

**STRUCTURAL AND FUNCTIONAL STUDIES ON HUMAN
COMPLEMENT FACTOR I**

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"If I have seen further it is by standing on ye shoulders of Giants"

Sir Isaac Newton, 5 Feb 1676

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II. PUBLICATIONS

PUBLICATIONS IN PEER-REVIEWED INTERNATIONAL SCIENTIFIC JOURNALS

ARTICLES

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2. Kawamura, A., J. Graham, A. Mushtaq, **S. A. Tsiftoglou**, G. M. Vath, P. E. Hanna, C. R. Wagner, and E. Sim. 2005. Eukaryotic arylamine N-acetyltransferase Investigation of substrate specificity by high-throughput screening. *Biochem Pharmacol* 69:347.
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4. Sim, R. B., and **S. A. Tsiftoglou**. 2004. Proteases of the complement system. *Biochem Soc Trans* 32:21.

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1. **Tsiftoglou, S. A.**, and R. B. Sim. 2004. Human complement factor I substrate specificities and inhibitor susceptibility, *Mol Immunol* 41(2-3):320, XXth International Complement Workshop, Honolulu, HI, USA.
2. Roversi, P., **S. A. Tsiftoglou**, R. Donev, F. Gagg, R. A. G. Smith, R. B. Sim, B. P. Morgan, and S. M. Lea. 2004. Crystallisation and preliminary characterisation of crystals of complement component proteins, *Mol Immunol* 41(2-3):302, XXth International complement Workshop, Honolulu, HI, USA.
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MEETING REPORTS

1. **Tsiftoglou, S. A.** 2004. Complements trip. In *The Biochemist*, Vol. 26, p. 37.

III. THESIS ABSTRACT

Structural and functional studies on human complement factor I

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The complement system is considered as the chief recognition and effector component of innate immunity; it is involved in inflammation and enhances the adaptive immune response. Factor I (fI) is a heterodimeric serine protease consisting of a heavy (HC) and a light-catalytic (LC) chain; it circulates in an active form regulating complement by selectively cleaving only C3b or C4b in the presence of a cofactor such as factor H (fH), CR1, MCP or C4bp. The cleavage of C3b occurs through a ternary complex formed between fI, C3b and a cofactor like fH and yields iC3b, a major opsonin.

The structural and functional properties of fI were investigated. The interchain disulphide bond formed between C³⁰⁹-C⁴³⁵ that links the HC and LC of fI as well as the composition of the *N*-linked carbohydrates of fI were determined. By using two independent assays, the proteolytic and amidolytic assays, the catalytic properties of human fI were characterised in detail. The catalytic subunit, the SP domain, was shown to have a native conformation that accommodates substrate recognition and cleavage. fI has specificity similar to thrombin, but exhibits lower catalytic activity. fI amidolytic activity reaches optimum at pH 8.25 and is insensitive to ionic strength. This is in contrast to its proteolytic activity within the fI-C3b-fH reaction, in which the pH optimum for C3b cleavage is <5.5 and the reaction rate is highly dependent on ionic strength. The rate of cleavage of tripeptide AMC substrates by fI was unaffected by fH or C3(NH₃) at optimum pH.

fI and the isolated SP domain were found to have similar amidolytic activities, but strikingly different proteolytic activities on C3(NH₃). fI did not cleave C3(NH₃) in the absence of fH, but cleaved it rapidly at two sites in its presence. The SP domain however, cleaved C3(NH₃) slowly in the absence of fH, at more than two sites. Cleavage by the SP domain was inhibited, not stimulated, by fH. These results suggested that the HC domains and/or the cofactor must orient the natural substrates and restrict cleavage by fI to the two sites which yield iC3b. The implications of these findings are discussed.

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VI. ABBREVIATIONS

Å	Angstrom Unit: 0.1 x 10 ⁻⁹ m
A	Ampere, SI unit of electric current resulting from a power production rate of 1 Watt per Volt of potential
2-AB	2-aminobenzamide
a ₂ M	alpha 2-macroglobulin
ABS	sialidase from <i>Arthrobacter urefaciens</i>
Ac	N-acetyl
aFGF	acidic Fibroblast Growth Factor
AGE	Agarose Gel Electrophoresis
AMC	7-amino-4-methylcoumarin
bFGF	basic Fibroblast Growth Factor
BKF	Fucosidase from bovine kidney
Boro MPG	Z-D-Phe-Pro-methoxypropylboroglycinepinanediol Ester
BSA	Bovine Serum Albumin
BTG	β-galactosidase from bovine testis
Bz	Na-benzoyl
°C	Degree Celsius. A non-SI unit of temperature
C4bp	C4-binding protein
CCP	Complement Control Protein
CD	Cluster of Differentiation
cDNA	complementary DNA; Single-stranded DNA synthesized in the laboratory using messenger RNA as a template and the enzyme reverse transcriptase.
CIP	Calf Intestinal Alkaline Phosphatase
CT	Contaminant
C1inh	C1 inhibitor
C3Ina	C3 Inactivator
C3(H ₂ O)/	C3/C4 with hydrolysed thioester
C4(H ₂ O)	
C3(NH ₃)	A structural analog of C3(H ₂ O). C3(NH ₃) is formed when the C3 thioester is cleaved by nucleophilic attack by NH ₃ .
cpm	counts per minute
CR	Complement Receptor
CUB	C1r/s Uegf Bone morphogenetic protein-1
CV	Column Volume
CVF	Cobra Venom Factor
CYT	Cytoplasmic Tail
Da	Dalton. A non-SI unit of mass, equal to the <i>unified atomic mass unit</i> (atomic mass

	constant)
DAA	Decay Accelerating Activity
DAF	Decay Accelerating Factor
DEAE	Diethylaminoethyl cellulose
DFP	di-isopropyl fluorophosphate
DNA	Deoxyribonucleic acid
dpm	disintegrations per minute
DSC	Differential Scanning Calorimetry
DTT	1, 4-dithiothreitol
εACA	ε-amino caproic acid
EDTA	Ethylene Diamine Tetra Acetic acid
EGF	Epidermal Growth Factor
ELISA	Enzyme Linked Immunosorbent Assay
Et	Ethyloxy
fB	complement factor B
fD	complement factor D
fH	complement factor H
FHL	fH-like
FHR	fH-related
fI	complement factor I
FIMAC	Factor I Membrane Attack Complex
FPLC	Fast Performance Liquid Chromatography
FS	Follistatin
GAGs	Glucosaminoglycans
GlcNAc	N-acetylglucosamine
GU	Glucose Unit
GuH	recombinant β- <i>N</i> -acetylglucosaminidase from <i>Streptococcus pneumonia</i>
HBS buffer	HEPES-buffered saline buffer (25 mM HEPES, 145 mM NaCl, 0.5 mM EDTA, pH 7.4)
HC	Heavy chain
HEPES	2-[4-(2-hydroxyethyl-1-piperazine)]ethanesulphonic acid
HPLC	High Performance Liquid Chromatography
HSA	Human Serum Albumin
aHUS	atypical Hemolytic Uremic Syndrome
IAM	Iodoacetamide
IB	Inclusion Body
iC3b	Inactivated C3b (cleaved by factor I)
IEF	Isoelectric focusing
IFN-γ	Interferon-γ
IgA/G/M	Immunoglobulin A/G/M
IL-1	Interleukin-1

IL-4	Interleukin-4			
IL-6	Interleukin-6			
Iodogen	1,3,4,6-tetrachloro-3 α ,6 α -diphenylglycoluril			
IPTG	Iso-propylthio- β -galactopyranoside (IPTG)			
KAF	Konglutinogen Activating Factor			
kDa	Kilodalton			
LB	Luria Broth			
LBTI	Lima Bean Trypsin Inhibitor type IIL			
LC	Light chain			
LDLR-A	Low Density Lipoprotein Receptor type A			
LHR	Long Homologous Repeat			
LPS	Lipopolysaccharide			
MAC	Membrane Attack Complex			
MASP	Mannan-Binding Lectin-Associated Serine Protease			
MBL	Mannan-Binding Lectin			
MCP	Membrane Cofactor Protein			
MD	Methylsulfonyl-D			
Me	Methyloxy			
MES	2-(N-morpholino)ethane sulphonic acid			
MIDAS	Metal Ion Dependent Adhesion Site			
MRC	Medical Research Council (U.K.)			
mRNA	messenger ribonucleic acid			
MV	Measles Virus			
MW	Molecular Weight			
NanI	recombinant sialidase from <i>Streptococcus pneumonia</i>			
nfl	Native human fl			
NMR	Nuclear Magnetic Resonance			
N ^x	Asparagine with attached carbohydrate			
NP-HPLC	Normal Phase High Performance Liquid Chromatography			
OD	Optical Density			
OG	Octyl- β -D-glucopyranoside			
OVA	Ovalbumin			
PACE	Paired Amino acid Cleaving Enzyme			
PAMPs	Pathogen-Associated Molecular Patterns			
PBS	Phosphate-Buffered Saline (Dulbecco A formula: 8.2 mM Na ₂ HPO ₄ , 1.5 mM KH ₂ PO ₄ , 139 mM NaCl, 3 mM KCl, pH 7.4)			
PBS buffer	PBS, 0.5 mM EDTA, pH 7.4			
PDGF	Platelet-Derived Growth Factor			
Pefabloc-SC	4-(2-aminoethyl)-benzenesulphonylfluoride.HCl			
Pefabloc-TH	Na-(2-Naphthylsulfonylglycyl)-4-amidino-(D,L)-phenylalanine	piperidide	acetate	

	(NAPAP)
Pefabloc-Xa	Na-tosylglycyl-3-DL-amidinophenylalanine methyl ester
PDGF	Platelet Derived Growth Factor
PEG	Polyethylene glycol
phNO ₂	p-Nitrophenyloxy
pI	Isoelectric point. At the pI the net charge of a protein or a polypeptide is zero.
PMSF	Phenylmethyl sulphonyl fluoride
PNGase F	Peptide-N ⁴ -(acetyl-beta-glucosaminyl)-asparagine amidase
PRRs	Pattern Recognition Receptor (PRRs)
PT	Pro-thrombin
PVDF	Polyvinylidene fluoride
RCA	Regulator of Complement Activation
rhfI	recombinant human factor I
RT	Room Temperature
sample buffer	0.2 M Tris, 8 M urea, 2% (w/v) SDS, 2 mM EDTA, pH 8.0 with 0.001% (w/v) Bromophenol Blue and 40 mM 1,4-dithiothreitol (reducing conditions) or 20 mM iodoacetamide (alkylating conditions)
SBTI	soybean trypsin inhibitor (Kunitz inhibitor)
S-Bzl	thiobenzyl
SCR	Short Consensus Repeat
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
SERPIN	Serine Protease Inhibitor
SI	International System of Units
SLE	Systemic Lupus Erythromatosus
SP	Serine Protease
SRCR	Scavenger Receptor Cysteine Rich
STP	Serine-Threonine-Proline
Suramin	hexasodium <i>sym</i> -bis [<i>m</i> -aminobenzoyl- <i>m</i> -amino- <i>p</i> -methylbenzoyl-1-aminonaphthalene-4,6,8-trisulfonate]carbamide
TBE	Tris Borate EDTA
TEMED	N, N, N', N'-tetramethylethylenediamine
Tm	melting temperature
TNF-α	Tumour Necrosis Factor α
Tos	p-tosyl
tPA	tissue-type Plasminogen Activator
Tris	tris(hydroxymethyl)aminoethane
Tween-20	poly(oxyethylene) sorbitan monolaurate
U	Unit
uPA	urokinase-type Plasminogen Activator

UT	untranslated
UV	ultraviolet
V	Volt. An SI unit of potential, equal to the potential of 1 joule per coulomb of charge.
Vh	Volt-hour
v/v	volume to volume
vWFA	von Willebrand Factor type A
W	Watt. An SI unit of power, equal to the power of 1 joule of work per second of time.
w/v	weight to volume
w/w	weight to weight
Z	N-carboxybenzyloxy

Chapter One

CHAPTER 1: INTRODUCTION

1.1 INNATE IMMUNITY AND THE COMPLEMENT SYSTEM

The term 'Immunity' has been established to characterise the global activity of the host to resist the predation of non-self organisms that would cause its destruction. Immunity, which is nowadays characterised by many facets, is scientifically specified through the terms 'Adaptive or Acquired' and 'Innate or Natural' immune responses. This terminology was adopted at the turn of the twentieth century by scientists who were investigating the immune response and belonged to two different camps of thinking; the humoralists who believed that humoral substances, like antitoxins, composed the basis for adaptive immunity and the cellularists who believed that immunity was innate or natural, mainly phagocytic and was therefore mediated by cells.

Innate immunity is nowadays considered as the frontline defence of multi-cellular organisms (Song et al., 2000). Broadly speaking it encompasses a broad range of passive and active mechanisms of defence against infection. It is considered more ancient than adaptive immunity since it is used by invertebrates such as insects and echinoderms, as well as by higher animals. In contrast, adaptive immunity is probably restricted to vertebrates. Although innate immunity is widely considered as a less specific and less sophisticated mechanism of host defence, it provides the host resistance at the first phase of infection in contrast to the adaptive immune system that is effective at later phases of infection and uses recognition molecules that obtain their diversity through ontogenic rearrangements of the genes.

Two major components of innate immunity are the phagocytic cells (such as neutrophils and macrophages) and natural killer cells. Such cells have the ability to identify and destroy invading pathogens using cell surface protein receptors that are germ-line encoded and have evolved under the process of natural selection to recognise pathogen-associated molecular patterns (PAMPs) such as mannose and lipopolysaccharides (Barton and Medzhitov, 2002; Janeway and Medzhitov, 1999).

While phagocytes combat free-living pathogens in blood stream or tissues, the natural killer cells focus on the fight against virus-infected cells. Innate immunity has the drawback of being capable of recognizing the relatively few microbial structures that are highly conserved, and of being unable to evolve as rapidly as microbes do. In contrast, adaptive immunity involves antigen-specific (target specific) receptors that are generated by somatic rearrangement mechanisms and relies on clonal expansion of subsets of T- and B- lymphocytes that carry the antigen-specific receptors. These rearrangement mechanisms are powerful since they can support the creation of an enormous repertoire of antigen binding structures that broaden the spectrum of protection. Throughout the years however, another major component of the innate immune system, the Complement system, has been discovered and characterised. Complement has obtained a considerable level of sophistication through evolution, and is nowadays recognised to function as a major mediator of the innate immune response. It is known to interact critically with other mechanisms of innate immunity as well as with the adaptive immunity to strengthen host defence.

In 1792 J. Hunter observed that blood resisted bacterial putrefaction for considerably longer periods of time than other tissues did. At the end of the nineteenth century it was noticed that immune haemolysis and immune bacteriolysis needed, in addition to the antibody, a heat labile component that was present in non-immunised serum. Although Buchner in 1889 was likely to have been the first to record this phenomenon, it was in 1895 that J. Bordet introduced the term 'alexin' to describe this component. The name 'complement' was eventually given to this component that augmented/'complemented' the identification of bacterial pathogens in the bloodstream by antibodies. The 'complement' was, therefore, regarded as an important factor for humoral responses.

