 0.0 5.0
assign (resid  91 and  name HG1# )(resid  90 and  name  HN ) 0.0 1.5 6.5
assign (resid  91 and  name HG2# )(resid  90 and  name  HN ) 0.0 1.5 6.5
assign (resid  91 and  name  HN )(resid  90 and  name  HA ) 0.0 0.0 3.3
assign (resid  91 and  name  HN )(resid  90 and  name  HB ) 0.0 0.0 3.3
assign (resid  91 and  name  HN )(resid  90 and  name HG1# ) 0.0 1.5 4.8
assign (resid  91 and  name  HN )(resid  90 and  name HG2# ) 0.0 1.5 4.8
assign (resid  91 and  name  HN )(resid  90 and  name  HN ) 0.0 0.0 3.3
assign (resid  92 and  name  HN )(resid  91 and  name  HA ) 0.0 0.0 3.3
assign (resid  92 and  name  HN )(resid  91 and  name  HB ) 0.0 0.0 3.3
assign (resid  92 and  name  HN )(resid  91 and  name HG1# ) 0.0 1.5 4.8
assign (resid  92 and  name  HN )(resid  91 and  name HG2# ) 0.0 1.5 4.8
assign (resid  93 and  name  HN )(resid  92 and  name  HA ) 0.0 0.0 5.0
assign (resid  93 and  name  HN )(resid  92 and  name HB# ) 0.0 1.0 4.3
assign (resid  93 and  name  HN )(resid  92 and  name HG# ) 0.0 1.0 4.3
assign (resid  93 and  name  HN )(resid  92 and  name  HN ) 0.0 0.0 3.3
assign (resid  94 and  name  HN )(resid  93 and  name  HA ) 0.0 0.0 5.0
assign (resid  94 and  name  HA )(resid  93 and  name HD# ) 0.0 2.4 5.7
assign (resid  94 and  name  HN )(resid  93 and  name HD1# ) 0.0 1.5 4.8
assign (resid  94 and  name  HN )(resid  93 and  name HD2# ) 0.0 1.5 4.8
assign (resid  94 and  name  HN )(resid  93 and  name  HG ) 0.0 0.0 3.3
assign (resid  94 and  name  HN )(resid  93 and  name  HN ) 0.0 0.0 3.3
assign (resid  95 and  name  HN )(resid  94 and  name HD# ) 0.0 1.5 6.5
assign (resid  95 and  name  HN )(resid  94 and  name HG2# ) 0.0 1.5 6.5
assign (resid  95 and  name  HN )(resid  94 and  name  HA ) 0.0 0.0 5.0
assign (resid  95 and  name  HN )(resid  94 and  name  HB ) 0.0 0.0 3.3
assign (resid  95 and  name  HN )(resid  94 and  name  HN ) 0.0 0.0 3.3
assign (resid  96 and  name  HN )(resid  95 and  name  HA ) 0.0 0.0 5.0
assign (resid  96 and  name  HN )(resid  95 and  name HB1 ) 0.0 0.0 5.0
assign (resid  96 and  name  HN )(resid  95 and  name HB2 ) 0.0 0.0 5.0
assign (resid  96 and  name  HN )(resid  95 and  name  HN ) 0.0 0.0 3.3
assign (resid  97 and  name  HN )(resid  96 and  name  HA ) 0.0 0.0 5.0
assign (resid  97 and  name  HN )(resid  96 and  name HB# ) 0.0 1.0 4.3
assign (resid  97 and  name  HN )(resid  96 and  name  HN ) 0.0 0.0 3.3
assign (resid  98 and  name  HN )(resid  97 and  name  HA ) 0.0 0.0 5.0
assign (resid  98 and  name  HN )(resid  97 and  name  HN ) 0.0 0.0 3.3
assign (resid  99 and  name  HN )(resid  98 and  name HB# ) 0.0 1.0 6.0
assign (resid  99 and  name  HN )(resid  98 and  name HG# ) 0.0 1.0 6.0
assign (resid  99 and  name  HN )(resid  98 and  name  HN ) 0.0 0.0 3.3
assign (resid 100 and  name  HN )(resid  99 and  name  HA ) 0.0 0.0 5.0
assign (resid 100 and  name  HN )(resid  99 and  name HB1 ) 0.0 0.0 3.3
assign (resid 100 and  name  HN )(resid  99 and  name HB2 ) 0.0 0.0 3.3
assign (resid 100 and  name  HN )(resid  99 and  name  HN ) 0.0 0.0 3.3
assign (resid 101 and  name  HN )(resid 100 and  name  HA ) 0.0 0.0 3.3
assign (resid 101 and  name  HN )(resid 100 and  name HB1 ) 0.0 0.0 3.3
assign (resid 101 and  name  HN )(resid 100 and  name HB2 ) 0.0 0.0 3.3
assign (resid 101 and  name  HN )(resid 100 and  name HG# ) 0.0 1.0 4.3
assign (resid 101 and  name  HN )(resid 100 and  name  HN ) 0.0 0.0 5.0
assign (resid 102 and  name  HN )(resid 101 and  name  HA ) 0.0 0.0 3.3
assign (resid 102 and  name  HN )(resid 101 and  name HB1 ) 0.0 0.0 5.0
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assign (resid  102 and  name   HN )(resid  101 and  name  HB2 ) 0.0 0.0 5.0
assign (resid  103 and  name   HN )(resid  102 and  name  HD# ) 0.0 2.4 7.4
assign (resid  103 and  name   HN )(resid  102 and  name   HA ) 0.0 0.0 3.3
assign (resid  103 and  name   HN )(resid  102 and  name   HN ) 0.0 0.0 5.0
assign (resid  104 and  name   HN )(resid  103 and  name   HA ) 0.0 0.0 3.3
assign (resid  104 and  name   HN )(resid  103 and  name  HB# ) 0.0 1.5 4.8
assign (resid  104 and  name   HN )(resid  103 and  name   HN ) 0.0 0.0 3.3
assign (resid  105 and  name   HN )(resid  104 and  name   HA ) 0.0 0.0 5.0
assign (resid  105 and  name   HN )(resid  104 and  name  HB# ) 0.0 1.0 4.3
assign (resid  105 and  name   HN )(resid  104 and  name  HG# ) 0.0 1.0 6.0
assign (resid  105 and  name   HN )(resid  104 and  name   HN ) 0.0 0.0 3.3
assign (resid  106 and  name   HN )(resid  105 and  name   HA ) 0.0 0.0 3.3
assign (resid  106 and  name   HN )(resid  105 and  name  HB# ) 0.0 1.0 4.3
assign (resid  107 and  name  HD1 )(resid  106 and  name   HA ) 0.0 0.0 2.8
assign (resid  107 and  name  HD2 )(resid  106 and  name   HA ) 0.0 0.0 2.8
assign (resid  109 and  name   HN )(resid  108 and  name   HA ) 0.0 0.0 3.3
assign (resid  109 and  name   HN )(resid  108 and  name  HB# ) 0.0 1.0 4.3
assign (resid  109 and  name   HN )(resid  108 and  name  HG# ) 0.0 1.0 6.0
assign (resid  109 and  name   HN )(resid  108 and  name   HN ) 0.0 0.0 3.3
assign (resid  111 and  name   HN )(resid  110 and  name   HA ) 0.0 0.0 5.0
assign (resid  111 and  name   HN )(resid  110 and  name  HB1 ) 0.0 0.0 5.0
assign (resid  111 and  name   HN )(resid  110 and  name  HB2 ) 0.0 0.0 5.0
assign (resid  112 and  name   HN )(resid  111 and  name   HB ) 0.0 0.0 3.3
assign (resid  112 and  name   HN )(resid  111 and  name  HG# ) 0.0 2.4 5.7
assign (resid  112 and  name   HN )(resid  111 and  name   HN ) 0.0 0.0 5.0
assign (resid  112 and  name   HN )(resid  111 and  name   HA ) 0.0 0.0 2.8
assign (resid  113 and  name   HN )(resid  112 and  name   HN ) 0.0 0.0 5.0
assign (resid  113 and  name   HN )(resid  112 and  name   HA ) 0.0 0.0 3.3
assign (resid  114 and  name   HN )(resid  113 and  name  HD# ) 0.0 2.4 7.4
assign (resid  114 and  name   HN )(resid  113 and  name   HN ) 0.0 0.0 5.0
assign (resid  114 and  name   HN )(resid  113 and  name   HA ) 0.0 0.0 3.3
assign (resid  115 and  name   HN )(resid  114 and  name HD1# ) 0.0 1.5 6.5
assign (resid  115 and  name   HN )(resid  114 and  name HD2# ) 0.0 1.5 6.5
assign (resid  115 and  name   HN )(resid  114 and  name   HN ) 0.0 0.0 5.0
assign (resid  115 and  name   HN )(resid  114 and  name   HA ) 0.0 0.0 3.3
assign (resid  116 and  name  HD1 )(resid  115 and  name   HA ) 0.0 0.0 2.8
assign (resid  116 and  name  HD2 )(resid  115 and  name   HA ) 0.0 0.0 2.8
assign (resid  116 and  name  HG1 )(resid  115 and  name   HA ) 0.0 0.0 5.0
assign (resid  116 and  name  HG2 )(resid  115 and  name   HA ) 0.0 0.0 5.0
assign (resid  118 and  name   HN )(resid  117 and  name   HB ) 0.0 0.0 3.3
assign (resid  118 and  name   HN )(resid  117 and  name  HG# ) 0.0 2.4 5.7
assign (resid  118 and  name   HN )(resid  117 and  name   HN ) 0.0 0.0 5.0
assign (resid  118 and  name   HN )(resid  117 and  name   HA ) 0.0 0.0 2.8
assign (resid  119 and  name   HN )(resid  118 and  name  HB1 ) 0.0 0.0 3.3
assign (resid  119 and  name   HN )(resid  118 and  name  HB2 ) 0.0 0.0 3.3
assign (resid  119 and  name   HN )(resid  118 and  name   HA ) 0.0 0.0 2.8
assign (resid  120 and  name   HN )(resid  119 and  name  HB1 ) 0.0 0.0 5.0
assign (resid  120 and  name   HN )(resid  119 and  name  HB2 ) 0.0 0.0 5.0
assign (resid  120 and  name   HN )(resid  119 and  name  HD# ) 0.0 1.0 6.0
assign (resid  120 and  name   HN )(resid  119 and  name  HG# ) 0.0 1.0 6.0
assign (resid  120 and  name   HN )(resid  119 and  name   HN ) 0.0 0.0 3.3
assign (resid  120 and  name   HN )(resid  119 and  name   HA ) 0.0 0.0 3.3
assign (resid   21 and  name   HN )(resid   22 and  name  HD1 ) 0.0 0.0 5.0
assign (resid   21 and  name   HN )(resid   22 and  name  HZ2 ) 0.0 0.0 5.0
assign (resid   23 and  name  HD# )(resid   22 and  name   HA ) 0.0 2.0 7.0
assign (resid   23 and  name   HN )(resid   22 and  name   HN ) 0.0 0.0 2.8
assign (resid   23 and  name   HA )(resid   24 and  name  HD1 ) 0.0 0.0 5.0
assign (resid   44 and  name  HD# )(resid   45 and  name   HN ) 0.0 2.0 7.0
assign (resid   56 and  name   HA )(resid   57 and  name  HD# ) 0.0 2.0 4.8
assign (resid   57 and  name  HD# )(resid   58 and  name   HN ) 0.0 2.0 7.0
assign (resid   66 and  name  HD# )(resid   67 and  name   HN ) 0.0 1.0 4.3
assign (resid   73 and  name  HD# )(resid   74 and  name   HN ) 0.0 2.0 7.0
assign (resid   75 and  name   HN )(resid   74 and  name  HD2 ) 0.0 0.0 3.3
assign (resid   79 and  name  HD# )(resid   78 and  name   HA ) 0.0 2.0 4.8
```

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assign (resid   79 and   name  HE# )(resid   78 and   name   HA ) 0.0 2.0 5.3
assign (resid   79 and   name  HD# )(resid   80 and   name   HN ) 0.0 2.0 7.0
assign (resid   81 and   name  HD# )(resid   82 and   name   HN ) 0.0 2.0 7.0
assign (resid   87 and   name  HD# )(resid   86 and   name   HN ) 0.0 2.0 7.0
assign (resid  105 and   name  HD# )(resid  106 and   name   HN ) 0.0 2.0 5.3
assign (resid  105 and   name  HE# )(resid  106 and   name   HN ) 0.0 2.0 7.0
assign (resid  115 and   name  HD# )(resid  114 and   name HD1# ) 0.0 3.5 7.8
assign (resid  115 and   name  HE# )(resid  114 and   name HD1# ) 0.0 3.5 6.3
assign (resid  115 and   name  HD# )(resid  114 and   name HD2# ) 0.0 3.5 7.8
assign (resid  115 and   name  HE# )(resid  114 and   name HD2# ) 0.0 3.5 6.3
assign (resid  116 and   name  HD1 )(resid  115 and   name  HD# ) 0.0 2.0 5.3
assign (resid  116 and   name  HD2 )(resid  115 and   name  HD# ) 0.0 2.0 5.3
assign (resid  120 and   name  HD# )(resid  119 and   name   HN ) 0.0 2.0 7.0
assign (resid   24 and   name   HN )(resid   23 and   name  HD# ) 0.0 2.0 7.0
assign (resid   31 and   name   HN )(resid   32 and   name  HG# ) 0.0 2.4 7.4
assign (resid   35 and   name   HN )(resid   36 and   name  HD# ) 0.0 2.4 7.4
assign (resid   45 and   name   HN )(resid   44 and   name  HD1 ) 0.0 0.0 5.0
assign (resid   45 and   name   HN )(resid   44 and   name  HD2 ) 0.0 0.0 5.0
assign (resid   60 and   name   HN )(resid   59 and   name  HD# ) 0.0 2.4 7.4
assign (resid   88 and   name   HN )(resid   87 and   name  HD# ) 0.0 2.0 7.0
! SHORT RANGE RESTRAINTS FOR SH2 MODULE
assign (resid   20 and   name   HN )(resid   17 and   name  HB# ) 0.0 1.0 6.0
assign (resid   20 and   name  HB# )(resid   17 and   name   HA ) 0.0 1.5 4.3
assign (resid   20 and   name   HN )(resid   17 and   name   HA ) 0.0 0.0 3.3
assign (resid   20 and   name   HA )(resid   17 and   name  HD# ) 0.0 2.4 5.7
assign (resid   20 and   name  HB# )(resid   17 and   name  HD# ) 0.0 3.9 6.7
assign (resid   20 and   name   HN )(resid   18 and   name  HB# ) 0.0 1.0 6.0
assign (resid   20 and   name  HB# )(resid   18 and   name   HN ) 0.0 1.5 6.5
assign (resid   20 and   name   HN )(resid   18 and   name   HN ) 0.0 0.0 3.3
assign (resid   17 and   name  HB# )(resid   19 and   name   HA ) 0.0 1.5 6.5
assign (resid   22 and   name   HN )(resid   20 and   name  HB# ) 0.0 1.5 4.8
assign (resid   23 and   name   HA )(resid   20 and   name  HB# ) 0.0 1.5 6.5
assign (resid   23 and   name  HB1 )(resid   20 and   name  HB# ) 0.0 1.5 4.3
assign (resid   23 and   name  HB2 )(resid   20 and   name  HB# ) 0.0 1.5 4.3
assign (resid   23 and   name   HN )(resid   20 and   name  HB# ) 0.0 1.5 4.8
assign (resid   23 and   name   HN )(resid   21 and   name  HB# ) 0.0 1.0 6.0
assign (resid   23 and   name   HN )(resid   21 and   name   HA ) 0.0 0.0 5.0
assign (resid   23 and   name   HN )(resid   21 and   name  HG# ) 0.0 1.0 6.0
assign (resid   27 and   name  HD# )(resid   24 and   name   HA ) 0.0 1.5 6.5
assign (resid   27 and   name HG2# )(resid   24 and   name   HA ) 0.0 1.5 6.5
assign (resid   27 and   name  HD# )(resid   24 and   name  HB1 ) 0.0 1.5 4.8
assign (resid   27 and   name HG2# )(resid   24 and   name  HB1 ) 0.0 1.5 4.3
assign (resid   27 and   name  HD# )(resid   24 and   name  HB2 ) 0.0 1.5 4.8
assign (resid   27 and   name HG2# )(resid   24 and   name  HB2 ) 0.0 1.5 4.3
assign (resid   31 and   name   HN )(resid   27 and   name  HD# ) 0.0 1.5 6.5
assign (resid   31 and   name   HN )(resid   28 and   name   HN ) 0.0 0.0 5.0
assign (resid   31 and   name   HN )(resid   28 and   name   HA ) 0.0 0.0 5.0
assign (resid   31 and   name   HN )(resid   29 and   name   HA ) 0.0 0.0 5.0
assign (resid   31 and   name   HN )(resid   29 and   name   HN ) 0.0 0.0 5.0
assign (resid   32 and   name   HB )(resid   29 and   name   HN ) 0.0 0.0 5.0
assign (resid   32 and   name   HN )(resid   29 and   name   HN ) 0.0 0.0 5.0
assign (resid   32 and   name   HB )(resid   29 and   name   HA ) 0.0 0.0 2.8
assign (resid   32 and   name   HN )(resid   29 and   name   HA ) 0.0 0.0 5.0
assign (resid   33 and   name   HN )(resid   29 and   name   HA ) 0.0 0.0 2.8
assign (resid   32 and   name  HG# )(resid   30 and   name   HN ) 0.0 2.4 7.4
assign (resid   33 and   name  HB# )(resid   30 and   name   HA ) 0.0 1.0 3.8
assign (resid   33 and   name   HN )(resid   30 and   name   HA ) 0.0 0.0 5.0
assign (resid   32 and   name   HN )(resid   30 and   name   HN ) 0.0 0.0 5.0
assign (resid   34 and   name  HB# )(resid   31 and   name   HA ) 0.0 1.0 3.8
assign (resid   36 and   name   HN )(resid   32 and   name   HA ) 0.0 0.0 5.0
assign (resid   28 and   name   HN )(resid   32 and   name   HB ) 0.0 0.0 5.0
assign (resid   28 and   name   HN )(resid   32 and   name   HN ) 0.0 0.0 3.3
assign (resid   35 and   name  HB# )(resid   32 and   name   HA ) 0.0 1.0 4.3
assign (resid   35 and   name   HN )(resid   32 and   name   HA ) 0.0 0.0 5.0
```