It was soon discovered that complement was multifactorial. Ferrata in 1907 demonstrated that the euglobulin and pseudoglobulin components of the serum were essential for the action of the complement. By the 1920s it was known that at least four serum fractions were involved in the bactericidal activity of the complement (Osborn, 1937). As protein purification methods improved, knowledge about the composition of complement increased. In the 1950s, the isolation of a protein named 'properdin' associated with an antibody-independent mechanism of complement

action, the alternative pathway, was described (Pillemer et al., 1954). More complement components were identified in the following years. In the 1960s eleven distinct plasma proteins that are now known to participate in the classical pathway of activation were identified mainly in the research groups of R. A. Nelson and H. J. Müller - Eberhard (Nelson et al., 1966). As the characterisation of individual components was achieved, it started to become clearer that complement is a system that protects against pathogens and does not rely exclusively on the functions of antibodies. In the late 1960s the existence of an alternative pathway was finally accepted on scientific grounds (Lepow, 1980). Detailed structural and functional information about the various components of the complement pathways had begun to gather since the 1970s. With the use of molecular biology approaches for the cloning and sequencing of complement proteins and receptors, as well as the construction of animal models, knowledge about the structure and organisation of the complement system has increased significantly (Campbell et al., 1988; Walport, 2001a; Walport, 2001b). The importance of the complement system as an effector of humoral immunity was highlighted with the observations of opsonisation and contribution to cellular immunity (Hosea et al., 1980).

A more complete picture has begun to emerge in recent years after it has been shown that components of the innate immune response like complement play important roles in several processes of acquired immunity like the antigen presentation process and the differentiation of cell lines (Carroll, 2004a; Hoebe et al., 2004; Pepys, 1974). Nowadays it is believed that the innate immune system not only provides a direct immune response, but also has a critical role in determining which antigens the acquired immune system responds to and the nature of that response (Carroll and Janeway, 1999; Fearon and Locksley, 1996; Pepys, 1976)

1.1.1 Overview and organisation of the Complement System

The human complement system consists of about 35 serum and cell surface protein components which participate in a broad range of processes from direct cell lysis to the enhancement of B and T cell responses (Fearon and Carroll, 2000). Soluble complement proteins in human plasma constitute about 15 % of the globulin fraction.

They are also present, at lower concentrations, in other body fluids. Complement has a major role in controlling the uptake and removal, through phagocytosis, of materials like immune complexes, microbes, necrotic or apoptotic cells or cell debris that are recognised by, and activate complement. Multimeric complement proteins such as C1q, Mannan Binding Lectin (MBL) and ficolins act as Pattern Recognition Receptors (PRRs) to recognise and bind to patterns of features associated with targets like microorganisms and apoptotic cells. Interaction principally involves charge clusters or carbohydrate (neutral or charged) recognition (Lu et al., 2002; Medzhitov and Janeway, 2000). Other plasma proteins such as pentraxins or immunoglobulins can act as “adaptors” by binding to the target first and creating a surface pattern that is recognised by complement proteins (Nauta et al., 2003; Sim and Malhotra, 1994). The complement components are organised in three activating pathways and one terminal pathway. (see Fig. 1 for a diagram of the complement system).

Complement is activated by three distinct pathways, the classical, the lectin and the alternative pathways upon recognition of particulate non-self or altered-self materials by binding of complement proteins to charge or carbohydrate arrays (Sim, 1993) (Fig. 1). This results in the assembly of short-lived unstable complexes, the C3 convertases that have the ability to activate C3 which is the central component of the complement system (Sahu and Lambris, 2001). The major fragment which is produced from the activation reaction, C3b, binds covalently to complement-activating surfaces and is mainly responsible for the initiation of most of the effector mechanisms of the system (Law and Dodds, 1997). Both C3b and its cleaved and inactivated form, iC3b, act as opsonins, enhancing the phagocytosis of the activators. In addition, C3b can also form complexes with the C3 convertases to form C5 convertases that initiate the assembly of the Membrane Attack Complex (MAC) which can damage a lipid bilayer of the target cell promoting cell lysis. C3a, which is the minor fragment of C3 produced during its activation, is an anaphylatoxin and like C4a and C5a that fall into the same category, it can mediate inflammatory responses like smooth muscle contraction, an increase of vascular permeability and degranulation of basophils with consequent release of histamine and other vasoactive agents. Very recently, C3a has been shown to have antimicrobial properties, efficiently killing the Gram-negative bacteria *Escherichia coli*, *Pseudomonas aeruginosa* and the Gram-positive *Enterococcus faecalis*, while in mice a synthetic C3a-derived peptide was found to suppress

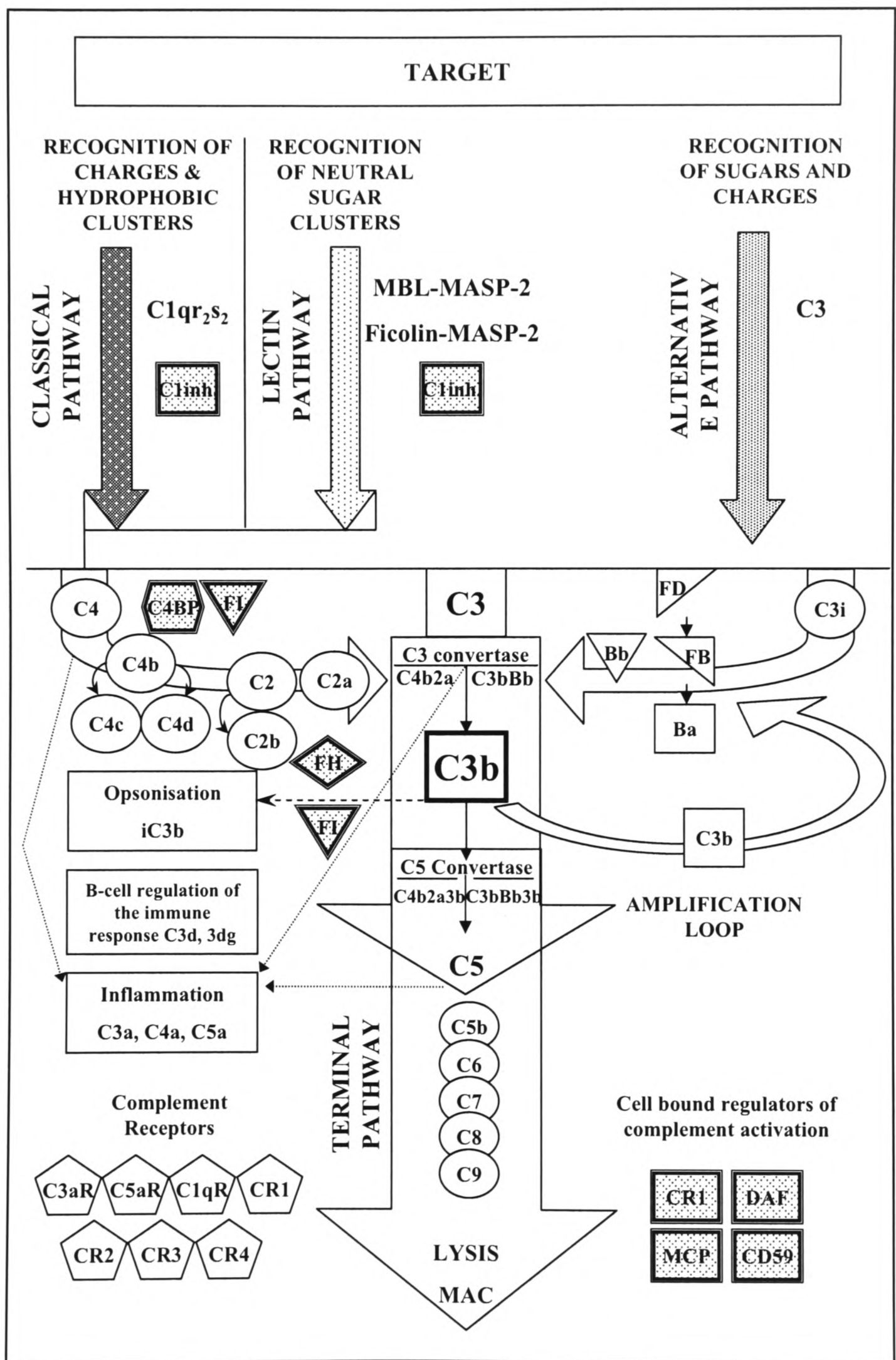


Fig. 1. A schematic representation of the human complement system. The three activation pathways of the system (classical, lectin and alternative) are represented together with the terminal pathway that eventually leads to the formation of the Membrane Attack Complex (MAC) after complement activation. Regulatory inhibitory proteins are shown in shaded boxes; complement receptors in pentagons. One positive regulator, properdin, which stabilizes C3bBb is not shown.

infection by *Streptococcus pyogenes* bacteria (Nordahl et al., 2004). Other effector mechanisms of complement have been reported, which include solubilisation of immune complexes and targeting of antigen to B-cells (Dempsey et al., 1996; Dempsey and Fearon, 1996).

Considering the multiple pathways of activation and the catalytic nature of many interactions, the regulation of the complement system is considered complex. Excessive or inappropriate activation of complement may damage host tissue. Examples of such damage are immune complex diseases like rheumatoid arthritis, certain types of glomerulonephritis like membranoproliferative glomerulonephritis and the atypical Hemolytic Uremic Syndrome (aHUS) (Goodship, 2004; Walport, 2001a) as well as autoimmune disorders like Systemic Lupus Erythematosus (SLE) (Carroll, 2004b) and ischaemia-reperfusion injury (Tsokos and Fleming, 2004). For protection against host destruction, the system uses both serum and cell surface regulators. Almost all mammalian cells express regulators of complement to protect the host against homologous attack events. The manifestation that some of the complement components also participate in adaptive immunity supported the view that the complement system plays a role in bridging innate and adaptive immunity. It was proposed that the complement system ‘instructs’ the humoral response (Fearon and Locksley, 1996).

1.1.1.1 The classical pathway

The classical pathway was the first explored in detail. It is activated by the binding of the first component of the cascade, the C1 complex, to immune complexes or aggregates that contain IgG (IgG1, IgG2 or IgG3) or IgM (Burton and Woof, 1992). Other substances can also activate the pathway without containing antibody. Such include the ligand-bound C-reactive protein (CRP), lipid A of Gram-negative bacteria, capsular polysaccharide of Gram-positive bacteria, mitochondrial cardiolipin and nucleic acids (Kishore and Reid, 2000; Sim, 1993). In these terms, the classical pathway may be dependent on or independent of antibody.

The C1 complex consists of C1q that is a recognition molecule and two serine proteases C1r and C1s, in the following stoichiometry: 1 C1q:2 C1r: 2 C1s (Gigli et al., 1976). C1q is a 450 kDa multimeric protein, shaped like a bunch of six tulips. The 'heads' of the tulips each consist of three globular domains through which C1q binds to targets. The 'stems' are collagen triple helices, to which are bound the serine protease C1r. C1s binds via C1r. Upon binding of the C1q to an activator, movement of the collagen stems cause C1r to autoactivate. The activated C1r subsequently cleaves and activates C1s (Dodds et al., 1978). The activated C1s cleaves C4. The main fragment C4b contains a highly labile internal thiol ester which has the ability to interact through its acyl group with nucleophiles like hydroxyl and amino groups (Law and Dodds, 1997). A small percentage, less than 10%, of C4b becomes covalently bound to the activator material via surface -OH or NH₂ groups, while the rest reacts with water. The generated C4b(H₂O) diffuses away from the site of complement activation. C2 binds to C4b, and the activated C1s of the proximal C1 complex cleaves C2 to generate the classical pathway C3 convertase C4bC2a along with the release of C2b. The C2a is a serine protease but is active only as part of the complex. C2a in C4b2a cleaves C3 to generate C3b that participates in further downstream activation reactions.

The conversion of C3 to C3b occurs with the release of C3a. C3b, like C4b contains an exposed reactive labile thiol ester that mediates covalent binding with any hydroxyl or amino groups or water. The thiol esters are short-lived once exposed ($t_{1/2} < 1$ s) and therefore will only react with the target, which is recognised by and remains bound by C1q on the surface. C3b is formed close to the target surface, as the C4b2a is localised on the surface. For each C1q molecule on an activating surface, approximately 230 C3b molecules are deposited (Ollert et al., 1994). If one of these C3b molecules binds covalently to C4b, then a C5 convertase, C4b2a3b is formed; the C4b and C3b in the complex are responsible for orientating C5 for cleavage by C2a in the convertase complex (Law and Reid, 1995).

The activation of C5 by the C5 convertase initiates a series of downstream events that form the lytic or terminal pathway that is common to all the complement system activation pathways. The cleavage of C5 produces C5a (Fig. 1) which is the most powerful complement-derived anaphylatoxin. C5a is 200-fold more potent than C3a

on a molar basis in causing histamine release and smooth muscle cell contraction. It is also a strong chemotactic agent for phagocytes, it increases neutrophil adhesiveness and promotes aggregation of platelets. The generated C5b does not contain a thiol ester as in C3b and C4b, but instead contains a metastable binding site for C6 that is formed during the cleavage. The C5b, still loosely bound to C3b, binds non-covalently to C6. This binding is followed by subsequent conformational changes that facilitate the interaction with C7, which alters the conformation of the complex. All these driven conformational changes that are part of the terminal reaction cascade, finally lead to the assembly of the Membrane Attack Complex (MAC). The resulting C5b-7 complex is said to undergo a hydrophilic-amphiphilic transition upon binding of C7. This generates a metastable binding site for the phospholipid bilayer of the target cell membrane and the dissociation from C3b. The free C5b-7 complex binds to the β subunit of C8. A conformational change in C8 allows the α chain of C8 to insert into the membrane. The complex C5b-8 then binds C9 and acts as a catalyst in the polymerisation of C9 to form the mature MAC. The MACs integrate into membranes forming ion-permeable channels (Bhakdi and Tranum-Jensen, 1991). The membrane-bound MAC complex has a MW of $1-2 \times 10^6$ Da. Any membrane disruption of this type may eventually lead to cell lysis under the pressure of physiological osmosis. Otherwise, the exchange of materials through the pores into the plasma, may activate a number of cellular metabolic events that will result in the synthesis and release of inflammatory mediators.