p85α-N SH2 Restraints

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assign (resid  37 and  name   HN ) (resid  33 and  name   HA ) 0.0 0.0 5.0
assign (resid  36 and  name  HB# ) (resid  33 and  name   HA ) 0.0 1.0 4.3
assign (resid  36 and  name   HN ) (resid  33 and  name   HA ) 0.0 0.0 3.3
assign (resid  35 and  name   HN ) (resid  33 and  name   HN ) 0.0 0.0 5.0
assign (resid  37 and  name  HB# ) (resid  34 and  name   HA ) 0.0 1.0 4.3
assign (resid  37 and  name   HN ) (resid  34 and  name   HA ) 0.0 0.0 5.0
assign (resid  38 and  name   HN ) (resid  35 and  name  HD# ) 0.0 1.0 6.0
assign (resid  39 and  name HG2# ) (resid  36 and  name   HA ) 0.0 1.5 4.3
assign (resid  39 and  name   HN ) (resid  37 and  name   HA ) 0.0 0.0 5.0
assign (resid  43 and  name HG2# ) (resid  39 and  name HG2# ) 0.0 3.0 5.8
assign (resid  60 and  name   HA ) (resid  58 and  name HG2# ) 0.0 1.5 4.8
assign (resid  65 and  name   HN ) (resid  63 and  name  HB# ) 0.0 1.0 6.0
assign (resid  65 and  name   HN ) (resid  63 and  name   HN ) 0.0 0.0 5.0
assign (resid  66 and  name   HN ) (resid  63 and  name   HN ) 0.0 0.0 3.3
assign (resid  67 and  name   HA ) (resid  63 and  name   HN ) 0.0 0.0 3.3
assign (resid  66 and  name   HN ) (resid  64 and  name  HA1 ) 0.0 0.0 5.0
assign (resid  66 and  name   HN ) (resid  64 and  name  HA2 ) 0.0 0.0 5.0
assign (resid  66 and  name   HN ) (resid  64 and  name   HN ) 0.0 0.0 5.0
assign (resid  70 and  name  HD# ) (resid  68 and  name   HN ) 0.0 1.5 6.5
assign (resid  78 and  name   HN ) (resid  74 and  name   HA ) 0.0 0.0 5.0
assign (resid  82 and  name   HN ) (resid  80 and  name  HA1 ) 0.0 0.0 5.0
assign (resid  82 and  name   HN ) (resid  80 and  name  HA2 ) 0.0 0.0 5.0
assign (resid  87 and  name   HN ) (resid  85 and  name   HA ) 0.0 0.0 5.0
assign (resid  89 and  name   HN ) (resid  87 and  name   HA ) 0.0 0.0 5.0
assign (resid  92 and  name  HB# ) (resid  89 and  name   HN ) 0.0 1.0 6.0
assign (resid  92 and  name  HG# ) (resid  89 and  name   HN ) 0.0 1.0 6.0
assign (resid  91 and  name   HN ) (resid  89 and  name   HA ) 0.0 0.0 5.0
assign (resid  91 and  name   HN ) (resid  89 and  name  HB1 ) 0.0 0.0 5.0
assign (resid  91 and  name   HN ) (resid  89 and  name  HB2 ) 0.0 0.0 5.0
assign (resid  92 and  name   HN ) (resid  90 and  name   HB ) 0.0 0.0 5.0
assign (resid  93 and  name   HN ) (resid  90 and  name  HG# ) 0.0 2.4 7.4
assign (resid  94 and  name   HN ) (resid  90 and  name  HG# ) 0.0 2.4 5.7
assign (resid  92 and  name   HN ) (resid  90 and  name HG1# ) 0.0 1.5 6.5
assign (resid  92 and  name   HN ) (resid  90 and  name HG2# ) 0.0 1.5 6.5
assign (resid  92 and  name   HN ) (resid  90 and  name   HA ) 0.0 0.0 5.0
assign (resid  93 and  name  HB# ) (resid  90 and  name   HA ) 0.0 1.0 4.3
assign (resid  93 and  name HD1# ) (resid  90 and  name   HA ) 0.0 1.5 6.5
assign (resid  93 and  name HD2# ) (resid  90 and  name   HA ) 0.0 1.5 6.5
assign (resid  93 and  name   HN ) (resid  90 and  name   HA ) 0.0 0.0 3.3
assign (resid  94 and  name   HN ) (resid  90 and  name   HA ) 0.0 0.0 5.0
assign (resid  92 and  name   HN ) (resid  90 and  name   HN ) 0.0 0.0 5.0
assign (resid  93 and  name   HN ) (resid  91 and  name   HA ) 0.0 0.0 5.0
assign (resid  94 and  name   HB ) (resid  91 and  name   HA ) 0.0 0.0 3.3
assign (resid  94 and  name  HD# ) (resid  91 and  name   HA ) 0.0 1.5 6.5
assign (resid  94 and  name   HN ) (resid  91 and  name   HA ) 0.0 0.0 5.0
assign (resid  95 and  name   HN ) (resid  91 and  name   HA ) 0.0 0.0 5.0
assign (resid  94 and  name HG2# ) (resid  91 and  name HG1# ) 0.0 3.0 6.3
assign (resid  94 and  name HG2# ) (resid  91 and  name HG2# ) 0.0 3.0 6.3
assign (resid  95 and  name  HB# ) (resid  92 and  name   HA ) 0.0 1.0 4.3
assign (resid  95 and  name   HN ) (resid  92 and  name   HA ) 0.0 0.0 5.0
assign (resid  96 and  name   HN ) (resid  92 and  name   HA ) 0.0 0.0 5.0
assign (resid  95 and  name   HN ) (resid  93 and  name   HN ) 0.0 0.0 5.0
assign (resid  96 and  name   HN ) (resid  93 and  name   HA ) 0.0 0.0 3.3
assign (resid  97 and  name   HN ) (resid  94 and  name  HD# ) 0.0 1.5 6.5
assign (resid  97 and  name   HN ) (resid  94 and  name HG2# ) 0.0 1.5 6.5
assign (resid  97 and  name  HB# ) (resid  94 and  name   HA ) 0.0 1.0 6.0
assign (resid  97 and  name   HN ) (resid  94 and  name   HA ) 0.0 0.0 3.3
assign (resid  98 and  name  HB# ) (resid  94 and  name  HD# ) 0.0 2.5 5.8
assign (resid  98 and  name  HD1 ) (resid  94 and  name  HD# ) 0.0 1.5 4.8
assign (resid  98 and  name  HD2 ) (resid  94 and  name  HD# ) 0.0 1.5 4.8
assign (resid  98 and  name  HG# ) (resid  94 and  name  HD# ) 0.0 2.5 5.8
assign (resid  98 and  name   HN ) (resid  95 and  name   HN ) 0.0 0.0 5.0
assign (resid  97 and  name   HN ) (resid  95 and  name   HA ) 0.0 0.0 5.0
assign (resid  98 and  name  HB# ) (resid  95 and  name   HA ) 0.0 1.0 4.3
```

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assign (resid   98 and   name  HD1 )(resid   95 and   name   HA ) 0.0 0.0 5.0
assign (resid   98 and   name  HD2 )(resid   95 and   name   HA ) 0.0 0.0 5.0
assign (resid   98 and   name   HN )(resid   95 and   name   HA ) 0.0 0.0 5.0
assign (resid   97 and   name   HN )(resid   95 and   name   HN ) 0.0 0.0 5.0
assign (resid   98 and   name   HN )(resid   96 and   name  HB# ) 0.0 1.0 6.0
assign (resid   98 and   name   HN )(resid   96 and   name   HA ) 0.0 0.0 5.0
assign (resid   99 and   name  HB# )(resid   96 and   name   HA ) 0.0 1.0 6.0
assign (resid   98 and   name   HN )(resid   96 and   name   HN ) 0.0 0.0 5.0
assign (resid   99 and   name   HN )(resid   97 and   name   HA ) 0.0 0.0 5.0
assign (resid  100 and   name  HB# )(resid   97 and   name   HA ) 0.0 1.0 4.3
assign (resid  100 and   name   HN )(resid   97 and   name   HA ) 0.0 0.0 3.3
assign (resid   99 and   name   HN )(resid   97 and   name   HN ) 0.0 0.0 5.0
assign (resid  103 and   name   HN )(resid  101 and   name  HB1 ) 0.0 0.0 5.0
assign (resid  103 and   name   HN )(resid  101 and   name  HB2 ) 0.0 0.0 5.0
assign (resid  103 and   name   HN )(resid  101 and   name  HB# ) 0.0 1.0 4.3
assign (resid  104 and   name   HN )(resid  102 and   name   HA ) 0.0 0.0 5.0
assign (resid  105 and   name   HN )(resid  102 and   name HD1# ) 0.0 1.5 6.5
assign (resid  105 and   name   HN )(resid  102 and   name HD2# ) 0.0 1.5 6.5
assign (resid  106 and   name   HN )(resid  103 and   name   HA ) 0.0 0.0 5.0
assign (resid  109 and   name   HN )(resid  107 and   name  HB1 ) 0.0 0.0 5.0
assign (resid  109 and   name   HN )(resid  107 and   name  HB2 ) 0.0 0.0 5.0
assign (resid  106 and   name   HN )(resid  109 and   name  HD# ) 0.0 2.4 7.4
assign (resid   85 and   name  HD# )(resid   81 and   name   HN ) 0.0 2.4 7.4
assign (resid   85 and   name  HD# )(resid   82 and   name   HN ) 0.0 2.4 7.4
assign (resid   23 and   name  HD# )(resid   20 and   name  HB# ) 0.0 3.5 6.3
assign (resid   23 and   name  HE# )(resid   20 and   name  HB# ) 0.0 3.5 6.3
assign (resid   22 and   name  HB# )(resid   24 and   name  HE1 ) 0.0 1.0 6.0
assign (resid   66 and   name  HD# )(resid   63 and   name   HN ) 0.0 1.0 6.0
assign (resid   78 and   name   HN )(resid   74 and   name  HD2 ) 0.0 0.0 5.0
assign (resid   81 and   name  HD# )(resid   79 and   name  HD# ) 0.0 4.0 7.3
assign (resid   81 and   name  HD# )(resid   79 and   name  HE# ) 0.0 4.0 9.0
assign (resid   97 and   name  HD# )(resid   93 and   name   HA ) 0.0 2.0 7.0
assign (resid   93 and   name HD1# )(resid   97 and   name  HD# ) 0.0 3.5 6.3
assign (resid   93 and   name HD2# )(resid   97 and   name  HD# ) 0.0 3.5 6.3
assign (resid  101 and   name   HN )(resid   97 and   name  HD# ) 0.0 2.0 5.3
assign (resid   93 and   name HD1# )(resid   97 and   name  HE# ) 0.0 3.5 6.3
assign (resid   93 and   name HD2# )(resid   97 and   name  HE# ) 0.0 3.5 6.3
assign (resid  101 and   name   HN )(resid   97 and   name  HE# ) 0.0 2.0 5.3
assign (resid  105 and   name  HE# )(resid  102 and   name HD1# ) 0.0 3.5 6.8
assign (resid  105 and   name  HE# )(resid  102 and   name HD2# ) 0.0 3.5 6.8
assign (resid   20 and   name   HN )(resid   17 and   name  HD# ) 0.0 2.4 7.4
assign (resid   27 and   name   HN )(resid   25 and   name  HA2 ) 0.0 0.0 5.0
assign (resid   30 and   name   HN )(resid   28 and   name   HA ) 0.0 0.0 5.0
assign (resid   34 and   name   HN )(resid   32 and   name  HG# ) 0.0 2.4 7.4
assign (resid   35 and   name   HN )(resid   37 and   name   HN ) 0.0 0.0 5.0
assign (resid   37 and   name   HN )(resid   34 and   name  HB# ) 0.0 1.0 6.0
assign (resid   37 and   name   HN )(resid   34 and   name  HG# ) 0.0 1.0 6.0
assign (resid   37 and   name   HN )(resid   35 and   name  HD# ) 0.0 1.0 6.0
assign (resid   37 and   name   HN )(resid   39 and   name HG2# ) 0.0 1.5 6.5
assign (resid   39 and   name   HN )(resid   36 and   name  HB# ) 0.0 1.0 6.0
assign (resid   39 and   name   HN )(resid   36 and   name  HD# ) 0.0 2.4 7.4
assign (resid   54 and   name   HN )(resid   51 and   name   HB ) 0.0 0.0 5.0
assign (resid   63 and   name   HN )(resid   66 and   name  HB# ) 0.0 1.0 6.0
assign (resid   75 and   name   HN )(resid   73 and   name  HB# ) 0.0 1.0 6.0
assign (resid   75 and   name   HN )(resid   78 and   name   HN ) 0.0 0.0 5.0
assign (resid   75 and   name   HN )(resid   79 and   name   HA ) 0.0 0.0 5.0
assign (resid   77 and   name   HN )(resid   75 and   name  HB# ) 0.0 1.0 6.0
assign (resid   87 and   name   HN )(resid   83 and   name  HB# ) 0.0 1.0 6.0
assign (resid   87 and   name   HN )(resid   85 and   name  HD# ) 0.0 2.0 7.0
assign (resid   89 and   name   HN )(resid   87 and   name  HD# ) 0.0 2.0 7.0
assign (resid   89 and   name   HN )(resid   91 and   name   HN ) 0.0 0.0 5.0
assign (resid   92 and   name   HN )(resid   89 and   name  HB# ) 0.0 1.0 4.3
assign (resid   95 and   name   HN )(resid   93 and   name  HD# ) 0.0 2.4 7.4
assign (resid  106 and   name   HN )(resid  103 and   name  HB# ) 0.0 1.5 6.5
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assign (resid  106 and   name   HN )(resid  110 and   name   HA ) 0.0 0.0 5.0
! LONG RANGE RESTRAINTS FOR SH2 MODULE
assign (resid   90 and   name   HN )(resid   17 and   name HD1# ) 0.0 1.5 6.5
assign (resid   92 and   name   HN )(resid   17 and   name  HD# ) 0.0 2.4 7.4
assign (resid   90 and   name   HN )(resid   17 and   name HD2# ) 0.0 1.5 6.5
assign (resid   90 and   name   HN )(resid   17 and   name  HB# ) 0.0 1.0 4.3
assign (resid   23 and   name  HB1 )(resid   17 and   name HD1# ) 0.0 1.5 6.5
assign (resid   23 and   name  HB2 )(resid   17 and   name HD1# ) 0.0 1.5 6.5
assign (resid   91 and   name HG1# )(resid   17 and   name HD1# ) 0.0 3.0 6.3
assign (resid   91 and   name HG2# )(resid   17 and   name HD1# ) 0.0 3.0 6.3
assign (resid   23 and   name  HB1 )(resid   17 and   name HD2# ) 0.0 1.5 6.5
assign (resid   23 and   name  HB2 )(resid   17 and   name HD2# ) 0.0 1.5 6.5
assign (resid   91 and   name HG1# )(resid   17 and   name HD2# ) 0.0 3.0 8.0
assign (resid   91 and   name HG2# )(resid   17 and   name HD2# ) 0.0 3.0 8.0
assign (resid   91 and   name  HG# )(resid   17 and   name  HT# ) 0.0 2.4 5.7
assign (resid   91 and   name HG1# )(resid   20 and   name   HA ) 0.0 1.5 4.8
assign (resid   91 and   name HG2# )(resid   20 and   name   HA ) 0.0 1.5 4.8
assign (resid   91 and   name   HA )(resid   20 and   name  HB# ) 0.0 1.5 6.5
assign (resid   91 and   name HG1# )(resid   20 and   name  HB# ) 0.0 3.0 5.8
assign (resid   91 and   name HG2# )(resid   20 and   name  HB# ) 0.0 3.0 5.8
assign (resid   45 and   name   HA )(resid   22 and   name  HB1 ) 0.0 0.0 5.0
assign (resid   45 and   name   HA )(resid   22 and   name  HB2 ) 0.0 0.0 5.0
assign (resid   45 and   name   HA )(resid   22 and   name   HA ) 0.0 0.0 5.0
assign (resid   46 and   name   HB )(resid   23 and   name   HA ) 0.0 0.0 3.3
assign (resid   46 and   name HG1# )(resid   23 and   name   HA ) 0.0 1.5 4.8
assign (resid   46 and   name HG2# )(resid   23 and   name   HA ) 0.0 1.5 4.8
assign (resid   46 and   name   HN )(resid   23 and   name   HA ) 0.0 0.0 5.0
assign (resid   46 and   name  HG# )(resid   23 and   name   HN ) 0.0 2.4 7.4
assign (resid   48 and   name   HN )(resid   26 and   name   HA ) 0.0 0.0 3.3
assign (resid   46 and   name   HB )(resid   26 and   name   HA ) 0.0 0.0 5.0
assign (resid   32 and   name  HG# )(resid   27 and   name  HD# ) 0.0 3.9 8.9
assign (resid   32 and   name   HN )(resid   27 and   name  HD# ) 0.0 1.5 6.5
assign (resid   48 and   name   HN )(resid   27 and   name  HD# ) 0.0 1.5 6.5
assign (resid   32 and   name   HN )(resid   27 and   name HG11 ) 0.0 0.0 5.0
assign (resid   32 and   name   HN )(resid   27 and   name HG12 ) 0.0 0.0 5.0
assign (resid   47 and   name   HN )(resid   27 and   name   HN ) 0.0 0.0 5.0
assign (resid   48 and   name   HN )(resid   27 and   name   HN ) 0.0 0.0 5.0
assign (resid   32 and   name   HA )(resid   27 and   name  HD# ) 0.0 1.5 6.5
assign (resid   47 and   name   HA )(resid   27 and   name  HD# ) 0.0 1.5 4.8
assign (resid   32 and   name   HA )(resid   27 and   name HG2# ) 0.0 1.5 6.5
assign (resid   47 and   name   HA )(resid   27 and   name HG2# ) 0.0 1.5 6.5
assign (resid   45 and   name   HN )(resid   32 and   name  HG# ) 0.0 2.4 7.4
assign (resid   48 and   name   HN )(resid   32 and   name  HG# ) 0.0 2.4 7.4
assign (resid   60 and   name   HN )(resid   32 and   name  HG# ) 0.0 2.4 7.4
assign (resid   45 and   name HD1# )(resid   32 and   name   HN ) 0.0 1.5 6.5
assign (resid   45 and   name HD2# )(resid   32 and   name   HN ) 0.0 1.5 6.5
assign (resid   45 and   name HD1# )(resid   32 and   name   HA ) 0.0 1.5 4.3
assign (resid   45 and   name HD2# )(resid   32 and   name   HA ) 0.0 1.5 4.3
assign (resid   45 and   name HD1# )(resid   32 and   name   HB ) 0.0 1.5 6.5
assign (resid   45 and   name HD2# )(resid   32 and   name   HB ) 0.0 1.5 6.5
assign (resid   60 and   name   HA )(resid   32 and   name  HG# ) 0.0 2.4 7.4
assign (resid   45 and   name HD1# )(resid   32 and   name HG1# ) 0.0 3.0 5.8
assign (resid   45 and   name HD2# )(resid   32 and   name HG1# ) 0.0 3.0 5.8
assign (resid   60 and   name   HB )(resid   32 and   name HG1# ) 0.0 1.5 4.8
assign (resid   45 and   name HD1# )(resid   32 and   name HG2# ) 0.0 3.0 5.8
assign (resid   45 and   name HD2# )(resid   32 and   name HG2# ) 0.0 3.0 5.8
assign (resid   60 and   name   HB )(resid   32 and   name HG2# ) 0.0 1.5 4.8
assign (resid   45 and   name HD1# )(resid   33 and   name   HN ) 0.0 1.5 6.5
assign (resid   45 and   name HD2# )(resid   33 and   name   HN ) 0.0 1.5 6.5
assign (resid   60 and   name HG2# )(resid   33 and   name   HA ) 0.0 1.5 6.5
assign (resid   44 and   name   HN )(resid   36 and   name  HD# ) 0.0 2.4 7.4
assign (resid   43 and   name HG2# )(resid   36 and   name   HA ) 0.0 1.5 4.8
assign (resid   43 and   name   HB )(resid   36 and   name  HD# ) 0.0 2.4 5.2
assign (resid   43 and   name HG2# )(resid   36 and   name  HD# ) 0.0 3.9 6.7
```

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assign (resid  44 and name  HA ) (resid  36 and name  HD# ) 0.0 2.4 5.2
assign (resid  62 and name  HB1 ) (resid  42 and name  HN ) 0.0 0.0 5.0
assign (resid  62 and name  HB2 ) (resid  42 and name  HN ) 0.0 0.0 5.0
assign (resid  62 and name  HG# ) (resid  42 and name  HN ) 0.0 1.0 6.0
assign (resid  64 and name  HN ) (resid  42 and name  HN ) 0.0 0.0 5.0
assign (resid 115 and name  HN ) (resid  42 and name  HN ) 0.0 0.0 5.0
assign (resid  62 and name  HN ) (resid  43 and name  HA ) 0.0 0.0 5.0
assign (resid 115 and name  HN ) (resid  43 and name  HB ) 0.0 0.0 5.0
assign (resid  61 and name  HB1 ) (resid  43 and name HG2# ) 0.0 1.5 6.5
assign (resid  61 and name  HB2 ) (resid  43 and name  HN ) 0.0 0.0 5.0
assign (resid  62 and name  HB1 ) (resid  43 and name  HN ) 0.0 0.0 5.0
assign (resid  62 and name  HB2 ) (resid  43 and name  HN ) 0.0 0.0 5.0
assign (resid  62 and name  HG# ) (resid  43 and name  HN ) 0.0 1.0 4.3
assign (resid 115 and name  HB# ) (resid  43 and name  HN ) 0.0 1.0 6.0
assign (resid 115 and name  HA ) (resid  43 and name  HA ) 0.0 0.0 5.0
assign (resid 115 and name  HB# ) (resid  43 and name  HA ) 0.0 1.0 3.8
assign (resid 117 and name  HG# ) (resid  43 and name  HA ) 0.0 2.4 5.7
assign (resid  62 and name  HA ) (resid  43 and name  HB ) 0.0 0.0 5.0
assign (resid 117 and name  HA ) (resid  43 and name HG2# ) 0.0 1.5 4.8
assign (resid 117 and name  HB ) (resid  43 and name HG2# ) 0.0 1.5 4.8
assign (resid 117 and name  HG# ) (resid  43 and name HG2# ) 0.0 3.9 6.7
assign (resid  62 and name  HN ) (resid  43 and name  HN ) 0.0 0.0 3.3
assign (resid  62 and name  HN ) (resid  44 and name  HB# ) 0.0 1.0 6.0
assign (resid 115 and name  HB# ) (resid  44 and name  HN ) 0.0 1.0 6.0
assign (resid 115 and name  HN ) (resid  44 and name  HN ) 0.0 0.0 5.0
assign (resid 117 and name  HN ) (resid  44 and name  HN ) 0.0 0.0 5.0
assign (resid  60 and name  HN ) (resid  44 and name  HA ) 0.0 0.0 5.0
assign (resid  61 and name  HA ) (resid  44 and name  HA ) 0.0 0.0 2.8
assign (resid  61 and name  HB# ) (resid  44 and name  HA ) 0.0 1.0 4.3
assign (resid  61 and name HD1# ) (resid  44 and name  HA ) 0.0 1.5 6.5
assign (resid  61 and name HD2# ) (resid  44 and name  HA ) 0.0 1.5 6.5
assign (resid  62 and name  HN ) (resid  44 and name  HA ) 0.0 0.0 5.0
assign (resid  61 and name  HA ) (resid  44 and name  HB# ) 0.0 1.0 4.3
assign (resid 117 and name  HN ) (resid  45 and name  HA ) 0.0 0.0 5.0
assign (resid 117 and name  HN ) (resid  45 and name  HB# ) 0.0 1.0 4.3
assign (resid 117 and name  HN ) (resid  45 and name  HD# ) 0.0 2.4 7.4
assign (resid 117 and name  HN ) (resid  45 and name  HG ) 0.0 0.0 5.0
assign (resid  60 and name  HB ) (resid  45 and name  HN ) 0.0 0.0 5.0
assign (resid  60 and name HG2# ) (resid  45 and name  HN ) 0.0 1.5 6.5
assign (resid 116 and name  HA ) (resid  45 and name  HA ) 0.0 0.0 5.0
assign (resid  60 and name  HB ) (resid  45 and name HD1# ) 0.0 1.5 6.5
assign (resid  60 and name HG2# ) (resid  45 and name HD1# ) 0.0 3.0 5.8
assign (resid  60 and name  HB ) (resid  45 and name HD2# ) 0.0 1.5 6.5
assign (resid  60 and name HG2# ) (resid  45 and name HD2# ) 0.0 3.0 5.8
assign (resid  57 and name  HN ) (resid  46 and name HG1# ) 0.0 1.5 6.5
assign (resid  57 and name  HN ) (resid  46 and name HG2# ) 0.0 1.5 6.5
assign (resid  59 and name  HA ) (resid  46 and name  HA ) 0.0 0.0 2.8
assign (resid  60 and name  HN ) (resid  46 and name  HA ) 0.0 0.0 3.5
assign (resid  59 and name  HA ) (resid  46 and name HG1# ) 0.0 1.5 6.5
assign (resid  59 and name  HA ) (resid  46 and name HG2# ) 0.0 1.5 6.5
assign (resid  60 and name  HB ) (resid  47 and name  HA ) 0.0 0.0 5.0
assign (resid  58 and name  HN ) (resid  47 and name  HN ) 0.0 0.0 3.3
assign (resid  58 and name  HB ) (resid  48 and name  HA ) 0.0 0.0 5.0
assign (resid  57 and name  HB1 ) (resid  49 and name  HN ) 0.0 0.0 5.0
assign (resid  57 and name  HB2 ) (resid  49 and name  HN ) 0.0 0.0 5.0
assign (resid  58 and name  HN ) (resid  49 and name  HB# ) 0.0 1.5 6.5
assign (resid  57 and name  HN ) (resid  49 and name  HN ) 0.0 0.0 5.0
assign (resid  58 and name  HN ) (resid  49 and name  HN ) 0.0 0.0 5.0
assign (resid  72 and name  HN ) (resid  57 and name  HB# ) 0.0 1.0 6.0
assign (resid  72 and name  HB ) (resid  57 and name  HN ) 0.0 0.0 3.3
assign (resid  72 and name  HN ) (resid  57 and name  HN ) 0.0 0.0 5.0
assign (resid  72 and name  HB ) (resid  58 and name  HN ) 0.0 0.0 5.0
assign (resid  72 and name HG11 ) (resid  58 and name  HN ) 0.0 0.0 5.0
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assign (resid  72 and  name HG12 )(resid  58 and  name  HN ) 0.0 0.0 5.0
assign (resid  72 and  name HG2# )(resid  58 and  name  HN ) 0.0 1.5 6.5
assign (resid  69 and  name  HD# )(resid  58 and  name  HA ) 0.0 2.4 7.4
assign (resid  69 and  name   HG )(resid  58 and  name  HA ) 0.0 0.0 5.0
assign (resid  71 and  name   HA )(resid  58 and  name  HA ) 0.0 0.0 2.8
assign (resid  70 and  name   HN )(resid  59 and  name HB# ) 0.0 1.0 6.0
assign (resid  70 and  name   HN )(resid  59 and  name HD# ) 0.0 2.4 7.4
assign (resid  71 and  name   HA )(resid  59 and  name  HN ) 0.0 0.0 5.0
assign (resid  72 and  name   HN )(resid  59 and  name  HN ) 0.0 0.0 5.0
assign (resid  70 and  name  HD# )(resid  59 and  name  HA ) 0.0 1.5 6.5
assign (resid  70 and  name HG2# )(resid  59 and  name  HA ) 0.0 1.5 6.5
assign (resid  72 and  name   HA )(resid  59 and  name HD1# ) 0.0 1.5 6.5
assign (resid  72 and  name   HA )(resid  59 and  name HD2# ) 0.0 1.5 6.5
assign (resid  70 and  name   HN )(resid  60 and  name  HB ) 0.0 0.0 5.0
assign (resid  68 and  name   HN )(resid  60 and  name HG2# ) 0.0 1.5 4.8
assign (resid  68 and  name   HN )(resid  60 and  name  HA ) 0.0 0.0 5.0
assign (resid  69 and  name   HA )(resid  60 and  name  HA ) 0.0 0.0 2.8
assign (resid  69 and  name  HB# )(resid  60 and  name  HA ) 0.0 1.0 6.0
assign (resid  69 and  name   HG )(resid  60 and  name  HA ) 0.0 0.0 5.0
assign (resid  70 and  name   HN )(resid  60 and  name  HA ) 0.0 0.0 5.0
assign (resid  67 and  name   HA )(resid  60 and  name HG2# ) 0.0 1.5 4.3
assign (resid  32 and  name  HG# )(resid  61 and  name  HN ) 0.0 2.4 7.4
assign (resid  68 and  name  HB# )(resid  61 and  name  HN ) 0.0 1.0 6.0
assign (resid  68 and  name  HD1 )(resid  61 and  name  HN ) 0.0 0.0 5.0
assign (resid  68 and  name  HD2 )(resid  61 and  name  HN ) 0.0 0.0 5.0
assign (resid  67 and  name   HA )(resid  61 and  name  HN ) 0.0 0.0 5.0
assign (resid  68 and  name   HN )(resid  61 and  name  HN ) 0.0 0.0 3.3
assign (resid  67 and  name   HA )(resid  62 and  name  HN ) 0.0 0.0 5.0
assign (resid  67 and  name   HA )(resid  62 and  name  HA ) 0.0 0.0 2.8
assign (resid  68 and  name   HN )(resid  62 and  name  HA ) 0.0 0.0 3.3
assign (resid  67 and  name   HA )(resid  62 and  name HD1 ) 0.0 0.0 3.3
assign (resid  67 and  name   HA )(resid  62 and  name HD2 ) 0.0 0.0 3.3
assign (resid  67 and  name   HA )(resid  62 and  name HG# ) 0.0 1.0 6.0
assign (resid  68 and  name   HN )(resid  63 and  name HD1 ) 0.0 0.0 5.0
assign (resid  68 and  name   HN )(resid  63 and  name HD2 ) 0.0 0.0 5.0
assign (resid  68 and  name   HN )(resid  63 and  name  HN ) 0.0 0.0 3.3
assign (resid  80 and  name   HN )(resid  72 and  name  HA ) 0.0 0.0 5.0
assign (resid  81 and  name   HA )(resid  72 and  name  HA ) 0.0 0.0 5.0
assign (resid  80 and  name   HN )(resid  72 and  name HG2# ) 0.0 1.5 4.8
assign (resid  85 and  name HD1# )(resid  73 and  name  HA ) 0.0 1.5 6.5
assign (resid  85 and  name HD2# )(resid  73 and  name  HA ) 0.0 1.5 6.5
assign (resid  81 and  name   HA )(resid  73 and  name  HN ) 0.0 0.0 5.0
assign (resid  85 and  name HD1# )(resid  73 and  name  HN ) 0.0 1.5 6.5
assign (resid  85 and  name HD2# )(resid  73 and  name  HN ) 0.0 1.5 6.5
assign (resid  80 and  name   HN )(resid  73 and  name  HN ) 0.0 0.0 5.0
assign (resid  79 and  name   HA )(resid  74 and  name  HA ) 0.0 0.0 2.8
assign (resid  80 and  name   HN )(resid  74 and  name  HA ) 0.0 0.0 5.0
assign (resid  85 and  name HD1# )(resid  74 and  name  HA ) 0.0 1.5 4.8
assign (resid  85 and  name HD2# )(resid  74 and  name  HA ) 0.0 1.5 4.8
assign (resid  88 and  name   HA )(resid  78 and  name  HA ) 0.0 0.0 5.0
assign (resid  88 and  name   HA )(resid  78 and  name HB1 ) 0.0 0.0 2.8
assign (resid  88 and  name   HA )(resid  78 and  name HB2 ) 0.0 0.0 3.3
assign (resid  85 and  name HD1# )(resid  79 and  name  HN ) 0.0 1.5 6.5
assign (resid  85 and  name HD2# )(resid  79 and  name  HN ) 0.0 1.5 6.5
assign (resid  85 and  name   HG )(resid  79 and  name  HN ) 0.0 0.0 5.0
assign (resid  88 and  name  HB# )(resid  79 and  name  HN ) 0.0 1.0 6.0
assign (resid  85 and  name HD1# )(resid  79 and  name  HA ) 0.0 1.5 6.5
assign (resid  85 and  name HD2# )(resid  79 and  name  HA ) 0.0 1.5 6.5
assign (resid  87 and  name   HN )(resid  79 and  name  HN ) 0.0 0.0 5.0
assign (resid  88 and  name   HA )(resid  79 and  name  HN ) 0.0 0.0 5.0
assign (resid  93 and  name  HD# )(resid  80 and  name  HN ) 0.0 2.4 7.4
assign (resid  85 and  name   HG )(resid  80 and  name  HN ) 0.0 0.0 5.0
assign (resid  93 and  name HD1# )(resid  81 and  name  HN ) 0.0 1.5 6.5
assign (resid  93 and  name HD2# )(resid  81 and  name  HN ) 0.0 1.5 6.5
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assign (resid   88 and   name   HN )(resid   83 and   name  HB# ) 0.0 1.0 6.0
assign (resid  102 and   name HD1# )(resid   94 and   name   HA ) 0.0 1.5 6.5
assign (resid  102 and   name HD2# )(resid   94 and   name   HA ) 0.0 1.5 6.5
assign (resid  102 and   name HD1# )(resid   94 and   name  HD# ) 0.0 3.0 5.8
assign (resid  102 and   name HD2# )(resid   94 and   name  HD# ) 0.0 3.0 5.8
assign (resid  102 and   name  HD# )(resid   97 and   name   HA ) 0.0 2.4 5.7
assign (resid  102 and   name  HD# )(resid   97 and   name  HB# ) 0.0 3.4 6.7
assign (resid  114 and   name HD1# )(resid  100 and   name   HN ) 0.0 1.5 6.5
assign (resid  114 and   name HD2# )(resid  100 and   name   HN ) 0.0 1.5 6.5
assign (resid  113 and   name  HD# )(resid  101 and   name   HN ) 0.0 2.4 7.4
assign (resid  112 and   name   HA )(resid  101 and   name   HA ) 0.0 0.0 2.8
assign (resid  111 and   name   HN )(resid  102 and   name   HN ) 0.0 0.0 5.0
assign (resid  111 and   name   HN )(resid  103 and   name  HB1 ) 0.0 1.5 6.5
assign (resid  111 and   name   HN )(resid  103 and   name  HB2 ) 0.0 1.5 6.5
assign (resid  110 and   name  HB1 )(resid  103 and   name   HN ) 0.0 0.0 5.0
assign (resid  110 and   name  HB2 )(resid  103 and   name   HN ) 0.0 0.0 5.0
assign (resid  111 and   name   HN )(resid  103 and   name   HN ) 0.0 0.0 5.0
assign (resid  110 and   name   HA )(resid  103 and   name   HA ) 0.0 0.0 3.3
assign (resid   44 and   name   HN )(resid  116 and   name   HA ) 0.0 0.0 3.3
assign (resid   44 and   name   HN )(resid  117 and   name HG1# ) 0.0 1.5 6.5
assign (resid   44 and   name   HN )(resid  117 and   name HG2# ) 0.0 1.5 6.5
assign (resid   85 and   name  HD# )(resid   78 and   name   HN ) 0.0 2.4 7.4
assign (resid  120 and   name  HD# )(resid   21 and   name   HN ) 0.0 2.0 7.0
assign (resid  120 and   name  HE# )(resid   21 and   name   HN ) 0.0 2.0 7.0
assign (resid   17 and   name HD1# )(resid   22 and   name  CZ3 ) 0.0 5.0 8.3
assign (resid   17 and   name HD2# )(resid   22 and   name  CZ3 ) 0.0 5.0 8.3
assign (resid   90 and   name   HA )(resid   22 and   name  CZ3 ) 0.0 2.5 7.5
assign (resid   90 and   name HG1# )(resid   22 and   name  CZ3 ) 0.0 5.0 8.3
assign (resid   90 and   name HG2# )(resid   22 and   name  CZ3 ) 0.0 5.0 8.3
assign (resid   91 and   name   HA )(resid   22 and   name  CZ3 ) 0.0 2.5 5.3
assign (resid   91 and   name HG1# )(resid   22 and   name  CZ3 ) 0.0 5.0 8.3
assign (resid   91 and   name HG2# )(resid   22 and   name  CZ3 ) 0.0 5.0 8.3
assign (resid   94 and   name   HA )(resid   22 and   name  CZ3 ) 0.0 2.5 7.5
assign (resid   94 and   name  HD# )(resid   22 and   name  CZ3 ) 0.0 5.0 8.3
assign (resid   94 and   name HG2# )(resid   22 and   name  CZ3 ) 0.0 5.0 7.8
assign (resid   44 and   name  HD2 )(resid   22 and   name  HD1 ) 0.0 0.0 5.0
assign (resid   44 and   name  HE2 )(resid   22 and   name  HD1 ) 0.0 0.0 3.3
assign (resid   44 and   name   HZ )(resid   22 and   name  HD1 ) 0.0 0.0 2.8
assign (resid   44 and   name   HZ )(resid   22 and   name  HE1 ) 0.0 0.0 5.0
assign (resid   91 and   name  HG# )(resid   22 and   name  HE1 ) 0.0 2.4 7.4
assign (resid   94 and   name  HD# )(resid   22 and   name  HE1 ) 0.0 1.5 6.5
assign (resid   94 and   name HG2# )(resid   22 and   name  HE1 ) 0.0 1.5 4.8
assign (resid   91 and   name   HA )(resid   22 and   name  HZ2 ) 0.0 0.0 3.3
assign (resid   17 and   name HD1# )(resid   23 and   name  HE# ) 0.0 3.5 8.5
assign (resid   17 and   name HD2# )(resid   23 and   name  HE# ) 0.0 3.5 8.5
assign (resid   45 and   name   HN )(resid   23 and   name  HE# ) 0.0 2.0 7.0
assign (resid   46 and   name   HN )(resid   23 and   name  HE# ) 0.0 2.0 7.0
assign (resid   90 and   name HG1# )(resid   23 and   name  HE# ) 0.0 3.5 6.8
assign (resid   90 and   name HG2# )(resid   23 and   name  HE# ) 0.0 3.5 6.8
assign (resid   91 and   name  HG# )(resid   23 and   name  HE# ) 0.0 4.4 9.4
assign (resid   94 and   name  HD# )(resid   23 and   name  HE# ) 0.0 3.5 8.5
assign (resid   94 and   name HG2# )(resid   23 and   name  HE# ) 0.0 3.5 8.5
assign (resid   46 and   name   HA )(resid   23 and   name  HD# ) 0.0 2.0 7.0
assign (resid   46 and   name   HB )(resid   23 and   name  HD# ) 0.0 2.0 5.3
assign (resid   46 and   name HG1# )(resid   23 and   name  HD# ) 0.0 3.5 6.8
assign (resid   46 and   name HG2# )(resid   23 and   name  HD# ) 0.0 3.5 6.8
assign (resid   46 and   name   HA )(resid   23 and   name  HE# ) 0.0 2.0 7.0
assign (resid   46 and   name   HB )(resid   23 and   name  HE# ) 0.0 2.0 5.3
assign (resid   46 and   name HG1# )(resid   23 and   name  HE# ) 0.0 3.5 6.8
assign (resid   46 and   name HG2# )(resid   23 and   name  HE# ) 0.0 3.5 6.8