1.1.1.2 The lectin pathway

The lectin pathway is highly analogous to the classical pathway and its activation leads to the assembly of a C3 convertase, C4b2a. The recognition molecule for this pathway is Mannan-Binding Lectin (MBL), which is a C-type lectin and a member of the collectin family of proteins that includes both collagen and globular domains. In quaternary structure, MBL is similar to the “bunch of tulips” structure of C1q. MBL binds in a Ca^{2+} -dependent manner to the target via its globular heads (C-type lectin domains) that recognise neutral sugars, with preference for mannose, N-acetyl glucosamine (GlcNAc) and fucose. This is in contrast to C1q that recognises charge clusters. Such carbohydrates occur on a wide range of microorganisms (Lu et al.,

2002). In circulation MBL is complexed with a group of proteases, the MBL-Associated Serine Proteases (MASPs) which activate C2 and C4 leading to the central C3 turnover step. The MASPs 1-3 (discussed in detail in section 1.2.4) are homologues of C1r and C1s. Mannan-Binding Lectin also recognises carbohydrate structures on antibodies, including the common IgG glycosylation variant IgG-G0 (Malhotra et al., 1995) and polymeric IgA (Roos et al., 2001). Recent evidence suggests that recognition of carbohydrates among the Ig classes may be highly selective (Arnold et al., 2004). Considering the nature of the carbohydrate recognition domains, the lectin pathway is probably the oldest in evolutionary terms. Recently another class of molecules, the ficolins were shown to activate complement by circulating, like MBL, in complex with MASPs (Lu et al., 2002). The ficolins resemble C1q and MBL in quaternary structure (Holmskov et al., 2003; Matsushita et al., 2000; Matsushita et al., 2001; Matsushita et al., 1996) by having globular domains which make up their head region, and collagenous stalks. In contrast to MBL, ficolin sequences lack an α -helical region and their globular region is a fibrinogen-like domain rather than a C-type lectin. Three human ficolins have been discovered so far: L- and H-ficolin found in serum and M-ficolin on the surface of non-differentiated blood monocytes (Endo et al., 1996b). The last is unlikely to be associated with the activation of complement. Like MBL, L-ficolin and H-ficolin have the capability of forming oligomers that are made of up to four and six trimers, respectively (Lu and Le, 1998; Yae et al., 1991). Recent data show striking differences in selectivity between MBL and the ficolins (Krarup et al., 2004). Binding of MBL to sugar arrays is inhibited by all carbohydrates with equatorial C3-OH and C4-OH groups. L-ficolin binding can be inhibited by an acetyl group independently of whether it is attached to a carbohydrate hexose ring (*N*-acetylmannosamine, *N*-acetylglucosamine and *N*-acetylgalactosamine) or is part of *N*-acetylcysteine, *N*-acetylglycine or *N*-acetylcholine. The selectivity of H-ficolin requires further investigation as recent evidence (Krarup et al., 2004) shows that although it binds to *Aerococcus viridans*, it cannot be inhibited by *N*-acetylglucosamine as was previously reported (Sugimoto et al., 1998).

Ficolins, like MBL in circulation, are complexed with the MBL-Associated Serine Proteases (MASPs). The MASPs were discovered quite recently and all three, MASP-1, MASP-2 and MASP-3 (an alternative splice product of the *MASP-1* gene), have the

same domain organisation as C1r and C1s (Schwaebler et al., 2002). Another molecule, MASP-2 which is an alternative splice product of the *MASP-2* gene lacking a serine protease domain is also complexed with MBL (Schwaebler et al., 2002). Studies have revealed that MASP-2 activates C2 and C4 therefore acting like C1s (Ambrus et al., 2003). The roles of MASP-1, MASP-3 and MASP-2 are unknown, but they do not seem to be involved in complement activation.

1.1.1.3 The alternative pathway

The alternative pathway recognises targets with clusters of both charged and neutral carbohydrates on their surfaces. In addition, it also has both antibody-dependent and antibody-independent modes of activation. It relies on the spontaneous hydrolysis of the thiol ester of C3 (Pangburn et al., 1980) as described by the “C3 tick-over” hypothesis (Nicol and Lachmann, 1973). The thiol ester bond can be hydrolysed by water or nucleophiles like ammonia or ethanolamine at a low rate, so that C3 undergoes a conformational change into C3(H₂O). With the normal plasma concentrations of ammonia (12 µM), ethanolamine (1.7 µM) and methylamine (0.1 µM) causing an estimated spontaneous hydrolysis rate of 0.005% C3/hour, it is believed that water must be the most effective nucleophile *in vivo* (Pangburn and Muller-Eberhard, 1980). C3(H₂O) resembles C3b in structure and function. C3(H₂O) has the ability to bind to factor B (fB) in a Mg²⁺-dependent manner, and the resulting C3(H₂O)B complex is recognised by factor D (fD). Factor D circulates in an active form and cleaves a bond in factor B to release the smaller fragment Ba, yielding the C3(H₂O)Bb complex that is an unstable fluid-phase C3 convertase of the complement system. This convertase can activate C3 to release C3a and C3b. The C3b formed can bind randomly and covalently to any macromolecular surface. It is thought that C3 binds mainly to OH groups on the surface of carbohydrates. Once C3b is deposited it can bind more factor B that is cleaved by factor D, forming more of the alternative pathway C3 convertase. On non-activating surfaces however, the first-deposited C3b can form a complex with a regulator molecule like factor H (fH), or with its cell surface homologues like Complement Receptor 1 (CR1), Membrane Cofactor Protein or Decay Accelerating Factor, instead of fB. On these surfaces fH has the ability to bind to C3b and to sialylated oligosaccharides or other polyanions that are present on

non-activating particles. The complexes C3bH or C3bCR1 are broken down by complement factor I (fI) which is one of the key regulators of the complement system. Cleaved C3b, iC3b functions as an opsonin. If fH binds before fB, no amplification of C3b fixation occurs, so that the surface is not opsonised. The C3bBb complex can be stabilised by properdin (section 1.1.2.1).

It is clear that the specificity of this pathway is based on the carbohydrate content and charge distribution of the microbial surface. These parameters along with the activities of soluble down-regulators and the presence or not of complement-regulatory cell surface proteins, regulate the function of this pathway. The alternative pathway has been described as being activated by a number of substances including antigen-bound IgG, IgA, LPS from Gram-negative bacteria, teichoic acid from Gram-positive bacteria, influenza and Epstein Barr viruses, virus-infected cells, yeasts, protozoans and helminths (Sim, 1993; Sim and Laich, 2000).

1.1.2 Regulation of the Complement System

The effective role of complement as a major mediator of the innate immune response requires a fine balance of control to prevent host tissue damage. The strict regulation of the amplification mechanisms is achieved via the existence of serum and cell surface control proteins.

Among the proteins that can regulate the classical and the lectin pathways is the serpin (serine protease inhibitor) C1inhibitor (C1inh). C1inh can bind covalently and inhibit irreversibly C1r, C1s, MASP-1 and MASP-2 serine proteases (Davis, 2004; Sim and Tsiftoglou, 2004). MASP-3 is thought to be an exception (Dahl et al., 2001). In addition MASP-1 and MASP-2 can also be inhibited by α_2 macroglobulin (Presanis et al., 2004). Similarly to many of the coagulation proteases, these particular serine proteases circulate as proenzymes, they can be autoactivated by autolysis or proteolysis and can be inactivated by a serpin. In contrast to C1r, C1s and the MASP group, C2 and fB do circulate as proenzymes, but are not controlled by serpins or α_2 M. As they get activated they participate in the formation of active, but unstable convertase complexes with C4b or C3b, that have short half lives of less than five

minutes. The formation of the Mg^{2+} -dependent C4b2a complex can also be regulated by the effects of the soluble C4bp and the cell-surface complement receptor 1 (CR1) and decay-accelerating factor (DAF). In similar terms, the alternative pathway C3 convertase can dissociate under the effect of fH, CR1 or DAF. There is evidence that the active site of the serine protease domain of Bb is irreversibly disorientated when Bb is dissociated from C3b (Xu et al., 2001). The dissociated C3b or C4b are cleaved by fI in the presence of a cofactor molecule like CR1, MCP, fH (for C3b) or C4bp, CR1 and MCP (for C4b). The cleavage of C3b yields iC3b and the cleavage of C4b, C4c and C4d respectively. DAF and MCP are widely present on human tissues, while CR1 has more limited distribution. The amplification of C3b fixation is prevented in the host tissues that express these regulators.

1.1.2.1 Regulation in the fluid phase

The major regulatory components of complement that are in circulation are Properdin, fH, C4bp and fI. Properdin was first purified in the 1950s and was found to be associated with activation of the alternative pathway (Pillemer et al., 1954). Hence the earlier name of the alternative pathway, the properdin pathway. It is a 56 kDa protein known to exist in various oligomeric forms like dimers, trimers, tetramers and higher oligomers. The trimeric forms are the most common. The importance of properdin lies in its ability to stabilise the C3 convertase, C3bBb (Volanakis, 1990) by causing a decrease in the dissociation rate of Bb from C3b. A synthetic peptide corresponding to the region 1402-1435 of C3 can bind properdin, block the binding of properdin to C3 and inhibit the lysis of rabbit erythrocytes via the alternative pathway (Daoudaki et al., 1988). Mapping studies have indicated that the binding site for properdin lies on the 42 kDa C-terminal α chain fragment of C3 (Lambris et al., 1984). It has also been proposed that fB has binding sites for properdin (DiScipio, 1981; Farries et al., 1988; Volanakis, 1990).

The Regulators of Complement Activation (RCA) gene cluster on human chromosome region 1q32 encodes several complement regulators and receptors (Rodriguez de Cordoba et al., 1999) including complement receptors 1 (CR1/CD35)(Krych-Goldberg and Atkinson, 2001) and 2 (CR2/CD21)(Ahearn and

Fearon, 1989), decay-accelerating factor (DAF/CD55)(Lublin and Atkinson, 1989), membrane cofactor protein (MCP/CD46) (Riley-Vargas et al., 2004) (all cell membrane-bound) and fH, fH-related proteins (FHRs) (Rodriguez de Cordoba et al., 2004) and C4bp (Blom et al., 2004) (in circulation) (Fig. 2). They are homologous, being made up almost entirely of Complement Control Protein (CCP) or Short Consensus Repet (SCR) modules (section 1.2.6.2) and have related functions. There is variation in the number of CCP domains. Among the cell-surface bound components, MCP and DAF contain 4, while CR1 has up to 44 depending on the allelic variant.

Factor H can regulate complement both in fluid-phase and on cell surfaces. It is involved as the major co-factor in the fI-mediated cleavage of C3b, while it prevents the assembly of and dissociates the alternative complement pathway C3 convertase, C3bBb complex (Sim et al., 1993a). It is also very important for the discrimination of activators from non-activators of complement that is determined by its avidity for surface-associated C3b (Pangburn, 2000). fH can regulate the alternative pathway by a) inactivation of C3b in the presence of factor I, b) acceleration of the decay of the alternative pathway C3 convertase (C3bBb) and c) competition with factor B for binding to C3b (Seya et al., 1995). The physiology and function of fH are discussed more extensively in section 1.5.

The human gene that encodes fH, *HFI*, is closely linked to the *FHR1*, *FHR2*, *FHR3*, *FHR4* and *FHR5* genes encoding five factor-H related human plasma proteins (Diaz-Guillen et al., 1999). The genes identified contain a number of large genomic duplications that include several exons and encode for six proteins that are structurally related and immunochemically cross-reactive with fH. The fHR proteins are likely synthesized in the liver and their native concentrations are much less than that of fH (Zipfel et al., 1999). Structurally they are all composed of CCPs with various degrees of similarity with CCPs of fH. They do not contain any CCPs homologous to the fH domains that exhibit decay acceleration, fI-cofactor or complement regulatory activities. However, binding to C3b has been shown for FHR3, FHR4 and FHR5 (Rodriguez de Cordoba et al., 2004). The functional properties of FHR1-5 have not been fully defined yet, but raise considerable interest as they could be associated with pathological conditions and perhaps complement regulation.

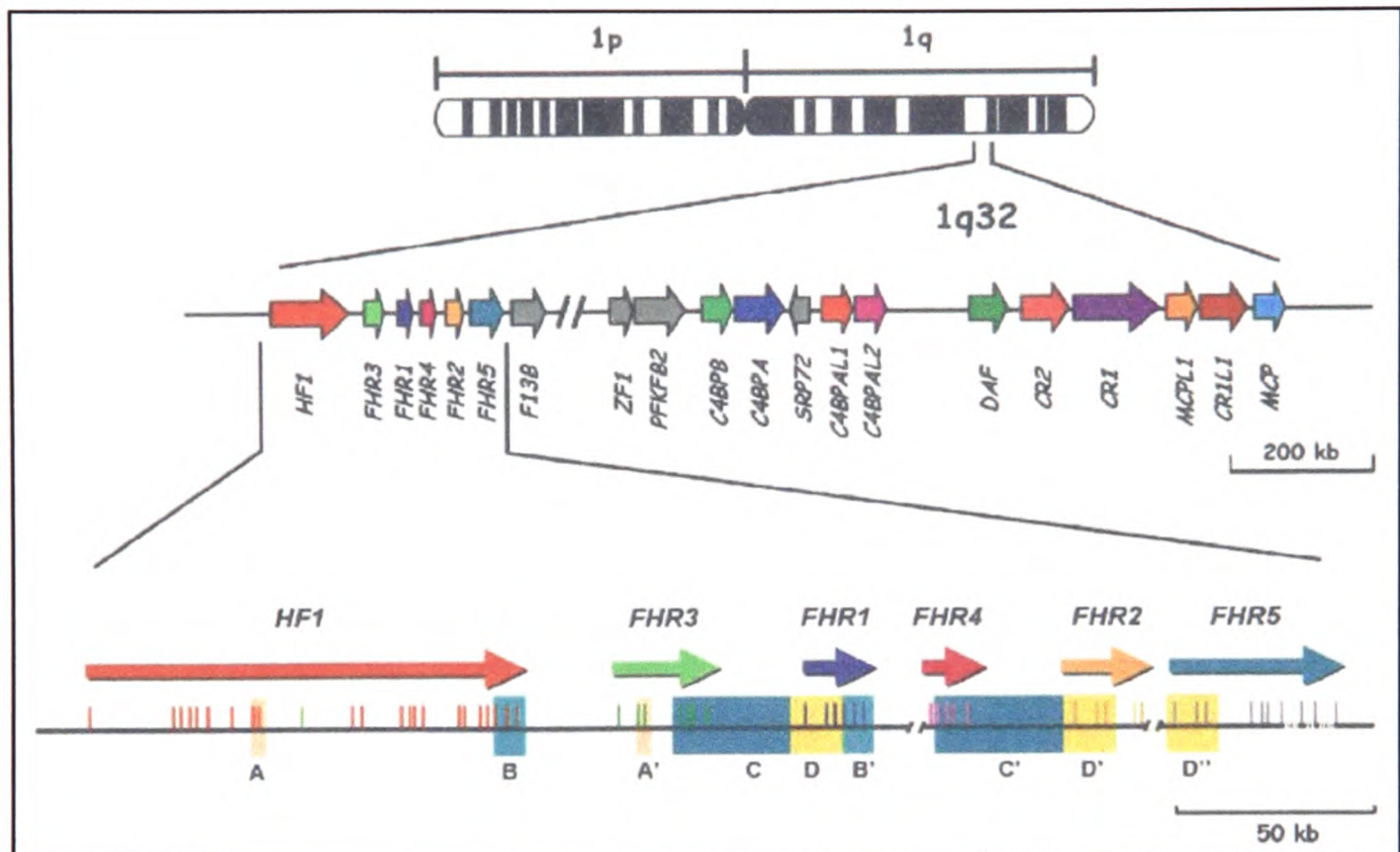


Fig. 2. A detailed diagrammatical representation of the human RCA gene cluster map. The human RCA gene cluster is located on human chromosome 1q32 and contains more than 60 genes of which 15 are complement related. All the complement related genes are arranged in tandem fashion in two regional groups. A 900 kbp long telomeric region that contains the *C4BPB*, *C4BPA*, *SRP72*, *C4BPAL1*, *C4BPAL2*, *DAF*, *CR2*, *CR1*, *MCPL1*, *CRIL1* and *MCP* genes and a 650 kbp long centromeric region that contains the *HF1*, *FHR3*, *FHR1*, *FHR4*, *FHR2*, *FHR5* genes and the gene that encodes the B subunit of the coagulation Factor XIII, the *F13B*. Sequence analyses have suggested that the *C4BPAL1*, *C4BPAL2*, *MCPL1* and *CRIL1* genes are currently pseudogenes in humans and arose through duplication events from the original genes encoding for the complement regulators. The space between the two regions contains several non-complement related genes. The lower part of the diagram shows the presence of large genomic duplications (A-D) that contain various exons of the *HF1/FHR1-5* genes.

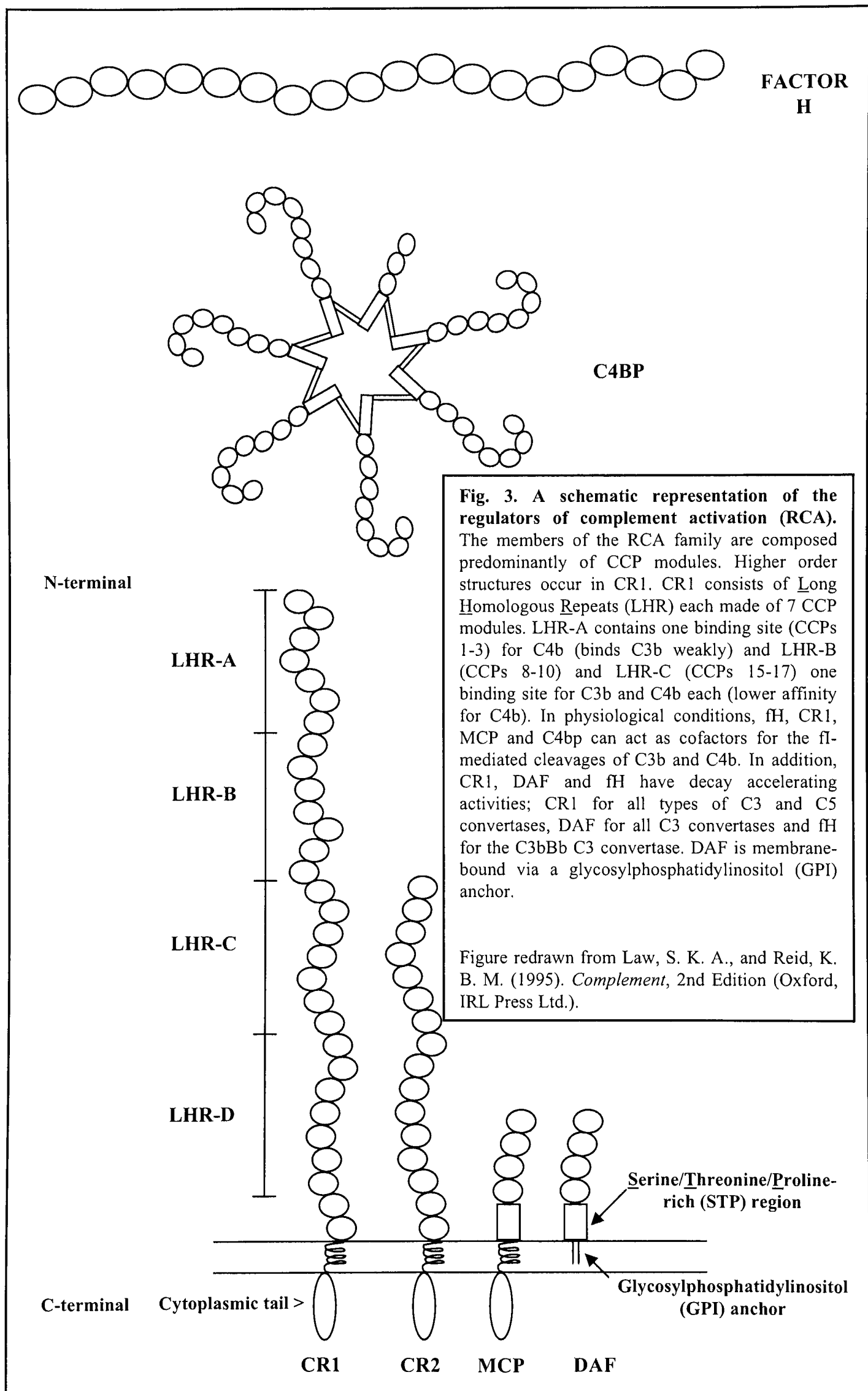
Figure from Rodriguez de Cordoba, S., Esparza-Gordillo, J., Goicoechea de Jorge, E., Lopez-Trascasa, M., and Sanchez-Corral, P. (2004). *The human complement factor H: functional roles, genetic variations and disease associations*. Mol Immunol 41, 355-367.

C4bp is a large glycoprotein of up to 570 kDa with an estimated plasma concentration of 200 µg/ml (Dahlback, 1983). From the several isoforms that have been characterised, the major one (75-80% of total C4bp plasma population) is composed of seven identical α -chains and one β -chain that are linked through their C-termini (Hillarp and Dahlback, 1990; Scharfstein et al., 1978a) (Fig. 3). The α - and β -chains contain eight and three CCP domains respectively. The C-terminal extensions (60 aa) contain two cysteines that participate in disulphide linkages between the chains. In addition each extension contains an amphipathic α -helix region that is required for intracellular polymerisation of the molecule (Kask et al., 2002).

C4bp is an inhibitor of the classical complement pathway where it controls C4b-mediated reactions. C4bp acts as a cofactor for the fl mediated cleavage of C4b that prevents the assembly of the classical pathway C3 convertase C4bC2a (Scharfstein et al., 1978b) (Fig. 1). C4bp also prevents the assembly of the classical pathway C3 convertase C4bC2a by binding nascent C4b and accelerating the natural decay of the complex (Gigli et al., 1979). In addition, C4bp also acts as a cofactor for the fl mediated cleavage of C3b in fluid phase, but only in non-physiological conditions (Seya et al., 1985; Seya et al., 1995; Sim et al., 1981a). It seems that C4bp is not an inhibitor of the alternative pathway C3 convertase since it cannot inhibit it in the fluid phase (Seya et al., 1985) and it does not reduce the hemolytic activity of cell-bound C3b unless present in very high concentrations (Fujita and Nussenzweig, 1979).

1.1.2.2 Surface-associated regulation

Complement Receptor 1 (CR1, CD35 or immune adherence receptor) is a single chain, type 1 transmembrane glycoprotein expressed on red blood cells and most leukocytes. In CR1 all CCPs, with the exception of two at the C-terminal, are part of larger units that are called Long Homologous Repeats (LHRs), each composed of seven CCPs (Fig. 3). This pattern suggests that seven CCPs form an ancient unit which underwent duplication and that high homology was conserved by concerted evolution (Clemenza et al., 1997). Structural duplication supports not only functional duplication but also synergy. For the case of CR1 duplication and divergence created



a large, multifunctional protein capable of binding both C3b and C4b and having both decay accelerating activity and cofactor activity.

To date four allelic variants of CR1 [average natural frequencies] have been described (CR1*3 [0.01], CR1*1 [0.83], CR1*2 [0.15] and CR1*4 [<0.01]; from the smallest to the largest) with MW ranging from 190 to 250 kDa in 30 kDa differences, respectively. Correspondingly, the number of the LHRs varies from three to six. The level of this polymorphism between these alleles is believed to have resulted from unequal crossing-over events through the highly homologous regions. Despite their large differences in MW, all variants have been shown to have similar functional properties, as cofactors for the fI activity as well as in the inhibition of both the classical and alternative pathway C3 convertases (Seya et al., 1985). Further polymorphisms have been observed regarding the copy number on erythrocytes which is under the control of high (H) and low (L) copy number alleles (Wilson et al., 1986). Homozygotes for the L allele have fewer than 200 copies, while the homozygotes for the H allele have 3-4 times more copies per red blood cell (Wilson et al., 1986).

CR1 is expressed on the surface of all leukocytes, except natural killer cells and the majority of T cells (Fearon, 1980; Tedder et al., 1983). It is not expressed on the surface of primate platelets. Nearly 90 % of CR1 *in vivo* is found on erythrocytes (population numerical predominance), while B cells and monocytes express 20,000-40,000 copies per cell. The identification of CR1 on some tissues, like on follicular dendritic cells in the germinal centres of the lymph nodes, remains to be explored in more detail (Reynes et al., 1985).

CR1 serves several functions. The erythrocyte CR1 serves as an immune adherence receptor for C3b/C4b-opsonised immune complexes. These bind to CR1 on erythrocytes and are moved to the liver and spleen where they are processed by tissue macrophages. CR1 has decay-accelerating activity for all types of C3 and C5 convertases, while with its co-factor activity properties, CR1 participates in the cleavage and inactivation of C3b and C4b. The presence of CR1 on the B cell surface has been implicated in their proliferation and differentiation (Hivroz et al., 1991; Weiss et al., 1987), while on the surface of neutrophils and monocytes CR1 mediates phagocytosis when these cells are activated by cytokines and other mediators of

inflammation (Wright and Silverstein, 1982). The stimulation of CR1 on phagocytes leads to the secretion of interleukin IL-1 α , IL-1 β (Bacle et al., 1990) and prostaglandins (Thieblemont et al., 1993). It has also been suggested that CR1 binds to the collagenous regions of C1q (Klickstein et al., 1997) and MBL (Ghiran et al., 2000).

CR1 has three sites of interaction with C3b/C4b and with the convertases (Krych et al., 1998). Each of the LHR A, B and C has a single site in its three amino-terminal CCPs of each LHR. Site 1 which is located in CCPs 1-3 is unique and binds C4b and very weakly C3b. It has high decay accelerating activity for the C3 convertases, but low activity as cofactor for the breakdown of C3/C4b by factor I. Site 2 located in CCPs 8-10 and nearly identical in sequence to site 1, binds both C3b and C4b efficiently (with lower affinity for C4b) and has high cofactor activity for both, but low decay accelerating activity for the C3 convertases. Site 3 has similar properties to site 2 and is located in CCPs 15-17. The distinctive difference in selectivity between sites 1 and 2, 3 lies in amino acid differences between CCPs 1 and 2, CCPs 8 and 9 and CCPs 15 and 16. Binding sites for C1q (Klickstein et al., 1997) and MBL (Ghiran et al., 2000) have been identified in LHR D, but their location in respect to CCP modules has not been fully characterised.

Since CR1 is a pivotal complement regulator, it has excited interest as a therapeutic target for the inhibition of undesired or excessive complement activation. Recombinant soluble forms of the protein lacking the transmembrane and the cytoplasmic tail have been produced and used successfully in animal models of complement mediated tissue injury (Klickstein et al., 2000).

The Decay Accelerating Factor (DAF) or CD55 (Fig. 3) was first isolated in 1981 from guinea pig serum (membrane protein shed into fluid phase) as a single chain protein with a MW of 60 kDa as judged by SDS-PAGE under reducing conditions (Nicholson-Weller et al., 1981). Characterisation studies, including the early staining of human DAF purified from human erythrocyte stroma with periodic acid Schiff reagent (Nicholson-Weller et al., 1982), demonstrated that DAF is a glycoprotein. Up to date, the role of DAF is well established; its primary function is to inhibit reversibly the assembly of the C3 convertases which act as the amplification steps in

the complement cascade, but it lacks any cofactor activity for fI-mediated cleavage of C4b or C3b (Lublin and Atkinson, 1989; Lukacik et al., 2004). DAF does not actually prevent the initial binding of C2 or fB to the cell surface where C3b or C4b is present, but it rapidly dissociates C2a or Bb from their binding sites, thus blocking the convertase formation.

DAF is present in nearly all peripheral blood cells: erythrocytes, granulocytes, T and B lymphocytes, monocytes and platelets (Kinoshita et al., 1985; Nicholson-Weller et al., 1985) and also in bone marrow mononuclear cells and erythroid progenitors (Moore et al., 1985). Quantification studies have shown 3,000 DAF molecules per erythrocyte, 85,000 per neutrophil, 68,000 per monocyte, 33,000 per lymphocyte (B cell more than T cell) and 2,000 per platelet (Kinoshita et al., 1985). The presence of DAF on several tissues and in extracellular fluids is indicative of its wide distribution and is supportive of its major physiological role in the control of the complement response (Medof et al., 1987). In addition, DAF also participates in many other non-complement interactions, including the ones with a range of viral and bacterial pathogens and the one with CD97. Viral and bacterial pathogens such as several echoviruses, enteroviruses and *E. coli*, respectively, have evolved to use CD55 for cell adhesion and invasion to host cells (Lea, 2002). CD55 also binds to CD97 that is expressed restrictively on leucocytes and is rapidly up-regulated on the activation of T and B cells; the precise biological role of this interaction is not yet clear, but it is suggested that it can be important for mediating adhesion and signalling within inflammatory and immune responses (Lea, 2002). The Human DAF from erythrocytes has a MW of approximately 74,000 Da.

Structurally, DAF has a C-terminal glycosylphosphatidylinositol (GPI) adduct that functions as an anchor to the cell membrane (Fig. 3). This anchor provides also good flexibility and mobility that enhance the response of DAF molecules against a high localised presence of C3b and C4b fragments on the cell surface. DAF consists of a serine/threonine/proline-rich (STP) region that is heavily glycosylated with highly sialylated O-linked carbohydrates and four consecutive, membrane-distal, CCP domains that are characteristic of complement regulators. In addition, the mature form of DAF also contains one N-linked carbohydrate (Lukacik et al., 2004). The characterisation of the structure of DAF (Lukacik et al., 2004) will support further the

understanding of its role in regulating the convertases through the construction of models. The mechanisms of decay acceleration by CD55 of the C3 convertases are not well understood at the molecular level. Despite similar affinities of DAF for the complexes C3bB and C3bBb, DAF accelerates the decay only of the active C3bBb convertases and not of its precursor (Hourcade et al., 1999). The data available suggest that DAF must bind sites in both of the components of the convertases. Bound CD55 promotes the dissociation of the convertase only after the activation cleavage of intact fB by fD; DAF accelerates the decay of the C3bBb convertase by weakening the association between C3b and Bb. The dissociation of the convertase reduces the affinity of DAF for the convertase components.

The Membrane Cofactor Protein (MCP) or CD46 is another well characterised member of the RCA family (Fig. 3). CD46 was first identified in 1985 as a C3b and C4b binding protein of human peripheral blood mononuclear cells (for a review see (Riley-Vargas et al., 2004). CD46 is known to be expressed on most cells in all tissues as four isoforms that are derived through alternative splicing of a single ~46 kbp gene. Structurally CD46 shares core architecture with DAF. The N-terminus of each isoform consists of four CCP domains with CCPs 1, 2 and 4 each containing one N-linked carbohydrate. The isoforms exhibit variability in the serine/threonine/proline-rich (STP) region that is heavily O-glycosylated and in the C-terminus that contains a distinct cytoplasmic tail (CYT). The STP region is a site of alternative splicing arising from three separate exons. The most common isoforms in normal human tissue contain the C region, while the B region is alternatively spliced giving rise to either a BC or C STP region. CD46 is not present on human red blood cells (Cole et al., 1985), but it is found on the erythrocytes of most other primates (Nickells and Atkinson, 1990).

CD46 protects host cells from complement attack by serving as a cofactor for the factor I mediated cleavage of C3b and C4b bound to cells. CD46 is an intrinsic cofactor for the cleavage of C3b and C4b deposited on the same cell surface as is CD46. The cleavage of C3b produces iC3b, while of C4b produces C4c and C4d (Seya et al., 1986). C4c is liberated into circulation, while C4d remains attached to the cell surface. CD46 has been shown to be most effective in the control of the alternative pathway amplification loop (Barilla-LaBarca et al., 2002; Devaux et al.,

1999). The data suggest that CD46 acts as a scanner and controller monitoring the deposition of mainly C3b on the cell surface and blocking the initiation of the alternative complement pathway. The linkage of CD46 deficiency to some occurrences of the atypical Hemolytic Uremic Syndrome highlights the important role of CD46 in the control of complement on injured tissue (Goodship et al., 2004; Richards et al., 2003).