assign (resid  117 and   name   HA )(resid   24 and   name  HE1 ) 0.0 0.0 5.0
assign (resid  117 and   name   HB )(resid   24 and   name  HE1 ) 0.0 0.0 3.3
assign (resid  117 and   name  HG# )(resid   24 and   name  HE1 ) 0.0 2.4 5.2
assign (resid  118 and   name   HA )(resid   24 and   name  HE1 ) 0.0 0.0 3.3
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assign (resid  119 and  name  HG# )(resid   24 and  name  HE1 ) 0.0 1.0 6.0
assign (resid   45 and  name   HA )(resid   24 and  name  HE1 ) 0.0 0.0 5.0
assign (resid   45 and  name  HB# )(resid   24 and  name  HE1 ) 0.0 0.0 3.3
assign (resid   45 and  name HD1# )(resid   24 and  name  HE1 ) 0.0 1.5 6.5
assign (resid   45 and  name HD2# )(resid   24 and  name  HE1 ) 0.0 1.5 6.5
assign (resid   45 and  name   HG )(resid   24 and  name  HE1 ) 0.0 0.0 5.0
assign (resid   45 and  name  HD# )(resid   24 and  name  HZ2 ) 0.0 2.4 5.7
assign (resid  115 and  name  HD# )(resid   40 and  name  HB# ) 0.0 3.5 6.3
assign (resid  115 and  name  HE# )(resid   40 and  name  HB# ) 0.0 3.5 6.3
assign (resid  115 and  name  HD# )(resid   43 and  name   HA ) 0.0 2.0 4.8
assign (resid  115 and  name  HE# )(resid   43 and  name   HA ) 0.0 2.0 7.0
assign (resid  115 and  name  HD# )(resid   43 and  name HG2# ) 0.0 3.5 6.8
assign (resid  114 and  name   HN )(resid   44 and  name  HD# ) 0.0 2.0 7.0
assign (resid  115 and  name   HN )(resid   44 and  name  HD# ) 0.0 1.0 6.0
assign (resid  117 and  name   HN )(resid   44 and  name  HD# ) 0.0 2.0 7.0
assign (resid  113 and  name  HD# )(resid   44 and  name  HD2 ) 0.0 2.4 5.7
assign (resid  116 and  name   HA )(resid   44 and  name  HD2 ) 0.0 0.0 5.0
assign (resid  115 and  name   HN )(resid   44 and  name  HE# ) 0.0 2.0 7.0
assign (resid  113 and  name  HD# )(resid   44 and  name  HE1 ) 0.0 2.4 5.7
assign (resid  114 and  name   HA )(resid   44 and  name  HE1 ) 0.0 0.0 3.3
assign (resid  116 and  name   HA )(resid   44 and  name  HE1 ) 0.0 0.0 3.3
assign (resid  113 and  name HD1# )(resid   44 and  name  HE2 ) 0.0 1.5 4.8
assign (resid  113 and  name HD2# )(resid   44 and  name  HE2 ) 0.0 1.5 4.8
assign (resid  116 and  name   HA )(resid   44 and  name  HE2 ) 0.0 0.0 5.0
assign (resid  113 and  name  HD# )(resid   44 and  name   HZ ) 0.0 2.4 5.2
assign (resid  114 and  name   HA )(resid   44 and  name   HZ ) 0.0 0.0 5.0
assign (resid  116 and  name   HA )(resid   44 and  name   HZ ) 0.0 0.0 5.0
assign (resid  116 and  name   HA )(resid   44 and  name  HD1 ) 0.0 0.0 5.0
assign (resid   94 and  name  HD# )(resid   44 and  name  HE2 ) 0.0 1.5 4.8
assign (resid   94 and  name  HD# )(resid   44 and  name   HZ ) 0.0 1.5 4.8
assign (resid  116 and  name  HB1 )(resid   44 and  name   HZ ) 0.0 0.0 5.0
assign (resid  116 and  name  HB2 )(resid   44 and  name   HZ ) 0.0 0.0 5.0
assign (resid  116 and  name  HG1 )(resid   44 and  name   HZ ) 0.0 0.0 5.0
assign (resid  116 and  name  HG2 )(resid   44 and  name   HZ ) 0.0 0.0 5.0
assign (resid   23 and  name  HD# )(resid   45 and  name   HA ) 0.0 2.0 5.3
assign (resid   57 and  name  HD# )(resid   46 and  name HG1# ) 0.0 3.5 6.3
assign (resid   57 and  name  HE# )(resid   46 and  name HG1# ) 0.0 3.5 6.3
assign (resid   57 and  name  HD# )(resid   46 and  name HG2# ) 0.0 3.5 6.3
assign (resid   57 and  name  HE# )(resid   46 and  name HG2# ) 0.0 3.5 6.3
assign (resid   57 and  name  HD# )(resid   48 and  name   HA ) 0.0 2.0 4.8
assign (resid   57 and  name  HE# )(resid   48 and  name   HA ) 0.0 2.0 4.8
assign (resid   57 and  name  HD# )(resid   48 and  name  HB# ) 0.0 3.0 6.3
assign (resid   79 and  name  HD# )(resid   57 and  name   HN ) 0.0 2.0 7.0
assign (resid   49 and  name  HB# )(resid   57 and  name  HD# ) 0.0 3.5 6.3
assign (resid   49 and  name  HB# )(resid   57 and  name  HE# ) 0.0 3.5 6.8
assign (resid   49 and  name   HN )(resid   57 and  name  HE# ) 0.0 2.0 5.3
assign (resid   81 and  name  HD# )(resid   59 and  name HD1# ) 0.0 3.5 6.3
assign (resid   81 and  name  HE# )(resid   59 and  name HD1# ) 0.0 3.5 6.3
assign (resid   81 and  name   HZ )(resid   59 and  name HD1# ) 0.0 1.5 4.8
assign (resid   81 and  name  HD# )(resid   59 and  name HD2# ) 0.0 3.5 6.3
assign (resid   81 and  name  HE# )(resid   59 and  name HD2# ) 0.0 3.5 6.3
assign (resid   81 and  name   HZ )(resid   59 and  name HD2# ) 0.0 1.5 4.8
assign (resid   81 and  name  HD# )(resid   70 and  name HG2# ) 0.0 3.5 6.8
assign (resid   81 and  name  HE# )(resid   70 and  name HG2# ) 0.0 3.5 6.8
assign (resid   81 and  name   HZ )(resid   70 and  name HG2# ) 0.0 1.5 4.8
assign (resid  105 and  name  HD# )(resid   70 and  name HG2# ) 0.0 3.5 6.8
assign (resid  105 and  name  HE# )(resid   70 and  name HG2# ) 0.0 3.5 6.3
assign (resid   81 and  name  HD# )(resid   72 and  name   HA ) 0.0 2.0 4.8
assign (resid   81 and  name  HE# )(resid   72 and  name   HA ) 0.0 2.0 5.3
assign (resid   81 and  name   HZ )(resid   72 and  name   HA ) 0.0 0.0 5.0
assign (resid   81 and  name  HE# )(resid   73 and  name   HZ ) 0.0 2.0 4.8
assign (resid   81 and  name   HZ )(resid   73 and  name   HZ ) 0.0 0.0 5.0
assign (resid   85 and  name HD1# )(resid   73 and  name  HD# ) 0.0 3.5 6.8
assign (resid   85 and  name HD2# )(resid   73 and  name  HD# ) 0.0 3.5 6.8
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assign (resid  79 and  name  HD# )(resid  74 and  name   HA ) 0.0 2.0 4.8
assign (resid  79 and  name  HE# )(resid  74 and  name   HA ) 0.0 2.0 7.0
assign (resid  79 and  name  HD# )(resid  74 and  name  HB# ) 0.0 3.0 5.8
assign (resid  79 and  name  HE# )(resid  74 and  name  HB# ) 0.0 3.0 6.3
assign (resid  79 and  name  HD# )(resid  74 and  name  HD2 ) 0.0 2.0 5.3
assign (resid  79 and  name  HE# )(resid  74 and  name  HD2 ) 0.0 2.0 5.3
assign (resid  89 and  name   HA )(resid  79 and  name  HD# ) 0.0 2.0 5.3
assign (resid  90 and  name HG1# )(resid  79 and  name  HD# ) 0.0 3.5 6.8
assign (resid  90 and  name HG2# )(resid  79 and  name  HD# ) 0.0 3.5 6.8
assign (resid  90 and  name   HN )(resid  79 and  name  HD# ) 0.0 2.0 5.3
assign (resid  89 and  name   HA )(resid  79 and  name  HE# ) 0.0 2.0 5.3
assign (resid  90 and  name  HG# )(resid  79 and  name  HE# ) 0.0 4.4 7.2
assign (resid  90 and  name   HN )(resid  79 and  name  HE# ) 0.0 2.0 5.3
assign (resid  72 and  name  HD# )(resid  79 and  name  HD# ) 0.0 3.5 6.8
assign (resid  72 and  name HG2# )(resid  79 and  name  HD# ) 0.0 3.5 6.8
assign (resid  72 and  name  HD# )(resid  79 and  name  HE# ) 0.0 3.5 6.3
assign (resid  72 and  name HG2# )(resid  79 and  name  HE# ) 0.0 3.5 6.8
assign (resid  73 and  name  HD# )(resid  81 and  name   HA ) 0.0 2.0 5.3
assign (resid  93 and  name   HG )(resid  81 and  name  HD# ) 0.0 2.0 7.0
assign (resid  80 and  name   HN )(resid  87 and  name  HD# ) 0.0 2.0 7.0
assign (resid  80 and  name   HN )(resid  87 and  name  HE# ) 0.0 2.0 7.0
assign (resid  79 and  name   HN )(resid  87 and  name  HD# ) 0.0 2.0 5.3
assign (resid  79 and  name   HN )(resid  87 and  name  HE# ) 0.0 2.0 5.3
assign (resid  79 and  name   HN )(resid  87 and  name   HZ ) 0.0 0.0 5.0
assign (resid  81 and  name  HD# )(resid  93 and  name HD1# ) 0.0 3.5 6.3
assign (resid  81 and  name  HE# )(resid  93 and  name HD1# ) 0.0 3.5 6.8
assign (resid  81 and  name   HZ )(resid  93 and  name  HD# ) 0.0 2.4 7.4
assign (resid  81 and  name  HD# )(resid  93 and  name HD2# ) 0.0 3.5 6.3
assign (resid  81 and  name  HE# )(resid  93 and  name HD2# ) 0.0 3.5 6.8
assign (resid  97 and  name  HD# )(resid 102 and  name HD1# ) 0.0 3.5 6.3
assign (resid  97 and  name  HE# )(resid 102 and  name HD1# ) 0.0 3.5 6.3
assign (resid  97 and  name  HD# )(resid 102 and  name HD2# ) 0.0 3.5 6.3
assign (resid  97 and  name  HE# )(resid 102 and  name HD2# ) 0.0 3.5 6.3
assign (resid  97 and  name  HD# )(resid 113 and  name   HN ) 0.0 2.0 7.0
assign (resid  27 and  name   HN )(resid  47 and  name  HG# ) 0.0 1.0 6.0
assign (resid  27 and  name   HN )(resid  48 and  name  HB# ) 0.0 1.0 6.0
assign (resid  33 and  name   HN )(resid  27 and  name  HD# ) 0.0 1.5 6.5
assign (resid  35 and  name   HN )(resid  45 and  name  HD# ) 0.0 2.4 7.4
assign (resid  45 and  name   HN )(resid  60 and  name   HN ) 0.0 0.0 5.0
assign (resid  45 and  name   HN )(resid  61 and  name   HA ) 0.0 0.0 5.0
assign (resid  46 and  name   HN )(resid  23 and  name  HD# ) 0.0 2.0 7.0
assign (resid  49 and  name   HN )(resid  57 and  name   HA ) 0.0 0.0 3.3
assign (resid  49 and  name   HN )(resid  57 and  name  HB# ) 0.0 1.0 6.0
assign (resid  49 and  name   HN )(resid  57 and  name  HD# ) 0.0 2.0 7.0
assign (resid  56 and  name   HN )(resid  49 and  name  HB# ) 0.0 1.5 6.5
assign (resid  59 and  name   HN )(resid  71 and  name   HN ) 0.0 0.0 5.0
assign (resid  60 and  name   HN )(resid  46 and  name   HN ) 0.0 0.0 5.0
assign (resid  61 and  name   HN )(resid  68 and  name  HB1 ) 0.0 0.0 5.0
assign (resid  61 and  name   HN )(resid  68 and  name  HB2 ) 0.0 0.0 5.0
assign (resid  62 and  name   HN )(resid  44 and  name   HN ) 0.0 0.0 5.0
assign (resid  63 and  name   HN )(resid  68 and  name  HG1 ) 0.0 0.0 5.0
assign (resid  72 and  name   HN )(resid  57 and  name  HD# ) 0.0 2.0 7.0
assign (resid  73 and  name   HN )(resid  85 and  name  HD# ) 0.0 2.4 7.4
assign (resid  74 and  name   HN )(resid  85 and  name  HD# ) 0.0 2.4 7.4
assign (resid  75 and  name   HN )(resid  85 and  name  HD# ) 0.0 2.4 5.7
assign (resid  79 and  name   HN )(resid  72 and  name HG2# ) 0.0 1.5 6.5
assign (resid  80 and  name   HN )(resid  85 and  name HD1# ) 0.0 1.5 4.8
assign (resid  80 and  name   HN )(resid  85 and  name HD2# ) 0.0 1.5 4.8
assign (resid  80 and  name   HN )(resid  73 and  name  HB1 ) 0.0 0.0 5.0
assign (resid  80 and  name   HN )(resid  73 and  name  HB2 ) 0.0 0.0 5.0
assign (resid  80 and  name   HN )(resid  85 and  name  HB# ) 0.0 1.0 6.0
assign (resid  87 and  name   HN )(resid  78 and  name  HB1 ) 0.0 0.0 5.0
assign (resid  87 and  name   HN )(resid  78 and  name  HB2 ) 0.0 0.0 5.0
assign (resid 101 and  name   HN )(resid 114 and  name  HD# ) 0.0 2.4 7.4
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assign (resid  102 and   name   HN ) (resid  114 and   name  HD# ) 0.0 2.4 7.4
assign (resid  111 and   name   HN ) (resid  102 and   name  HB# ) 0.0 1.0 6.0
assign (resid  111 and   name   HN ) (resid  103 and   name   HA ) 0.0 0.0 5.0
assign (resid  115 and   name   HN ) (resid   44 and   name  HD1 ) 0.0 0.0 5.0
assign (resid  115 and   name   HN ) (resid   44 and   name  HD2 ) 0.0 0.0 5.0
assign (resid  117 and   name   HN ) (resid   43 and   name HG2# ) 0.0 1.5 6.5
assign (resid  120 and   name   HN ) (resid   21 and   name  HG# ) 0.0 1.0 6.0
! HYDROGEN BOND RESTRAINTS FOR SH2 MODULE
! H-BONDS
! 21-23
assign (resid   23 and   name    N ) (resid   21 and   name     O ) 0.0 0.0 3.3
assign (resid   23 and   name   HN ) (resid   21 and   name     O ) 0.0 0.0 2.3
! 28-31
assign (resid   31 and   name    N ) (resid   28 and   name     O ) 0.0 0.0 3.3
assign (resid   31 and   name   HN ) (resid   28 and   name     O ) 0.0 0.0 2.3
! 28-32
assign (resid   32 and   name    N ) (resid   28 and   name     O ) 0.0 0.0 3.3
assign (resid   32 and   name   HN ) (resid   28 and   name     O ) 0.0 0.0 2.3
! 29-33
assign (resid   33 and   name    N ) (resid   29 and   name     O ) 0.0 0.0 3.3
assign (resid   33 and   name   HN ) (resid   29 and   name     O ) 0.0 0.0 2.3
! 30-34
assign (resid   34 and   name    N ) (resid   30 and   name     O ) 0.0 0.0 3.3
assign (resid   34 and   name   HN ) (resid   30 and   name     O ) 0.0 0.0 2.3
! 31-35
assign (resid   35 and   name    N ) (resid   31 and   name     O ) 0.0 0.0 3.3
assign (resid   35 and   name   HN ) (resid   31 and   name     O ) 0.0 0.0 2.3
! 32-36
assign (resid   36 and   name    N ) (resid   32 and   name     O ) 0.0 0.0 3.3
assign (resid   36 and   name   HN ) (resid   32 and   name     O ) 0.0 0.0 2.3
! 33-37
assign (resid   37 and   name    N ) (resid   33 and   name     O ) 0.0 0.0 3.3
assign (resid   37 and   name   HN ) (resid   33 and   name     O ) 0.0 0.0 2.3
! 37-39
assign (resid   39 and   name    N ) (resid   37 and   name     O ) 0.0 0.0 3.3
assign (resid   39 and   name   HN ) (resid   37 and   name     O ) 0.0 0.0 2.3
! 61-68
assign (resid   68 and   name    N ) (resid   61 and   name     O ) 0.0 0.0 3.3
assign (resid   68 and   name   HN ) (resid   61 and   name     O ) 0.0 0.0 2.3
assign (resid   61 and   name    N ) (resid   68 and   name     O ) 0.0 0.0 3.3
assign (resid   61 and   name   HN ) (resid   68 and   name     O ) 0.0 0.0 2.3
! 59-70
assign (resid   70 and   name    N ) (resid   59 and   name     O ) 0.0 0.0 3.3
assign (resid   70 and   name   HN ) (resid   59 and   name     O ) 0.0 0.0 2.3
assign (resid   59 and   name    N ) (resid   70 and   name     O ) 0.0 0.0 3.3
assign (resid   59 and   name   HN ) (resid   70 and   name     O ) 0.0 0.0 2.3
! 47-58
assign (resid   58 and   name    N ) (resid   47 and   name     O ) 0.0 0.0 3.3
assign (resid   58 and   name   HN ) (resid   47 and   name     O ) 0.0 0.0 2.3
assign (resid   47 and   name    N ) (resid   58 and   name     O ) 0.0 0.0 3.3
assign (resid   47 and   name   HN ) (resid   58 and   name     O ) 0.0 0.0 2.3
! 45-60
assign (resid   60 and   name    N ) (resid   45 and   name     O ) 0.0 0.0 3.3
assign (resid   60 and   name   HN ) (resid   45 and   name     O ) 0.0 0.0 2.3
assign (resid   45 and   name    N ) (resid   60 and   name     O ) 0.0 0.0 3.3
assign (resid   45 and   name   HN ) (resid   60 and   name     O ) 0.0 0.0 2.3
! 43-62
assign (resid   62 and   name    N ) (resid   43 and   name     O ) 0.0 0.0 3.3
assign (resid   62 and   name   HN ) (resid   43 and   name     O ) 0.0 0.0 2.3
assign (resid   43 and   name    N ) (resid   62 and   name     O ) 0.0 0.0 3.3
assign (resid   43 and   name   HN ) (resid   62 and   name     O ) 0.0 0.0 2.3
! 66-63
assign (resid   63 and   name    N ) (resid   66 and   name     O ) 0.0 0.0 3.3
assign (resid   63 and   name   HN ) (resid   66 and   name     O ) 0.0 0.0 2.3
```

```
! 73-80
assign (resid   80 and   name    N )(resid   73 and   name    O ) 0.0 0.0 3.3
assign (resid   80 and   name   HN )(resid   73 and   name    O ) 0.0 0.0 2.3
assign (resid   73 and   name    N )(resid   80 and   name    O ) 0.0 0.0 3.3
assign (resid   73 and   name   HN )(resid   80 and   name    O ) 0.0 0.0 2.3
! 89-93
assign (resid   93 and   name    N )(resid   89 and   name    O ) 0.0 0.0 3.3
assign (resid   93 and   name   HN )(resid   89 and   name    O ) 0.0 0.0 2.3
! 90-94
assign (resid   94 and   name    N )(resid   90 and   name    O ) 0.0 0.0 3.3
assign (resid   94 and   name   HN )(resid   90 and   name    O ) 0.0 0.0 2.3
! 91-95
assign (resid   95 and   name    N )(resid   91 and   name    O ) 0.0 0.0 3.3
assign (resid   95 and   name   HN )(resid   91 and   name    O ) 0.0 0.0 2.3
! 92-96
assign (resid   96 and   name    N )(resid   92 and   name    O ) 0.0 0.0 3.3
assign (resid   96 and   name   HN )(resid   92 and   name    O ) 0.0 0.0 2.3
! 93-97
assign (resid   97 and   name    N )(resid   93 and   name    O ) 0.0 0.0 3.3
assign (resid   97 and   name   HN )(resid   93 and   name    O ) 0.0 0.0 2.3
! 94-98
assign (resid   98 and   name    N )(resid   94 and   name    O ) 0.0 0.0 3.3
assign (resid   98 and   name   HN )(resid   94 and   name    O ) 0.0 0.0 2.3
! 95-99
assign (resid   99 and   name    N )(resid   95 and   name    O ) 0.0 0.0 3.3
assign (resid   99 and   name   HN )(resid   95 and   name    O ) 0.0 0.0 2.3
! 96-100
assign (resid  100 and   name    N )(resid   96 and   name    O ) 0.0 0.0 3.3
assign (resid  100 and   name   HN )(resid   96 and   name    O ) 0.0 0.0 2.3
! 102-111
assign (resid  111 and   name    N )(resid  102 and   name    O ) 0.0 0.0 3.3
assign (resid  111 and   name   HN )(resid  102 and   name    O ) 0.0 0.0 2.3
assign (resid  102 and   name    N )(resid  111 and   name    O ) 0.0 0.0 3.3
assign (resid  102 and   name   HN )(resid  111 and   name    O ) 0.0 0.0 2.3
! DIHEDRAL ANGLES RESTRAINTS FOR SH2 MODULE
constraints dihedral reset