CD46, due to its broad cell surface expression, its complement regulation functions and its cell signalling capabilities, has evolved as a target for at least seven human pathogens (Riley-Vargas et al., 2004). So far CD46 is known to interact with the Measles virus (MV), *Neisseria gonorrhoeae* and *Neisseria meningitidis*, Human Herpesvirus 6, *Streptococcus pyogenes* and Adenoviruses groups B and D. Although detailed mapping of the sites of interaction has not yet been performed, domains necessary for these interactions have been identified. Adhesion of *Neisseria gonorrhoeae* via the pilus and interactions of *Streptococcus pyogenes* via M protein, both require regions in CCPs 3 and 4, while human herpesvirus 6 requires CCPs 2 and 3 (Riley-Vargas et al., 2004). The CD46-MV interaction is the most thoroughly studied. The engagement of CD46 by pathogens has further implications. Binding of Measles virus to CD46 induces a reduction in IL-12 expression in human macrophages (Karp et al., 1996), an increase in nitric oxide production in macrophages of mice (Katayama et al., 2000) and variations in the internalization pathways of CD46 in non-lymphoid cell lines (Crimeen-Irwin et al., 2003). CD46 is downregulated following the initial attachment of MV (Bartz et al., 1996). In addition, MV-induced CD-46 down regulation leads to an increased sensitivity of the infected cell to complement (Schnorr et al., 1995). Although further work is necessary for exploring and refining further the interactions of the pathogens with CD46, it is clear that a number of microbes with diverse disease manifestations have evolved different strategies to exploit the interaction with CD46 to enhance the susceptibility of the host to infection.

1.1.3 The Membrane Attack Complex

Lysis is considered an important part of the response to some infectious agents, not just in the early phases of infection, but also once adaptive immunity has been established (Barrington et al., 2001). The Membrane Attack complex (MAC) is the mediator of complement dependent cell lysis and although its components have been well characterised, the exact nature of interactions with the membrane to cause lysis are still under detailed investigation (Cole and Morgan, 2003). Although so far the 'leaky-patch' hypothesis has been dominant (Esser, 1991) (Fig. 4), in the light of new evidence it appears that the MAC need not form pores, but when C9 is present in low copies, "functional" channels rather than rigid, hollow protein-lined channels visible in electron microscopy may be formed (Bhakdi and Trantum-Jensen, 1991).

Experiments have shown that the efficiency with which lysis occurs depends on the number of MACs in the cell membrane, on the composition of the extracellular fluid and the type or metabolic condition of cell. The operation of a functional MAC is driven by the osmotic gradient that causes the target cell to swell and burst. The presence of Ca^{2+} in the extracellular matrix is important, too. During a MAC attack, an influx of calcium excess causes loss of the mitochondrial transmembrane potential, driving the cell to an energy crisis since the energy-demanding pumps are trying to redress the ionic balance (Papadimitriou et al., 1994). Calcium is believed to have a dual role, protecting the cells when MAC is present in sub-lytic doses, but contributing to cell death when damage is broader (Morgan et al., 1986). In addition, there is exhaustive loss of ATP and its precursors through the MAC complex to the extracellular environment (Papadimitriou et al., 1991).

The activity of the Membrane Attack Complex is controlled by two soluble proteins, the S protein (vitronectin) and SP-40 (clusterin) that inhibit the insertion of the MAC into the lipid bilayer. CD59, a widespread cell-surface protein, binds to the C5b-8 complex and prevents C9 binding for MAC assembly (Davies and Lachmann, 1993; Morley and Walport, 2000; Walport, 2001a).

The lysis of metabolically-active nucleated cells differs from that of non-nucleated erythrocytes and displays 'multi-hit' kinetics, supporting the requirement for assembly of several MACs. Nucleated cells are more difficult to lyse as they possess a variety of protective mechanisms: ion pumps that can rebalance the effects of

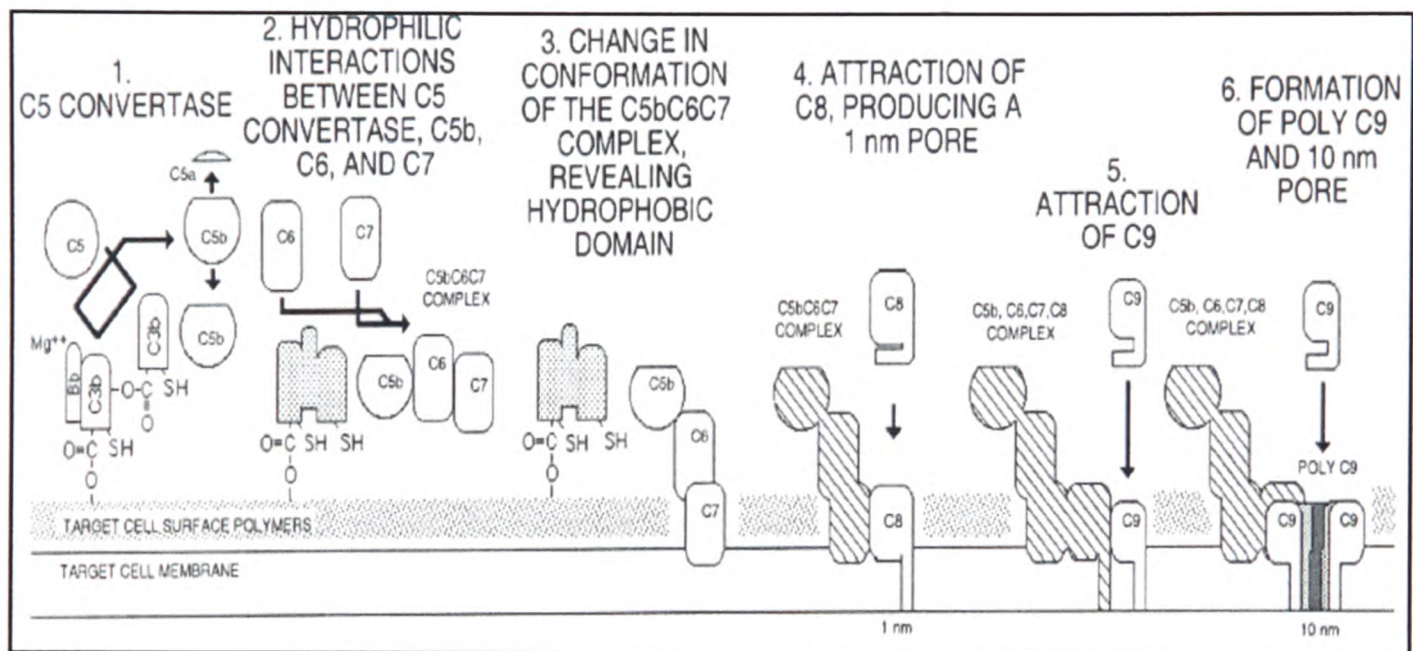


Fig. 4. A flow chart diagrammatical representation of the Membrane Attack Complex (MAC) assembly as described by the 'Leaky-patch' hypothesis. The association of all the terminal pathway components following complement activation, leads eventually to the lysis of the complement activator.

Figure from www.dent.ucla.edu/sod/courses/OB471b/Ch_03_-_Complement.pdf

membrane pores and provide the capacity to remove MACs in membrane vesicles (Morgan, 1989) and can express cell-bound regulators (CD59) or proteases or kinases that block the MAC assembly (Cole and Morgan, 2003). Gram-positive bacteria, in contrast to Gram-negative species like *Neisseria*, are more resistant to lysis as they possess a thick cell wall that prevents the MAC from reaching the inner bacterial plasma membrane (Cole and Morgan, 2003).

The MAC system has been implicated in a variety of other non-lytic effects that vary depending on the nature of target and the system involved. On cells that show resistance to lysis, like neutrophils and phagocytes, the MAC induces activation with secretion of pro-inflammatory mediators such as cytokines, prostaglandins, thromboxanes, leukotrienes and reactive oxygen species that stimulate the inflammatory response preparing the host immune system to fight pathogens (Hansch et al., 1984). Uncontrolled secretion of such compounds can have devastating effects as in the case of membranous glomerulonephritis (Takano et al., 2001) and dilated cardiomyopathy (Zwaka et al., 2002). Although there is still a complex picture, the MAC assembly has also been described to regulate intracellular signal transduction pathways that can affect the gene transcription process (Cole and Morgan, 2003).

In the past 15 years the MAC has been implicated to participate in the cell cycle as a stimulus for proliferation. Several models, including aortic smooth muscle cells (Niculescu et al., 1999) and Schwann cells (Dashiell et al., 2000), showed that non-lethal MAC attack did indeed stimulate cell proliferation. This was not the case for end-cell types like oligodendrocytes (Rus et al., 1996). The MAC appears to induce entry to the cell cycle in some cell types and leads the cell to division if the cell is capable of dividing. This understanding can have a great importance in exploring fundamental questions surrounding the understanding of tissue repair after inflammation. The area requires detailed refinement as cell cycle regulation and proliferation are multi-factorially controlled processes.

1.2 THE COMPLEMENT SERINE PROTEASES

The serine proteases are a family of proteolytic enzymes that utilize a uniquely activated serine residue in the substrate-binding pocket to catalyse the hydrolysis of peptide bonds. Serine proteases are broadly distributed and participate in several diverse but basic biological processes like digestion, cell differentiation, fertilisation and blood coagulation (Horl, 1989). From the approximately 400-500 proteases in the human genome, about 30% are predicted to be serine proteases (Southan, 2001). The serine proteases show a preference for the hydrolysis of peptide bonds adjacent to a particular class of amino acids. For the trypsin-like group members, there is a preference for basic amino acids such as R or K, since a D residue in the substrate binding pocket can form a strong electrostatic bond with these residues. On the other hand, the chymotrypsin-like members show a preference for an aromatic or bulky non-polar amino acid such as W, F, Y or L since they bear a non-polar binding pocket with a residue like S. The elastase-like enzymes, on the other hand, have bulky amino acids in their pockets and require small hydrophobic residues like A or V (Branden and Tooze, 1991; Styer, 1995).

Seven serine proteases, factor D (fD), MASP-2, C1s, C1r, factor B (fB), C2 and factor I (fI), play key roles in the generation of complement activities in the amplification and regulation of cascade reactions (Arlaud et al., 1998; Law and Reid, 1995; McAleer and Sim, 1993; Sim and Tsiftoglou, 2004) (Table 1). Two additional homologues of MASP-2, namely MASP-1 and MASP-3, have been identified, but they do not seem to have a role in the complement system.

The complement proteases exhibit very narrow trypsin-like specificities limited to the cleavage of a few specific arginyl bonds in their natural substrates. The majority of these proteolytic enzymes function within complex multimolecular assemblies with the support of cofactors in some cases. The catalytic subunits of the complement proteases are single serine protease domains which despite sharing a common architecture with other members of the chymotrypsin family, also contain some unique structural and functional characteristics that differentiate them from typical pancreatic serine proteases. In several structural aspects the complement serine

Table 1. Properties of the human complement serine proteases.

Properties		Average serum		Apparent mol. mass	Amino acids in mature enzyme	Genetic locus	N-glycosylation sites (position in mature enzyme)	Cofactors	Natural Substrates		Synthetic Substrates
Protease		Concentration (µg/mL)	(kDa)						P _n P ₁ P ₂ P ₃ P ₄ P ₁ †P ₁ †P ₂ †P ₃ †P ₄ †P _n †	P _n P ₃ P ₂ P ₁ †P ₁ †P ₂ †P ₃ †P ₄ †P _n †	
FACTOR D EC: [3.4.21.46]		1.5	25	228	228	19	0	C3b	<u>fB</u> : Q ²³¹ QK R†K I V L ²³⁸		Z-R † SBzl Z-K † SBzl
		200	93	739	739	6p21.3	4 (97, 117, 260, 353)	C3b	<u>C3</u> : G ⁷²³ L A R†S N L D ⁷³⁰ <u>C5</u> : Q ⁷³⁰ L G R†L H M K ⁷³⁷		Ac-GK † OMe Z-GLAR † SBzl Z-KR † SBzl
C2 EC: [3.4.21.43]		30	105	732	732	6p21.3	8 (9, 92, 270, 313, 447, 451, 601, 631)	C4b	As for factor B		Ac-GK † OMe Z-GLAR † SBzl Z-LGLAR † SBzl
FACTOR I EC: [3.4.21.45]		25-35	80	561	561	4q24-q25	6 (52, 85, 159, 446, 476, 528)	fH, MCP, CR1, C4bp	<u>C3b</u> : L ¹²⁷⁸ P S R†S S K I ¹²⁸⁵ <u>S</u> : S ¹²⁹⁵ L L R†S E E T ¹³⁰² <u>C4b</u> : S ¹³¹⁴ T G R†N G F K ¹³²¹ <u>H</u> : H ⁹³⁴ R G R†T L E I ⁹⁴¹		Boc-DPR † AMC Boc-VPR † AMC Z-GPR † AMC Z-LLR † AMC MD-FGR † AMC
		1.5-12	87	676	676	3q27-q28	4 Potential (30, 159, 366, 388)	NONE	N/A		Z-GR † SBzl Bz-R † OEt
C1r EC: [3.4.21.41]		50	88	688	688	12p13	4 (108, 204, 497, 564)	NONE	<u>C1r</u> : Q ⁴⁴³ R Q R†I I G G ⁴⁵⁰ <u>C1s</u> : E ⁴¹⁹ K Q R†I I G G ⁴²⁶		Ac-GK † Ome, Z-GR † SBzl
C1s EC: [3.4.21.42]		50	80	673	673	12p13	2 (159, 391)	NONE	<u>C2</u> : S ²²⁰ L G R†K I Q I ²²⁷ <u>C4</u> : G ⁷³⁴ L Q R†A L E I ⁷⁴¹		Tos-R † Ome Bz-R † OEt Ac-GK † Ome Z-GR † SBzl Z-Y † OphNO ₂
		0.5	78	668	668	1p36.3-p36.2	0	NONE	As for C1s		Ac-GK † Ome, Bz-R † OEt Z-GR † SBzl Tos-R † Ome Z-GR † SBzl
MASP-3 MEROPS: S01.132		1-2.5	105	709	709	3q27-q28	7 Potential (30, 159, 366, 388, 514, 580, 621)	N/A	N/A		Z-GR † SBzl Bz-I-E-G-R † pNA

*MEROPS database identification numbers are used for the enzymes which do not have an Enzyme Commission (EC) number. Data were obtained from 1) The MEROPS peptidase database (<http://merops.sanger.ac.uk/>), 2) The BRENDA Enzyme database (<http://www.brenda.uni-koeln.de>), 3) The SWISS-PROT (protein knowledge database) (<http://us.expasy.org/sprot/>), 4) Arlaud, G. J., Volanakis, J. E., Thielens, N. M., Narayana, S. V., Rossi, V., and Xu, Y. (1998). *The atypical serine proteases of the complement system*. Adv Immunol 69, 249-307, 5) Rossi, V., Cseh, S., Bally, I., Thielens, N. M., Jensenius, J. C., and Arlaud, G. J. (2001). *Substrate specificities of recombinant mannose-binding lectin-associated serine proteases-1 and -2*. J Biol Chem 276, 40880-40887 and 6) Ritchie, G. E., Moffatt, B. E., Sim, R. B., Morgan, B. P., Dwek, R. A., and Rudd, P. M. (2002). *Glycosylation and the complement system*. Chem Rev 102, 305-319. Chemical abbreviations: Z: N-carboxybenzyloxy, S-Bzl: thiobenzyl, Ac: N-acetyl, Me: methyl, Ome: methyloxy, AMC: 7-amino-4-methylcoumarin, MD: Methylsulfonyl-D, Bz: Na-benzoyl, Tos: p-tosyl, Et: ethyloxy, phNO₂: p-Nitrophenyloxy.

protease could be characterised as atypical compared to the other groups as they share features from both the trypsin and chymotrypsin groups.