!!23
assign (resid  22  and name  C  ) (resid  23  and name  N  )
      (resid  23  and name  CA  )(resid  23 and name  C  )  1 -120.0 30.0 2  ! 8.1Hz
!!24
assign (resid  23  and name  C  ) (resid  24  and name  N  )
      (resid  24  and name  CA  )(resid  24 and name  C  )  1 -120.0 30.0 2  ! 8.7Hz
!!46
assign (resid  45  and name  C  ) (resid  46  and name  N  )
      (resid  46  and name  CA  )(resid  46 and name  C  )  1 -120.0 30.0 2  ! 8.7Hz
!!47
assign (resid  46  and name  C  ) (resid  47  and name  N  )
      (resid  47  and name  CA  )(resid  47 and name  C  )  1 -120.0 30.0 2  ! 8.3Hz
!!55
assign (resid  54  and name  C  ) (resid  55  and name  N  )
      (resid  55  and name  CA  )(resid  55 and name  C  )  1 -120.0 30.0 2  ! 8.6Hz
!!57
assign (resid  56  and name  C  ) (resid  57  and name  N  )
      (resid  57  and name  CA  )(resid  57 and name  C  )  1 -120.0 30.0 2  ! 8.4Hz
!!58
assign (resid  57  and name  C  ) (resid  58  and name  N  )
      (resid  58  and name  CA  )(resid  58 and name  C  )  1 -120.0 30.0 2  ! 8.9Hz
!!60
assign (resid  59  and name  C  ) (resid  60  and name  N  )
      (resid  60  and name  CA  )(resid  60 and name  C  )  1 -120.0 30.0 2  ! 9.1Hz
!!61
assign (resid  60  and name  C  ) (resid  61  and name  N  )
      (resid  61  and name  CA  )(resid  61 and name  C  )  1 -120.0 30.0 2  ! 8.6Hz
```

```
!!66
assign (resid  65 and name  C  ) (resid  66  and name  N  )
      (resid  66  and name  CA  )(resid  66 and name  C  )  1 -120.0 30.0 2  ! 8.8Hz
!!68
assign (resid  67  and name  C  ) (resid  68  and name  N  )
      (resid  68  and name  CA  )(resid  68 and name  C  )  1 -120.0 30.0 2  ! 8.5Hz
!!69
assign (resid  68  and name  C  ) (resid  69  and name  N  )
      (resid  69  and name  CA  )(resid  69 and name  C  )  1 -120.0 30.0 2  ! 7.9Hz
!!70
assign (resid  69  and name  C  ) (resid  70  and name  N  )
      (resid  70  and name  CA  )(resid  70 and name  C  )  1 -120.0 30.0 2  ! 8.9Hz
!!73
assign (resid  72 and name  C  ) (resid  73  and name  N  )
      (resid  73  and name  CA  )(resid  73 and name  C  )  1 -120.0 30.0 2  ! 8.6Hz
!!74
assign (resid  73  and name  C  ) (resid  74  and name  N  )
      (resid  74  and name  CA  )(resid  74 and name  C  )  1 -120.0 30.0 2  ! 8.7Hz
!!76
assign (resid  75  and name  C  ) (resid  76  and name  N  )
      (resid  76  and name  CA  )(resid  76 and name  C  )  1 -120.0 30.0 2  ! 8.4Hz
!!79
assign (resid  78  and name  C  ) (resid  79 and name  N  )
      (resid  79  and name  CA  )(resid  79 and name  C  )  1 -120.0 30.0 2  ! 9.3Hz
!!100
assign (resid  99 and name  C  ) (resid 100  and name  N  )
      (resid 100  and name  CA  )(resid 100 and name  C  )  1 -120.0 30.0 2  ! 7.9Hz
!!109
assign (resid 108 and name  C  ) (resid 109  and name  N  )
      (resid 109  and name  CA  )(resid 109 and name  C  )  1 -120.0 30.0 2  ! 8.4Hz
!!112
assign (resid 111 and name  C  ) (resid 112  and name  N  )
      (resid 112  and name  CA  )(resid 112 and name  C  )  1 -120.0 30.0 2  ! 8.7Hz
!!113
assign (resid 112 and name  C  ) (resid 113  and name  N  )
      (resid 113  and name  CA  )(resid 113 and name  C  )  1 -120.0 30.0 2  ! 7.9Hz
!!114
assign (resid 113 and name  C  ) (resid 114  and name  N  )
      (resid 114  and name  CA  )(resid 114 and name  C  )  1 -120.0 30.0 2  ! 8.4Hz
!!115
assign (resid 114 and name  C  ) (resid 115  and name  N  )
      (resid 115  and name  CA  )(resid 115 and name  C  )  1 -120.0 30.0 2  ! 9.6Hz
!!29
assign (resid  28 and name  C  ) (resid  29  and name  N  )
      (resid   29  and name  CA  )(resid  29 and name  C  )  1 -60.0 30.0 2  ! 3.5Hz
!!30
assign (resid  29 and name  C  ) (resid  30  and name  N  )
      (resid  30  and name  CA  )(resid  30 and name  C  )  1 -60.0 30.0 2  ! 4.25Hz
!!33
assign (resid  32 and name  C  ) (resid  33  and name  N  )
      (resid   33  and name  CA  )(resid  33 and name  C  )  1 -60.0 30.0 2  ! 3.8Hz
!!45
assign (resid  44 and name  C  ) (resid  45  and name  N  )
      (resid 45  and name  CA  )(resid  45 and name  C  )  1 -120.0 30.0 2  ! 8.71Hz
!!49
assign (resid  48 and name  C  ) (resid  49  and name  N  )
      (resid  49  and name  CA  )(resid  49 and name  C  )  1 -120.0 30.0 2  ! 8.0Hz
!!93
assign (resid  92 and name  C  ) (resid  93  and name  N  )
      (resid  93  and name  CA  )(resid  93 and name  C  )  1 -60.0 30.0 2  ! 3.96Hz
!!95
assign (resid  94 and name  C  ) (resid  95  and name  N  )
      (resid  95  and name  CA  )(resid  95 and name  C  )  1 -60.0 30.0 2  ! 3.99Hz
  end
```

Appendix III

XPLOR Structure Calculation and Refinement Protocols

A. Simulated Annealing Protocol for Structure Calculation from Random Starting Configuration

Author: M. Nilges, Yale University (1990); modified by: T. S. Harvey, Oxford University (1991)

```
Remarks YASAP.inp
Remarks Yet Another Simulated Annealing Protocol

! Simplified version without all that shifting around of NOE restraints.
! Any initial structure works as long as the geometry is reasonable.
! Extended strands or randomly chosen torsion angles are fine.
!
!
! stage 0:  initial minimization
! stage 1:  9 ps high temperature dynamics
! stage 2:  increase vdW and ASYMptote
! stage 3:  cool to 300 K, increase vdW further
! stage 4:  minimize
!
! This input file makes use of the soft NOE energy term.
!   RSWItch   is set to 0.5 A initially and not modified.
!   ASYMptote is 0.1 in stage 1, increased to 1.0 in stage 2.
!   The nOe force constant is 50 throughout the calculation.
!
! The vdW repel constant is small in stage 1 and increased in stage 2
!   and cooling.
!
! Timing on Convex C2:
!   NP5 (484 atoms):      ~ 20 min CPU
!   Crambin (642 atoms): ~ 30 min CPU
!
! For larger systems the dynamics time in stage 1 may have to be increased.
!
! Note: This input file is for structure determination with NMR restraints,
!   NOT for refinement. There are NO energy terms used, only geometrical
!   restraints (lengths and angles), and in the sense that DISMAN is a
!   distance geometry program, this input file does distance geometry.
!   Lennart-Jones vdW energies of the structures generated are usually good.
!   But, as no electrostatic potential is used, one cannot expect too much
!   of the electrostatic energy.
!
! Author: Michael Nilges, Yale University, FEB-1990



!---------------
! used variables
!
! $11 {seed}
! $21 {root}
! $22 {file}
! $81 {rcon}
```

```
! $31 {knoe}
! $32 {kcdi}
! $33 {asym}
! $82 {kbon}
! $83 {kang}
! $84 {kchi}
! $85 {kpla}
! $86 {kdih}
! $91 {bath}


!-------------------------------------------------------------------------
! The following two statements could be executed 'outside' this file.
! this makes it easier to use the same input file several times
! when the only changes between runs are the filenames and the
! initial random number seed.

!evaluate ($11 {seed}  = 123456)
!evaluate ($21 {root}  = "filename")


!-------------------------------------------------------------------------
! actually set the initial seed

evaluate ($11 {seed} = $11 + 3)
set seed $11 {seed}  end


!-------------------------------------------------------------------------
! read in the PSF file, or generate the structure.
!
!structure reset @tpa_yasap.psf end


@tpa_bld.inp


!-------------------------------------------------------------------------
! read in an initial structure, or generate the coordinates
!
!--> coor @inistruct.pdb

set echo = false end

@random_struct.inp

set echo = true end
!-------------------------------------------------------------------------
! Set the initial weights for different energy terms
! All planarity and chirality terms are treated as improper torsion angles.
! The 'normal' dihedral angle potential (e.g. for phi, psi and chi) is
! switched off.
! The only weight that is much varied in this input file is the vdW weight.
! Initially, it is very low.
! Also, the weights for angles and impropers are initially reduced.
! This is necessary as otherwise the large timestep of 3 fs would cause
! the calculation to blow up.
! Note that the final weights for angles and especially for chirality and
! planarity are much higher than those in the normal parameter files.
! This is to ensure that the geometry of the structures is good.
! The ratio of the NOE weight to the softest geometry term 1:10. This is
! even lower than the ratio used in GROMOS (where it can be around 1:1;
! the softest geometry term being the planarity of peptide bonds).
```

```
evaluate ($81 {rcon}  = 0.002)   ! vdW

evaluate ($31 {knoe}  = 50.0)    ! noes
evaluate ($32 {kcdi}  = 200.0)   ! torsion angles
evaluate ($33 {asym}  = 0.1)     !
evaluate ($82 {kbon}  = 500)     ! bonds
evaluate ($83 {kang}  = 500)     ! angles
evaluate ($85 {kpla}  = 500)     ! planarity
evaluate ($84 {kchi}  = 500)     ! chirality
evaluate ($86 {kdih}  = 0)       ! dihedrals


!-------------------------------------------------------------------------
! The parameter file that is read in uses the 'variable' force constants
! defined above.

parameters
  reset
     @parallhsa.pro
end


!-------------------------------------------------------------------------
! The next statement makes sure the experimental energies are used in the
! calculation, and switches off the unwanted energy terms.

flags
  include noe cdih
  exclude elec hbon dihe
end
```

```
!-------------------------------------------------------------------------
! Read experimental restraints

!set echo = false end

noe
  reset
  nrestraints = 3000                    ! allocate space for NOEs

  class      all
  @tpa_all_corr.tbl
  averaging  all cent
  potential  all soft
  scale      all $31 {knoe}
  sqoffset   all 0.0
  sqconstant all 1.0
  sqexponent all 2
  soexponent all 1
  asymptote  all $33 {asym}
  rswitch    all 0.5

  class      hbo
  @tpa_hbonds.tbl
  averaging  hbo cent
  potential  hbo soft
  scale      hbo $31 {knoe}
  sqoffset   hbo 0.0
  sqconstant hbo 1.0
  sqexponent hbo 2
  soexponent hbo 1
  asymptote  hbo $33 {asym}
  rswitch    hbo 0.5
```

```
end

      @tpa_dihe.tbl

set echo = true end

      cons dihed
       scale $32 {kcdi}
      end end
!-------------------------------------------------------------------------
! set up large VHEAP
param nbonds cutnb=24 end end
    energy end
!-------------------------------------------------------------------------
! Nonbond parameters

evaluate ($81 {rcon}  = 0.002)
parameters
  nbonds
    toler = 1.0
    wmin=0.01     ! warning if two atoms come close basically switched off
    cutnb = 4.0   ! low nonbonded cutoff
    repel=1.0     ! scale factor for vdW radii = 1 ( L-J radii)
    rexp= 2       ! exponent in (r^2 - R0^2)^rexp
    rcon=$81 {rcon}     ! actually set the vdW weight
  end
end


!-------------------------------------------------------------------------
! The initial minimization ensures the stereochemistry of the initial
! structure is right (esp. compatible with the used topology).

mini powell nstep=50 drop= 10 end

!-------------------------------------------------------------------------
! Set a few dynamics parameters.

vector do (mass  =  10.0) (all)          ! uniform mass for all atoms
vector do (fbeta = 100.0) (all)          ! coupling to heat bath
evaluate  ($91 {bath}  = 1000.01)              ! heat bath temperature


!-------------------------------------------------------------------------
! Set SHAKE constraints. As mentioned above, the planarity and chirality
! force constants have to be reduced so that SHAKE and a timestep of
! 3 fs work at 1000 K.

evaluate ($83 {kang}  = 200)     ! angles
evaluate ($85 {kpla}  =  50)     ! planarity
evaluate ($84 {kchi}  =  50)     ! chirality
parameters
  reset
     @parallhsa.pro
end

shake
  reference = parameter bonds (all) (all)
  tolerance = 1.0e-2
end
```

```
!---------------------------------------------------------------------------
! STAGE 1:
! We simply do 9 ps of dynamics with the weights set above.
! The high value of NTRFr ensures that the center of mass motion is
! removed only once.

dynamics  verlet
      nstep=3000                        !
      timestep=0.003                    ! 9 ps of dynamics
      iasvel=maxwell                    ! initial velocities from maxwell distr.
      firsttemperature = $91 {bath}     ! at high temperature
      tcoupling = true                  ! temperature controlled with T-bath
      tbath = $91 {bath}                ! at high T, friction (fbeta) set above
      ntrfr = 999999999                 ! COM removal only once
      nprint=50                         ! energy output every 50 steps
      iprfrq=100                        ! energy statistics every 100 steps
end


!---------------------------------------------------------------------------
! STAGE 2: increase the vdW force constant (hardness) and tilt the asymptote

while ($81 {rcon}  < 0.1) loop stg2

   evaluate ($33 {asym}  = min(1.0, $33 {asym}  + 0.1))          ! tilt asymptote
   noe asymp all $33 {asym}  end
   noe asymp hbo $33 {asym}  end

   evaluate ($81 {rcon}  = min(0.10001, $81 {rcon}  * 1.5))      ! increase vdW
   parameters
     nbonds repel 1.0 rcon $81 {rcon}  end
   end

   dynamics  verlet
      nstep=300              !
      timestep=0.003         ! 1 ps of dynamics
      iasvel=current         ! take the old velocities
      tcoupling = true
      tbath = $91 {bath}
      nprint=50
      iprfrq=100
      ntrfr = 0              ! COM motion is not removed any more
   end

end loop stg2


!---------------------------------------------------------------------------
! STAGE 3: cool the system
! Here, we switch to the normal 'square-well' potential (which does not
! make too much difference at this point), and to smaller and harder spheres
! in the vdW repel potential. The suggested values (REPEl = 0.8 and
! RCONst = 4) have proven to be large enough (they seem to be a bit on the
! large side already). If one wants to increase the force constant further
! one should at the same time decrease the vdW radius by choosing a smaller
! value for REPEl to prevent the structures from being overly extended.