1.2.1 Factor D

The native concentration of factor D (fD) in serum is 1-2 µg/ml. This unglycosylated 25 kDa enzyme is encoded by a gene located on chromosome 19 (19p13.3). fD participates in the alternative activation pathway of complement (section 1.1.1.3) by catalysing the cleavage of C3b-bound factor B. fD cleaves only the R²³³-K²³⁴ bond of its natural substrate factor B. This cleavage activates the C3-convertase of the alternative pathway. Compared to the characterised blood serine proteases, it circulates in an active form and it is not inhibited by any of the known serpins. In parallel, the low reactivity of fD with synthetic substrates (Kam et al., 1987) and active site inhibitors (Kam et al., 1992) supported the hypothesis that apparently, the interaction with its natural substrate induces conformational changes rendering the enzyme capable of functioning. The knowledge about the structural transition came to light after structural and mutational studies (Volanakis and Narayana, 1996). It is now hypothesized that the C3bBb complex induces the conformational changes necessary for the realignment of the active center residues of fD. Following cleavage of C3b-bound fB, the active center of fD reverts to its native resting state conformation. This conformational change transition is a profound control of activity mechanism that does not require the existence of any zymogenic forms of fD or the presence of any native serpin-type inhibitor.

1.2.2 Factor B and C2

These two structurally and functionally similar single chain glycoproteins provide the catalytic subunits of the C3- and C5-convertases. Factor B (fB) and C2 are encoded by single genes that are present in the class III region of the major histocompatibility complex on chromosome 6 (Carroll et al., 1984). The 5' end of the C2 gene lies approximately 600 kbp centromeric of the 5'-end of the HLA-B gene (Carroll et al., 1987). The factor B gene (*Bf*) lies centromeric to the C2 gene and shares the same transcriptional orientation. Both genes contain 18 exons although they

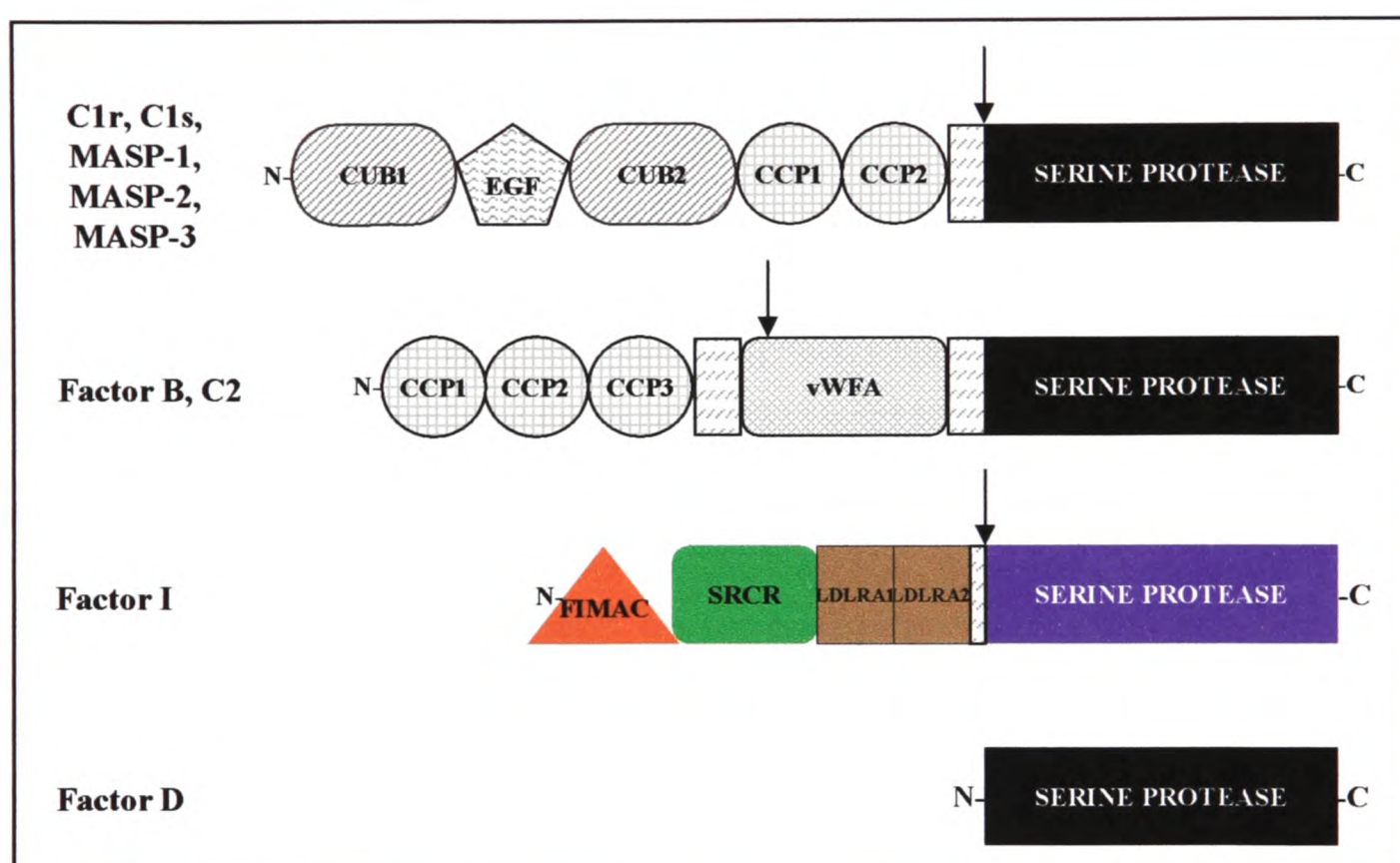


Fig. 5. Schematic representation of the modular organisation of the complement serine proteases. The size for each enzyme is approximate. Non-labelled portions of the molecules represent connecting segments. The arrows indicate the peptide bonds cleaved prior to the activation of the enzymes. Detailed information about the domain types can be found in section 1.2.6.

differ largely in size as the C2 gene (18 kbp) contains some large introns compared to the *Bf* gene (6 kbp).

The modular organisation of factor B and C2 is shown in Fig. 5. fB consists of 739 amino acids, in the mature secreted protein (Horiuchi et al., 1993), with an observed MW of 93 kDa (8.6 % w/w carbohydrate, 4 potential sites for N-linked glycosylation), while C2 has 732 amino acids (Horiuchi et al., 1989) with an observed MW of 105 kDa (15.9% w/w carbohydrate, 8 potential sites for N-linked glycosylation). The difference in MW between the observed and the estimated values (83 kDa for fB and 81,000 for C2) indicates that all sites are occupied by oligosaccharides (Tomana et al., 1985). For fB and C2, full expression of their proteolytic activities occurs only in the context of the C3/C5-convertase complexes (Arlaud et al., 1998). Uncomplexed Bb retains only about 1% of the proteolytic activity of the C3bBb complex (Fishelson and Muller-Eberhard, 1984). Both the uncleaved and the dissociated forms of fB (Gutierrez et al., 1983) and C2 (Caporale et al., 1981) also have some activity against synthetic substrates. In their native state fB (Ueda et al., 1987) and C2 (Smith et al., 1984) share a similar three-lobed structure as revealed by transmission electron micrographs. One lobe of the native enzymes corresponds to the N-terminal fragments Ba and C2b that are removed upon cleavage by fD and C1s, respectively. These fragments contain three CCP domains as well as seven residues from the N-terminal region of the vWFA domain. During convertase formation the conformation is altered to a two-lobed structure, containing the vWFA domain and a serine protease domain, bound to C3b/C4b through a single lobe (Smith et al., 1984).

The current hypothetical model for the assembly of the C3 convertases (Arlaud et al., 1998) proposes that the primary binding of fB or C2 to C3b or C4b, respectively, is mediated through two low affinity sites, one on Ba or C2b and the other on the vWFA modules. Mg^{2+} plays the role of an allosteric effector of the site on the vWFA module. The cleavage of fB or C2 by fD or C1s, respectively, causes a transient conformational change in the vWFA module site. This change causes an increase of the binding avidity for C3b or C4b, sequestration of Mg^{2+} and expression of proteolytic activity for C3 (Laich and Sim, 2001).

1.2.3 C1r and C1s

C1r and C1s are the serine proteases that are associated with C1q in the C1 complex. The genes for these proteases are encoded in close proximity in a tail-to-tail orientation, with an approximate distance of 9.5 kbp between their 3' ends, on human chromosome locus 12p13 (Kusumoto et al., 1988; Nguyen et al., 1988). The pro-active forms of C1r and C1s which are mainly synthesized in the liver are single polypeptide chains of 688 amino acids (92 kDa) and 673 amino acids (82 kDa), respectively (Table 1). The modular organisation of C1r and C1s is shown in Fig. 5.

Activated C1r and C1s have very limited specificities, similar to the other complement proteases. The physiologically relevant substrates for C1r are proenzymic C1r and proenzymic C1s, both cleaved at the conserved R-I bonds at the C-terminal ends of the linker regions (Arlaud et al., 2002). The activated C1s cleaves the R⁷³⁸-A⁷³⁹ bond in C4 (Press and Gagnon, 1981) and the R²²³-L²²⁴ bond in C2 (Kerr, 1979). C1s has also been suggested to cleave types I and II collagens, the MHC class I antigens and β_2 microglobulin (Arlaud et al., 1998). The serpin C1-inhibitor is the only physiological regulator of the C1 complex (Reboul et al., 1977). Regarding specificities, C1r has been found to have a limited esterolytic activity cleaving synthetic substrates with an R or K at the P₁ position, while C1s cleaves a wider range of synthetic substrates that contain either basic (R/K) or aromatic (Y) amino acids (Cooper, 1985), due to the presence of a hydrophobic area and an anionic binding site at its active site. On prolonged incubation C1r may be further autocleaved at R²¹¹-G²¹² and R²⁷⁹-G²⁸⁰ positions (Arlaud and Thielens, 1993) yielding the α fragment and the γ B fragment the latter of which is made of two CCPs and the SP domain disulphide linked together. The γ B fragment generated by autolysis or limited proteolysis has been found to retain the ability to cleave proenzymic C1s (Arlaud et al., 1986; Lacroix et al., 1989). Similar studies with the γ B fragment of C1s have shown that efficient cleavage of C4 requires substrate binding sites located in both the CCP modules (Rossi et al., 1998).

A basic characteristic of C1r and C1s is that during activation of the complement system, they function as a calcium-dependent tetramer C1r₂C1s₂ that binds to the

recognition molecule C1q to form the C1 complex. The formation of the C1 complex is based on an equilibrium between C1q and C1r₂C1s₂, a simple dissociation, reassociation equilibrium, described simply by the relation $C1 \rightleftharpoons C1q + C1r_2C1s_2$ (Tseng et al., 1997). Physiologically the C1 complex consists of one C1q molecule and one C1r₂C1s₂ heterodimer (Gigli et al., 1976). C1r, through its γ B fragment, forms non-covalent dimers in the absence of calcium at physiological pH (Arlaud et al., 2002). In the presence of C1r, C1s forms calcium dependent C1r₂s₂ complex, mediated through the CUB-EGF domains region, that binds to C1q through C1r (Zavodszky et al., 1993). For every dimer, three calcium ions are sequestered (Thielens et al., 1990). Assembly of the C1 complex appears as a multifactorial process which is proposed to involve a C1q binding site in the calcium binding EGF domain region of C1r.

1.2.4 The Mannan Binding Lectin Associated Serine Proteases

The Mannan Binding Lectin Associated Serine Proteases (MASPs) are the serine proteases that are associated with Mannan Binding Lectin or the ficolins (section 1.1.1.2). Their modular organisation is the same as for C1r and C1s (Fig. 5). The genes for MASPs are not closely associated as for the case of C1r and C1s: the *MASP-1* gene is located on the chromosome locus 3q27-q28 (Takada et al., 1995) and the *MASP-2* gene on chromosome locus 1p36.2-3 (Stover et al., 2001). Similarly to the majority of the complement components their primary site of synthesis is the liver. The *MASP-1* gene is encoded by a total of sixteen exons, six of which encode the linker region plus the serine protease domain (Endo et al., 1996a), while exons 1-10 encode the non-catalytic regions of MASP-1 and MASP-3. MASP-3 is another product of the same gene produced by alternative splicing and contains the same non-catalytic domains of MASP-1, but a different SP domain. Unlike MASP-1, the serine protease domain of MASP-3 is encoded by a single exon (exon 11) (Dahl et al., 2001). The *MASP-2* gene contains 12 exons from which 1-4 and 6-12 combine to generate the MASP-2 mRNA. Similarly to MASP-3, the SP domain of MASP-2 is single-exon (5) encoded (Stover et al., 2001). The combination of exons 1-5 of the gene generates the mRNA for MAP19 an alternative splice product of the *MASP-2* gene. Comparison studies of the *C1s* and *MASP2* genes have revealed identical

positions of introns supporting the hypothesis that both genes probably have arisen from an ancestral gene by gene duplication (Stover et al., 2001).

Each of MASP-1 (Sato et al., 1994), MASP-2 (Thiel et al., 1997) and MASP-3 (Dahl et al., 2001) are synthesized as single polypeptide pro-enzymes of 87, 78 and 105 kDa (as determined by SDS-PAGE under non-reduced conditions) with the mature enzymes containing 676 and 668 and 709 amino acid residues, respectively. The average concentrations of MASP-2 and MASP-3 in human serum have been estimated at 534 (Moller-Kristensen et al., 2003) and 100-250 (Dahl, 2004) ng/ml, respectively. Despite the structural similarities between the members of the MASPs/C1r/C1s family the overall amino acid sequence identity between them is only approximately 40% (Dahl et al., 2001) with extra variation arising from post-translational modifications. Initially it was thought that MASP-1 was responsible for complement activation, but it is now established that it is MASP-2 which, like C1s, cleaves C2 and C4. The mechanisms for MASP activation require further investigation, but, upon binding of MBL to a target surface, MASP-1, MASP-2 and MASP-3 can autoactivate independently, unlike C1r and C1s. The physiological roles of MASP-1 and MASP-3 remain uncertain, but they do not appear to activate complement (Presanis et al., 2004). MASP-19 has been implicated in the inhibition of calcium oxalate renal stone formation (Kang et al., 1999), while recombinant MASP-3 has been reported to inhibit the MASP-2 activity of recombinant MBL-recombinant MASP-2 or native MBL complexes from plasma, by competing with MASP-2 for MBL-binding site(s) and by inhibiting the activation of MASP-2 (Dahl et al., 2001).