noe
  potential all square
  potential hbo square
end

parameters
  nbonds
```

```
    repel 0.8                ! smaller spheres
    rconst 4                 ! harder spheres
    wmin 1.5                 ! switch nbond warning back on
  end
end

while ($91 {bath}  gt 300) loop stg3
  dynamics  verlet
     nstep=30
     timestep=0.003 {ps}
     iasvel=current
     tcoupling = true
     tbath = $91 {bath}
     nprint=30 iprfrq = 30
     ntrfrq = 0
  end

  evaluate ($91 {bath}  = $91 {bath}  - 25)        ! decrease finaltemperature
end loop stg3


!-----------------------------------------------------------------------
! STAGE 4: minimisation
! The SHAKE constraints are switched off, and the angle and improper
! force constants increased to their high final values.

shake reset end

evaluate ($83 {kang}  = 500)    ! angles
evaluate ($85 {kpla}  = 500)    ! planarity
evaluate ($84 {kchi}  = 500)    ! chirality

parameters
  reset
     @parallhsa.pro
  end

mini powell nstep=200 drop = 10.0 end


!-----------------------------------------------------------------------
! Write out the final structure.
! Whatever is written in REMARK records before writing the file ends up as
! remarks in the file.

evaluate ($22 {file}  = $21 {root}  + ".sa")
Remark $21  final structure, random seed $11
write coor output=$22 {file}  end
evaluate ( $22 {file} = $21 {root} + ".enr")
set display= $22 end
display energy= $ENER  noe energy= $NOE  VDW energy = $VDW
display dihedral energy  = $CDIH
close $22 end

evaluate ($24 = $21 + ".prt")
set print = $24 end
print threshold = 0.1 noe
close $24 end

!-----------------------------------------------------------------------
! If one wants to calculate several structures at once by executing this
! file several times, the STOP should be omitted.

stop
```

random_struct.inp

```
REMARK build a random structure using the internal coordinates
REMARK phi and psi are assigned randomly, the other torsion angles
REMARK keep their initial values (180 degrees).
REMARK
REMARK Michael Nilges, Yale University
!
! variables: $1 $2 $3 $4 $51 $52


!-------------------------------------------------------------------------
! undefined ICs (bonds, angles) are filled using the ideal values
! in the parameter file

ic
   fill reference=parameters end
end

coor init sele=(all) end


!-------------------------------------------------------------------------
! 1) get random structure

for $1 in id ( name ca ) loop main                    ! loop over all ca atoms
  vector show element (resid) ( id $1 )
  evaluate ($3 = $result)
  evaluate ($2 = decode($3) - 1)
  evaluate ($4 = decode($3) + 1)

!-------------------------------------------------------------------------
! check if the previous and the next residue are defined before trying
! to modify the torsion angles.
  vector show element (resid) ( resid $2  and  name ca  )
  if ( $select > 0 ) then
    vector show element (resid) ( resid $4  and  name ca  )
    if ( $select > 0 ) then


!-------------------------------------------------------------------------
! this is for a random initial structure (random in phi and psi)
! it uses a gaussian distribution with SD 360 degrees. Should be
! random enough.
      evaluate ($51 {phi} = gauss(360))
      evaluate ($52 {psi} = gauss(360))
! this is for an extended strand
!      evaluate ($51 {phi} = -139)
!      evaluate ($52 {psi} =  135)


!-------------------------------------------------------------------------
! noe modify the torsion anlges with the ic edit command

      ic
        edit dihe ( resid $2 and name C )  ! modify phi
                  ( resid $3 and name N )
                  ( resid $3 and name CA)
                  ( resid $3 and name C ) $51 {phi} end
        edit dihe ( resid $3 and name N )  ! modify psi
                  ( resid $3 and name CA)
                  ( resid $3 and name C )
                  ( resid $4 and name N ) $52 {psi} end
      end
```

```
    end if
  end if
end loop main


!-------------------------------------------------------------------------
! chose any three consecutive atoms as first three and build the coords

ic
  build seed=(resid 17 and name c )
             (resid 18 and name n )
             (resid 18 and name ca)
  end
end
!-------------------------------------------------------------------------
! build the hydrogens (which are not built by the ic command)

flags exclude vdw elec end
hbuild select= (hydrogen) phistep=360 end
flags include vdw elec end
```

tpa_bld.inp

```
REMARKS generate a structure including internal coordinates


!-------------------------------------------------------------------------
! initial force constants for parallhsa.pro - values don't not matter here

evaluate ($82 = 0.001)
evaluate ($83 = 0.001)
evaluate ($84 = 0.000)
evaluate ($85 = 0.001)
evaluate ($86 = 0.001)
evaluate ($81 = 0.0001)

structure reset end

ic reset end

rtf  reset   @topallhsa.pro
end

parameter
  reset
  @parallhsa.pro
  nbfix         S       S       462 13.6 462 13.6

  nbonds
    atom
    nbxmod    -3        ! include 1-4 interactions
    tolerance  0.5
    wmin       0.1
    repel      1.0
    rcon       $81
    rexp       2
    cutnb      4.0
   end
end
```

```
!-----------------------------------------------------------------------------
! generate the structure from the sequence and topology information.
! setup=true includes ICs for the builder.

segment
   name="tpa"
   setup=true
   chain
      LINK PEPP    HEAD - *     TAIL + PRO     END  { LINK to PRO }
      LINK PEPT    HEAD - *     TAIL + *       END
      FIRSt PROP                TAIL + PRO     END { nter for PRO }
      FIRSt NTER                TAIL + *       END
      LAST  CTER   HEAD - *                    END
      coor @/home/nmra/kristy/tpa/tpa.pdb

   end
end

!-----------------------------------------------------------------------------
! apply a special disulfide patch that only deletes the hydrogen
! but does not add a bond

patch disn reference=1=(resid  6) reference=2=(resid 36) end
patch disn reference=1=(resid 34) reference=2=(resid 43) end

!cis-prolines
!vector do (chem="NX") (name n and resid 16)
!vector do (chem="NX") (name n and resid 36)

Remark PSF file for tpa F1
Remark for simulated annealing calculations
Remark
! write psf output= tpa_yasap.psf end
```

B. Fast Simulated Annealing Structure Refinement Protocol

Author: T. S. Harvey, Oxford University (1991)

```
Remark  new_fast.inp  [$5, seed]

! Reference:
! M. Nilges, G.M. Clore, A.M. Gronenborn (1988), Determination of three-
!   dimensional structures of proteins from interproton distance data
!   by hybrid distance geometry-dynamical simulated annealing calculations,
!   FEBS Letters 229, 317-324.


! Variables to be set outside (i.e. before calling HYBRID.INP):
!   $5    is the NAME of the following files:
!           NAME.sa     SA final structure
!           NAME.enr    energy display file
!           NAME.prt    violations print file
!   SEED  the random number seed for assigning

! Variables used internally :
!   $1  residue number, vdW force constant
!   $2  temperature
!   $3  NOE scale
!   $4  CDIH scale


structure reset @tpa_yasap.psf end


evaluate ($21 = $5 + ".sa")
coor @$21 end                          !read input coordinates

!-----------------------------------
! Set up energy potential parameters

parameters
  nbonds
    nbxmod = 4              ! exclude 1-4 interactions
    wmin   = 0.5            ! warnings at 0.5 A distance
    cutnb  = 4.5            ! nonbonded cutoff distance
    toler  = 1.0            ! distance change update tolerance ! usually 0.5 TSH
    repel  = 1.0            ! turn on repel function; full L-J vdW radii
    rexpon = 2              ! repel exponent
    rconst = 0.001          ! repel energy constant; initially very small
  end
end

flags
  exclude *                                ! do not rely on defaults
  include bond angle improper vdw NOE cdih
end
!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!
!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!

! Read in all of the restraints
set echo = false end
noe
  reset
  nrestraints = 6000                                ! allocate space for NOEs

  class      all
  @tpa_all_corr.tbl
  averaging    all cent
```

```
  potential    all square
  scale        all 0.5                          ! initially very low scale
  sqconstant   all 1.0
  sqexponent   all 2

  class      hbo
  @tpa_hbonds.tbl
  averaging    hbo cent
  potential    hbo square
  scale        hbo 0.5                          ! initially very low scale
  sqconstant   hbo 1.0
  sqexponent   hbo 2

end
set echo = true end


@tpa_dihe.tbl           ! dihedral angle restraints

vector do (mass  =   9.0) (all)                ! uniform mass
vector do (mass  =   10.0) (name hn)
vector do (fbeta = 100.0) (all)                ! coupling to heatbath

vector do (vx = maxwell (2500.0)) (all)      ! starting velocities at 1000.0 K
vector do (vy = maxwell (2500.0)) (all)
vector do (vz = maxwell (2500.0)) (all)


!---------------------------------------------------------------------
! Dynamical simulated annealing at 1000 K

eval ($1 = 0.001)           ! initial force constant for vdw
eval ($3 = 0.5)             ! initial scale on NOE, hbond restraints (very low)
eval ($4 = 0.5)             ! initial scale on dihedral restraints (very low)

!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!
evaluate ($82 {kbon} = 500)
evaluate ($83 {kang} = 140)
evaluate ($84 {kpla} =  40)
evaluate ($85 {chi} =  40)
evaluate ($86 {kdih} = 00)
parameters reset @parallhsa.pro end

ic
  fill reference = parameters end
end

shake
  reference = parameter bonds (all) (all)
  tolerance = 1.0e-2 !!
end

!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!

  eval ($2 = 1000.0)
  dynamics verlet
    nstep     = 10                       ! 10 steps
    timestep  = 0.004                    ! time step 4 fs
    iasvel    = current                  ! use current velocities
    tcoupling = true                     ! Berendsen method
    tbath     = $2
    nprint    = 10                       ! output statistics at end of cycle
    iprfrq    = 10
   end
```

```
while ($1 < 0.25) loop anneal

  eval ($1 = $1 + .019)              ! increase vdw constant to 0.25
  eval ($1 = min($1, 0.25))
  parameters
    nbonds   rconst = $1    end
  end

  eval ($3 = $3 * 2.5)                 ! increase NOE scale to 50
  eval ($3 = min ($3, 50.0))
  noe
    scale all $3
    scale hbo $3
    end

  eval ($4 = $4 * 2.50)                ! increase cdih
  eval ($4 = min ($4, 200.0))          ! to 200 (!)


 constraints
    dihedrals
      scale = $4
    end
 end

  dynamics verlet
    nstep     = 20                     ! 20 steps
    timestep  = 0.003                  ! time step 3 fs
    iasvel    = current                ! use current velocities
    tcoupling = true                   ! Berendsen method
    tbath     = $2
    nprint    = 15
    iprfrq    = 15
  end

end loop anneal

  parameters
    nbonds
    repel = 0.9    ! larger spheres
    rconst = 4.0   ! harder spheres
    end
  end


  dynamics verlet
    nstep     = 60                     ! 60 steps
    timestep  = 0.004                  ! time step 4 fs
    iasvel    = current                ! use current velocities
    tcoupling = true                   ! Berendsen method
    tbath     = $2
    nprint    = 30
    iprfrq    = 30
  end

  eval ($2 = 300.0)

  dynamics verlet
    nstep     = 100                    ! 100 steps
    timestep  = 0.004                  ! time step 4 fs
    iasvel    = current                ! use current velocities
    tcoupling = true                   ! Berendsen method
```

```
    tbath      = $2                    ! at reduced temperature
    nprint     = 50
    iprfrq     = 50
  end

  parameters
    nbonds
    repel = 0.85
    end
  end

evaluate ($83 {kang} = 150)
evaluate ($84 {kpla} =  50)
evaluate ($85 {chi} =  50)


  dynamics verlet
    nstep      = 100                  ! 100 steps
    timestep   = 0.005                ! time step 5 fs
    iasvel     = current              ! use current velocities
    tcoupling  = true                 ! Berendsen method
    tbath      = $2                   ! at reduced temperature
    nprint     = 50
    iprfrq     = 50
  end


evaluate ($5 = $5 + ".enr")
set   display = $5    end                    ! energy log file name predefined
evaluate ($5 = $5 - ".enr")

display minimization, 500 cycles
display vdw NOE bond angle cdihedral improper total
display

shake reset end
  evaluate ($83 = 500.)          ! increase kang (100 to 500)
  evaluate ($84 = 500.)          ! increase kpla (90 to 500)
  evaluate ($85 = 500.)          ! increase kchi (90 to 500)
  evaluate ($82 = 500.)          ! increase kbon (100 to 500)
  parameters
    nbonds
    repel = 0.8                  ! final value
    end
  end
parameters reset @parallhsa.pro end


minimize powell
    nstep = 500
    drop  = 20.0
end
evaluate ($5 = $5 + "n")
display $vdw $noe $bond $angl $cdih $impr $ener
evaluate ($5 = $5 + ".sa")
write coordinates
  output = $5                          ! coordinate file name is predefined
end
close $5 end
evaluate ($5 = $5 - ".sa")
```