1.2.5 Factor I

Factor I is a key regulatory protein of complement in serum (section 1.1.2.1). It is an 88 kDa serine protease that cleaves two peptide bonds in the α' chain of C3b or C3(H₂O) (Davis and Harrison, 1982) and C4b (Fujita et al., 1978). Its action is limited to bimolecular complexes with C3b or C4b and a regulatory protein like fH, C4bp, MCP or CR1. The cleavage of C3b yields iC3b that is a major opsonin of the complement system (Hart et al., 2004). By proteolytically cleaving C3b and C4b, fI blocks the formation of the alternative pathway C3-convertase and both of the C5-

convertases of the alternative and classical pathways. fI is discussed in detail in sections 1.3 and 1.4.

1.2.6 Modular Organisation

A schematic representation of the human complement proteases is shown in Fig. 5. Factor B and C2 in one group and C1r/C1s/MASPs in another, exhibit similar modular organisation, while fD and fI have unique structures. With the exception of factor D (fD), that possesses only a serine protease domain, all complement proteases have additional protein polypeptide domains in their N-terminal regions that are organised in series of structural modules. To date, fI is the only serine protease of the human genome that has the FIMAC-SRCR-LDLR-A1-LDLR-A2-SP diverse domain organisation. The domains influence the orientation and localisation of protein substrates, and mediate complex formation through protein-protein interactions. All members of a domain protein family exhibit a similar architecture, but vary from each other in the size and occurrence of surface loops of variable size and in other features such as glycosylation (Bork and Koonin, 1996).

1.2.6.1 The Serine Protease (SP) domain

The chymotrypsin fold and the zymogen activation mechanisms have been thoroughly explored to date (Freer et al., 1970; Khan and James, 1998; Kossiakoff et al., 1977; Stroud et al., 1977). A basic characteristic of the chymotrypsin architecture fold is the presence of two antiparallel β -barrel-type domains. Each barrel contains six β -strands with the catalytic residues of the catalytic triad located at the interface (Perona and Craik, 1997).

In every serine protease domain there are four basic features that are essential for substrate recognition and subsequent catalysis; a catalytic triad, a primary specificity pocket, a less specific binding site for the P_1 - P_3 residues of the substrate and an oxyanion-binding hole (Perona and Craik, 1995)(Nomenclature: $P_n \dots P_2$, P_1 , P_1' , $P_2' \dots P_n'$ symbolizes the area around the cleavage site, while P_1 - P_1' indicates the bond that is hydrolysed (Schechter and Berger, 1967). The residues H^{57} , D^{102} and S^{195}

(chymotrypsinogen numbering) form the catalytic triad (Fig. 6) that facilitates the catalysis mechanism. Substrate hydrolysis occurs through nucleophilic attack on the carbonyl carbon of the scissile bond mediated by the γ -oxygen of S¹⁹⁵; the H⁵⁷ plays the role of a catalyst by increasing the nucleophilicity of S¹⁹⁵; D¹⁰² supports the tautomeric state of H⁵⁷ in the ground state and maintains the positive charge of the same residue in the transition phase during catalysis (Craik et al., 1987) (Fig. 7).

The ionisation states of the amino acid side chains are important for the catalytic activity mechanism. Initially as shown in Fig. 7 the –OH group of S¹⁹⁵ is free, not bonded to H⁵⁷. However upon the binding of a specific substrate, the side chain rotates around the C¹-C² axis and the oxygen atom of S¹⁹⁵ which, as a nucleophile at alkaline pH, reacts with the carbonyl carbon of the P₁ residue of the substrate. At alkaline pH the –OH proton of S¹⁹⁵ is accepted by H⁵⁷ as this is facilitated by the imidazole ring of H⁵⁷ which is ready to accept this proton. The acceptance of the proton shifts the imidazole ring closer to D¹⁰² so that a low energy H-bond can be formed which favours the formation of Ser-O. At alkaline pH the imidazole ring of H⁵⁷ is deprotonated, thus enabling the H⁵⁷ to act as a catalyst. At acidic pH as the imidazole ring is already protonated, H⁵⁷ cannot function as a base catalyst. The formed Ser-O subsequently attacks the carbonyl atom of the P₁ amino acid residue, forming a tetrahedral intermediate (Fig. 7). This is supported by the carbonyl oxygen of the substrate that is hydrogen bonded with the backbone –NH₂ groups of G¹⁹³ and S¹⁹⁵ forming the ‘oxyanion hole’. In the tetrahedral intermediate, the oxygen becomes fully charged and single bonded as the H bonds become shorter and stronger. The tetrahedral intermediate however does not accumulate in normal catalysis; the amine group of the P₂ residue of the substrate receives a proton from H⁵⁷ and leaves the carbonyl, while the carbonyl oxygen becomes a carbonyl again. The subsequent phase of deacetylation is considered as the reverse of acetylation. A water molecule removes a proton from H⁵⁷ and attacks the acyl-enzyme carbonyl as it is proximal to the –OH[–] group. The proton returns to S¹⁹⁵, which upon obtaining the positive charge leaves the tetrahedral intermediate, while the –OH[–] group is attached to the carbon atom of the carbonyl group of the P₁ of the substrate backbone group resulting in the release of the second peptide with a complete C-terminus.

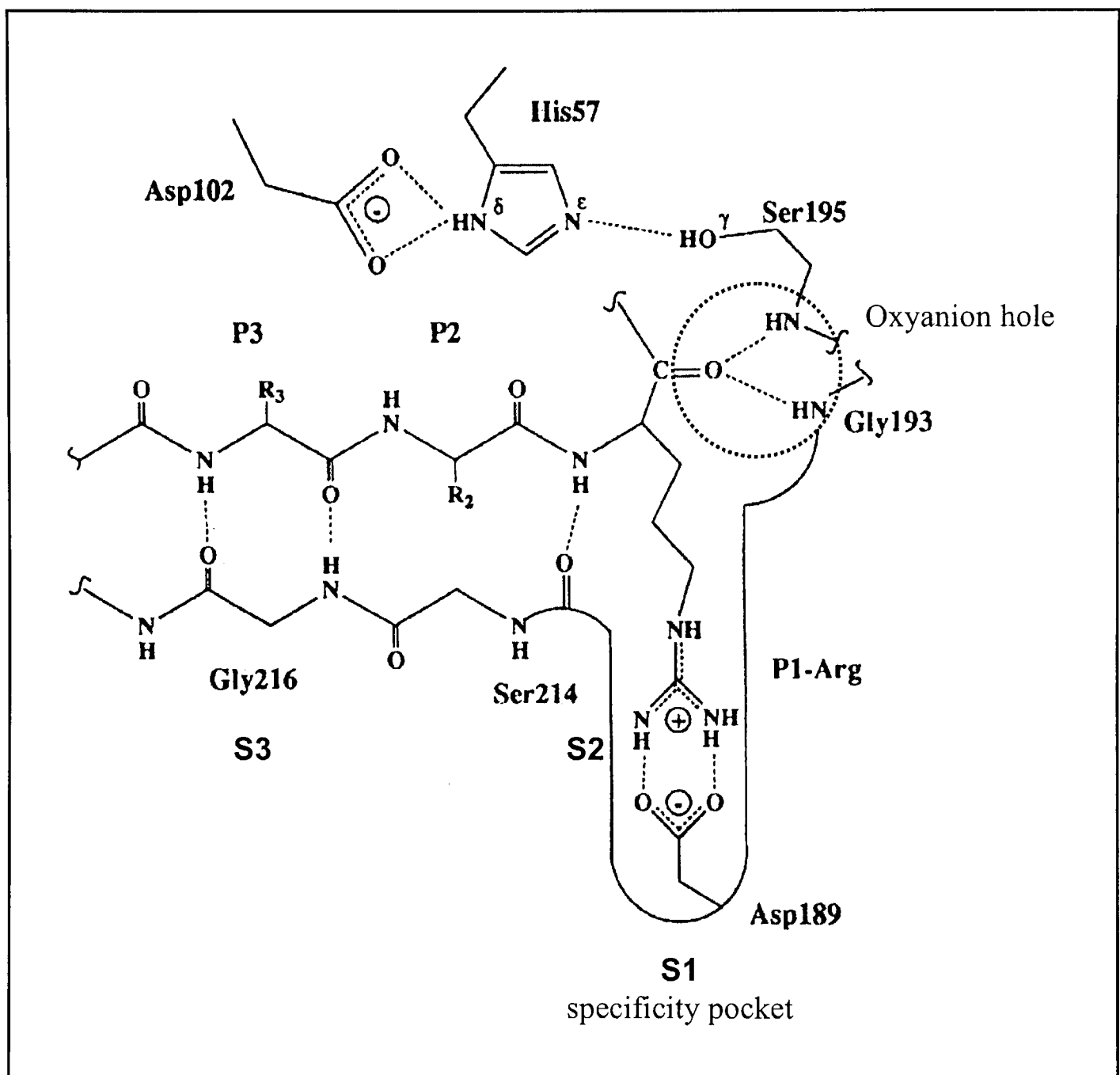


Fig. 6. The active site architecture of the serine proteases in the chymotrypsin family. The active site structure of serine proteases of the chymotrypsin family is illustrated, using chymotrypsin numbering. The substrate (with its residues denoted P_n...P₂, P₁, P₁', P₂'...P_n', where P₁-P₁' is the hydrolysed bond) forms multiple interactions with the proteases (with S_n...S₂, S₁, S₁', S₂'...S_n' denoting the corresponding enzyme binding sites). The presence of R¹⁸⁹ in the S₁ specificity pocket offers trypsin-like substrate specificity; a positive P₁-residue, such as R or K electrostatically interacts with the D¹⁸⁹. The S₂ and S₃ residues form hydrogen bonding with P₂ and P₃ of the substrate so that the scissile P₁-P₁' bond is positioned for attack by the γO of the active site serine (S¹⁹⁵). The carboxyl oxygen of the P₁ residue that is negatively charged after this attack is stabilized by the amide groups of S¹⁹⁵ and G¹⁹³ forming the oxyanion hole. H⁵⁷ accepts the S¹⁹⁵ hydroxyl proton in the transition state, and the positive charge of H⁵⁷ is stabilised by the negatively charged D¹⁰². An acyl-enzyme complex is established.

Figure adapted from Perona, J. J., and Craik, C. S. (1997). *Evolutionary divergence of substrate specificity within the chymotrypsin-like serine protease fold*. J Biol Chem 272, 29987-29990.

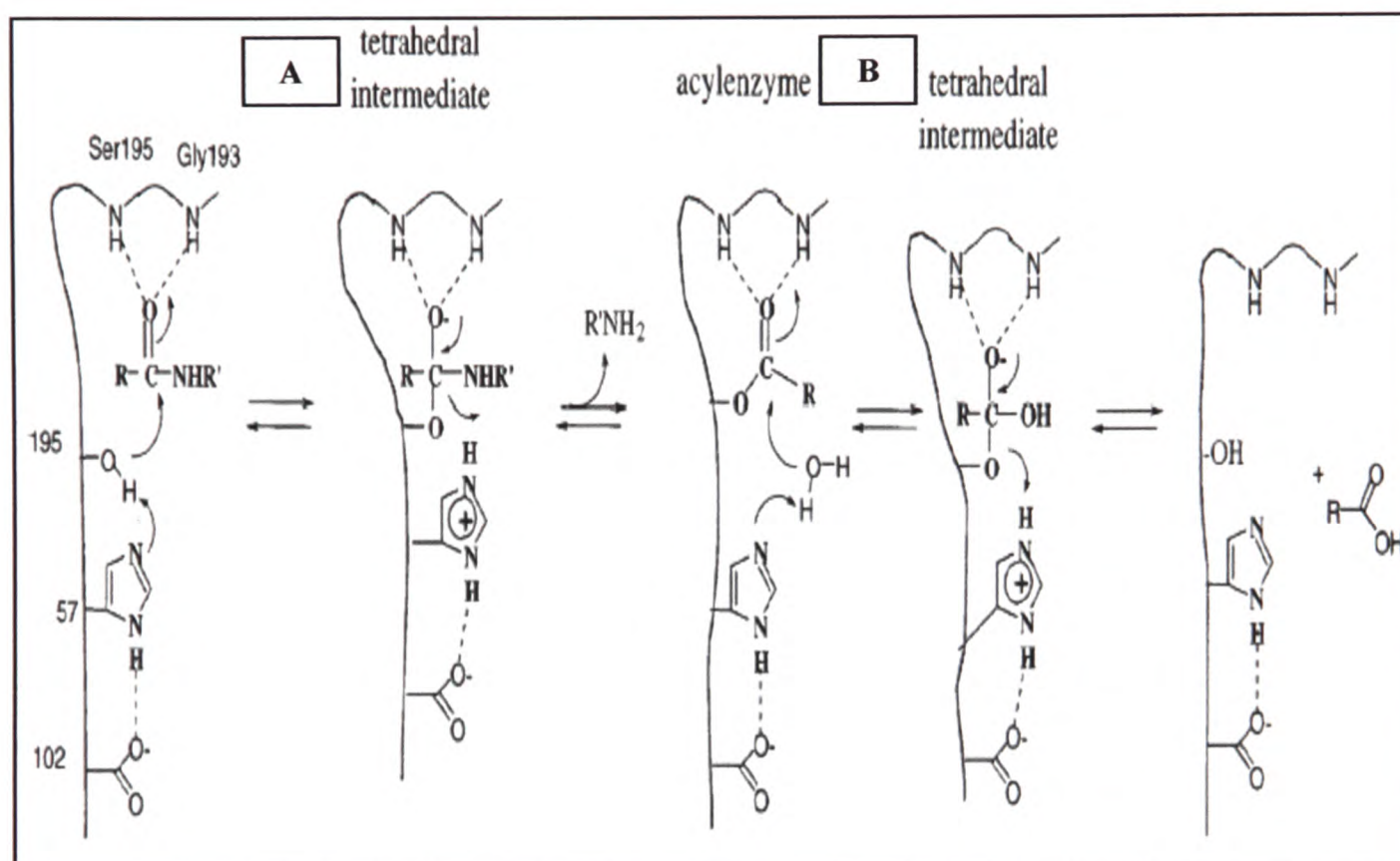


Fig. 7. The serine protease cleavage of substrate peptide bonds is achieved through the formation of tetrahedral transition states and occurs in two major steps. A) The formation of an acyl-enzyme intermediate, involving the reactive Ser¹⁹⁵, is the first step in the hydrolysis. The intermediate is formed where the scissile P₁-P_{1'} bond is positioned for attack by the γ O of the active Ser¹⁹⁵ and where the bonds of the C₁ carbon from the P₁ carboxyl group obtain a tetrahedral geometry through interactions with four groups including the amines Ser¹⁹⁵ and Gly¹⁹³. B) Deacylation of the acyl-enzyme intermediate is the second step. The formed acyl-enzyme intermediate is hydrolyzed by a water molecule leading to the release of the second product peptide with a complete C-terminus and to the restoration of the hydroxyl group of Ser¹⁹⁵.

Figure from www.dent.ucla.edu/sod/courses/OB471b/Ch_03_-_Complement.pdf

In γ -chymotrypsin the residues 189-195 (site S1), 214-220 (site S2) and 225-228 (site S3) form the walls of the specificity pocket. The specificity pocket is a basic requirement for substrate recognition and is present in all the serine proteases (Cohen et al., 1981) including the complement ones. Mutational studies have shown that in addition to the residues of the binding pocket, the surface loops that connect the walls of the pocket have a major effect on substrate specificity although they do not come in direct contact with recognised substrate (Hedstrom et al., 1994; Hedstrom et al., 1992). All the complement proteases contain all the three conserved catalytic triad residues and the highly conserved catalytic surrounding regions. A number of conserved motifs are present in the trypsin/chymotrypsin fold (Yousef et al., 2004). All the motifs around the catalytic triad WVLTAHC (positions 51-58), DIALLL (positions 102-108) and GDSGGP (positions 193-198) are highly conserved. Moreover, other short motifs like the VXGWG (X: Y/S/A) (positions 140-142), CGG(S/T)L(I/L/V) (positions 42-47), SWG (positions 214-216), P(W/Y)(Q/M)(V/A)X(L/I/V) (positions 28-33) and the (R/K)(I/V/L)(V/I/L)GG activation cleavage site (positions 15-19) are also conserved. The presence of I/V at the N-terminus of SP domains is generally considered essential for the formation of a salt bridge with D¹⁹⁴ (Khan and James, 1998; Stroud et al., 1977). In the zymogens of trypsin-like proteases, the oxyanion hole (the amides of G¹⁹³ and S¹⁹⁵) and about 50% of the substrate binding pocket residues are not in the catalytically competent conformation seen in the activated enzyme (Reiling et al., 2003). In contrast, the catalytic triad of the zymogens is arrayed as in the activated form. Upon zymogen activation a highly conserved new N-terminus (I-I/V-G-G) establishes a conserved and buried salt bridge between the newly formed amino terminus and D¹⁹⁴. The establishment of this salt bridge contributes to the refolding of the molecule into the conformation desired for catalysis. Within these frames the complement serine proteases have a similar basis for their catalytic mechanism. However, fD exhibits some variation, as in native conditions it has a “resting state conformation” in which the catalytic triad is thought to be in a non-functional state compared to the rest of the complement proteases (Volanakis and Narayana, 1996).

Apart from the catalytic triad a number of other structural features are considered core features of the chymotrypsin fold. A number of conserved cysteine residues are pivotal in supporting the fold, maintaining the SP domain in a catalytically active

conformation, as well as contributing to the overall structure of the multidomain enzymes (Yousef et al., 2004) (Table 2). The number and distribution of cysteines can vary among groups of proteases with some core residues however being conserved in trypsin and chymotrypsin folds. The serine protease domain cysteines can nowadays be exploited as markers for examining the evolution of proteolytic enzymes (section 1.3.6).

Apart from the residue 189 that is located in the bottom of the specificity pocket and contributes to the distinct specificity (Fig. 8), other residues are important for facilitating the recognition process; C2 and factor B for example have an N¹⁸⁹ but cleave R bonds. The residue at position 216 which is usually a G, guides the correct positioning of the scissile bond of the substrate relative to the oxyanion hole and the S¹⁹⁵ and H⁵⁷ pair. The G²¹⁶ forms two antiparallel β -strand H bonds with the P₃ residue of the substrate (Perona and Craik, 1995). In addition, the residues at positions 225 (Dang and Di Cera, 1996) and 214 (Krem et al., 2002) affect the binding of Na⁺ ions in a designated binding loop (220 loop) and the optimal contact of the substrate with the very stable active site at the specificity pocket site S1, respectively. Moreover, the C-terminal region of the serine protease domain has been implicated in governing the function and specificity properties, as residues 189-220 account for >95% of the area around the specificity pocket site S1 and the catalytic H⁵⁷ and >70% of the area around the specificity sites S2 and S3 (Krem et al., 2000). These sites contact the three residues of the substrate immediately upstream from the scissile bond; the C-terminal end contains the active site S¹⁹⁵, the specificity sites S1 and S3, the residue 225 that plays a crucial role in the architecture of the S1 site and residues 216 and 226 that affect the access to the active site and the specificity pocket.

1.2.6.2 The Complement Control Protein (CCP) domain

More than 140 such domains have been identified in multiple copies in 24 human proteins including members of the RCA family (section 1.1.2.1), complement proteases (section 1.2.6), MAC components (C6 and C7) and non-complement proteins. Non-complement proteins include the β_2 -glycoprotein-1, IL-2 receptor,

Table 2. Representation of the conserved SP domain cysteine residues among the human complement proteases ¹

	C ²²	C ⁴²	C ⁵⁸	C ¹²² (2)	C ¹³⁶	C ¹⁵⁷	C ¹⁶⁸	C ¹⁸²	C ¹⁹¹	C ²⁰¹	C ²²⁰
Chymotrypsin [S01.152] ³		*	*	*	*		*	*	*	*	*
Trypsin [S01.127]	*	*	*		*	*	*	*	*	*	*
Factor D [S01.191]		*	*		*		*	*	*	*	*
Pancreatic elastase [S01.153]		*	*		*		*	*	*	*	*
Factor I [S01.199]		*	*	*	*		*	*	*	*	*
t-Plasminogen activator [S01.232]		*	*	*	*		*	*	*	*	*
u-Plasminogen activator [S01.231]		*	*	*	*		*	*	*	*	*
Plasma kallikrein [S01.212]		*	*	*	*		*	*	*	*	*
Factor B [S01.196]		*	*	*			*	*	*		*
C2 [S01.194]		*	*	*			*	*	*		*
Plasmin [S01.233]		*	*	*	*		*	*	*	*	*
MASP-1 [S01.198]		*	*	*			*	*	*		*
C1r (activated) [S01.192]				*			*	*	*		*
C1s (activated) [S01.193]				*			*	*	*		*
MASP-2 [S01.229]				*			*	*	*		*
MASP-3 [S01.132]				*			*	*	*		*
Thrombin [S01.217]		*	*	*			*	*	*		*
Protein C (activated) [S01.218]		*	*	*			*	*	*		*
FIXa [S01.214]		*	*	*			*	*	*		*
FVIIa [S01.215]	*	*	*	*		*	*	*	*		*
FXa [S01.216]	*	*	*	*		*	*	*	*		*

Disulphide bond formation ⁴: C²²-C¹⁵⁷, C⁴²-C⁵⁸, C¹²²-C^x, C¹³⁶-C²⁰¹, C¹⁶⁸-C¹⁸² and C¹⁹¹-C²²⁰

1. This table was constructed using data derived from homology alignments and all the numbering is according to the chymotrypsinogen system.
2. The C¹²² has been identified to be disulphide bonded to another C^x N-terminal to the trypsin-like domain. It is therefore conserved in all the serine proteases that possess non-catalytic N-terminal domains necessary for their function, and whose active forms consist of multiple chains (e.g. complement proteases). Smaller enzymes like trypsin, upon activation replace their zymogenic N-terminal tail with an Ile (or equivalent) forming the new N-terminal end (section 1.2.2.1).
3. Complement proteases are highlighted in bold and are grouped with other proteases that share common evolutionary markers in their catalytic subunits. In brackets the [MEROPS ID] numbers.
4. All the listed cysteine residues, with the exception C¹²², participate in intradomain disulphide bonds that are pivotal for the maintenance of a catalytically active conformation.

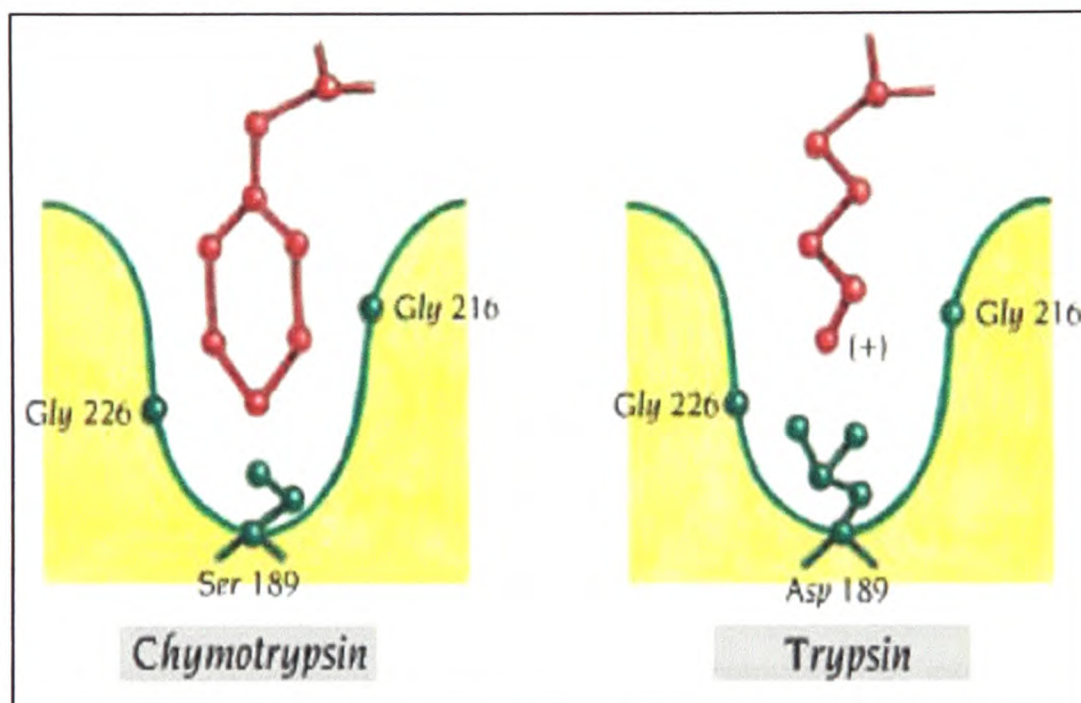


Fig. 8. Schematic representation of the specificity pockets of chymotrypsin and trypsin indicating the preference for a side chain adjacent to the scissile bond in the substrates. The occupancy of residue 189 by Ser in members of the chymotrypsin family or by Asp in members of the trypsin family, specifies different preferences; Chymotrypsin shows a preference for aromatic side chains, while trypsin prefers positively charged side chains that can interact with D¹⁸⁹ through electrostatic interactions.

Figure from Branden, C., and Tooze, J. (1991). *Introduction to Protein Structure* (New York, Garland Publishing, Inc.).

haptoglobin, the β subunit of blood clotting factor XIII and a number of cell adhesion proteins known as selectins (Sim and Perkins, 1990).

CCP domains are composed of approximately 60 amino acids and they exhibit a typical framework of highly conserved residues including two prolines, one tryptophan and four cysteine residues that form two disulphide bridges (C1-C3, C2-C4). Usually they are arranged in elongated structures that resemble “beads on a string” (RCA members) with each CCP domain separated from the next by a linker region of 2-8 residues. The solution structures of CCP module 5, 15 and 16 of human factor H as determined by NMR spectroscopy and simulated annealing, indicate that the CCP domain folds autonomously and exhibits a common overall topology, characterised by a β -sandwich arrangement with six β -strands enveloping a compact hydrophobic core, with the N and C termini located at opposite sides of the ellipsoid module (Barlow et al., 1992; Barlow et al., 1993; Norman et al., 1991). Variations in sequence and length are accommodated in external loops. A particularly variable region is located within an external loop that protrudes from the surface and is believed to provide ligand specificity (Barlow et al., 1993). The interface between two CCP domains exhibits a limited degree of rotation but can support folding and bending in proteins that contain several CCP domains. As the binding specificity of the CCP modules can vary within the same protein (fH), the folding and bending capacities are important for supporting diverse interactions of the proteins with various ligands.

1.2.6.3 The von Willebrand Factor type A (vWFA) domain

The von Willebrand Factor type A (vWFA) domain which consists of more than 200 amino acids and is present in C2 and fB, has been identified in homologous regions of several components of various systems covering areas of immunity, haemostasis, cell adhesion and the extracellular matrix. It is named from the von Willebrand Factor (vWF) protein in which this domain was first recognised. vWF contains three copies of the vWF type A domain, while copies of the same domain are also present in collagen types VI, VII, XII and XIV, cartilage matrix protein and the α subunits of integrins such as LFA-1, Mac-1, CR3, CR4, VLA-1 and VLA-2

(Colombatti and Bonaldo, 1991). The presence of the vWFA domain appears to mediate ligand binding important for cell-cell, cell-matrix or matrix-matrix interactions.

Despite the presence of cysteines there are no disulphide bridges formed. Structural information for CR3 (Lee et al., 1995) and LFA-1 (Qu and Leahy, 1995) derived from crystallographic studies of their α chains indicated the classic α/β open sheet (“Rossmann fold”) that consists of a central β -sheet core formed by parallel and one short antiparallel β strand, surrounded bi-phasically by seven amphipathic α helices. The discovery of Mg^{2+} or Mn^{2+} binding sites in CR3 and LFA-1, respectively, led to the characterisation of a motif called MIDAS (Metal Ion Dependent Adhesion Site) that is primarily characterised by the sequence D-X-S-X-S and the presence of T and D residues. Residues can coordinate the divalent ion binding through their side chains, directly or indirectly through hydrogen bonding to coordinating water molecules.

1.2.6.4 The CUB domain

The CUB domain obtained its name from the initials of the proteins in which it was first recognised: C1r/C1s, the sea urchin protein Uegf and the human Bone morphogenetic protein-1 (Bork and Beckmann, 1993). The CUB modules consist of about 110 amino acids from which four cysteines participate in disulphide bridging (C1-C2; C3-C4); the first CUB domain at the N-termini of C1r, C1s and the MASPs lacks the first two cysteines and therefore has a single disulphide bridge. Predictions of the secondary structure have suggested that the CUB modules have an antiparallel β -barrel fold comparable to that of the immunoglobulin module (Bork and Beckmann, 1993). The crystal structure of a CUB domain reveals a conformational folding in a compact ellipsoidal structure that is composed of the two 5-stranded β sheets which are stabilised by a tightly packed hydrophobic core and disulphide bonds (Feinberg et al., 2003; Romero et al., 1997). Studies using truncated proteins have shown that the N-terminal CUB and EGF-like (section 1.2.4) domains of MASP-1 and MASP-2 participate in Ca^{2+} dependent interactions with MBL (Thielens et al., 2001; Wallis and Dodd, 2000).

1.2.6.5 The Epidermal Growth Factor (EGF)-like domain

The (EGF)-like modules are very widespread with more than 600 copies identified to date in more than 70 different proteins which include members of the blood coagulation or fibrinolysis system or cell adhesion (Campbell and Bork, 1993). These modules usually contain about 50 amino acids from which six conserved cysteines participate in a 1-3, 2-4, 5-6 disulphide binding pattern. The bridging supports a strained structure with a minor and a major β -sheet that are connected by five loops of variable length (Rao et al., 1995).

The EGF modules of C1r, C1s and the MASPs belong to a particular subset that is involved in binding of calcium and is characterised by the consensus pattern D/N, D/N, Q/E, D^{*}/N^{*}, Y/F (asterisk denotes a β -hydroxylated residue): three of these residues have been shown to provide four coordination bonds through their side-chain carboxyl or hydroxyl oxygen atoms along with the backbone carbonyls of two nearby residues, while the other two residues (the second D/N and Y/F) serve important structural roles (Selander-Sunnerhagen et al., 1992). Hence, a single calcium-binding EGF-like domain contains six out of the seven ligands that contribute to a pentagonal bipyramidal coordination for a calcium ion. In addition, direct evidence that the EGF module of C1r has the ability to bind Ca^{2+} is provided by Nuclear Magnetic Resonance (NMR) spectroscopy (Hernandez et al., 1997). The seventh coordination bond is provided by an amino acid, yet unidentified, but proposed to be located in CUB1 (Hernandez et al., 1997).

1.2.6.6 The Factor I-Membrane Attack Complex (FIMAC) domain

The Factor I-Membrane Attack Complex (FIMAC) is a 70 amino acid module which apart from fI has also been identified only in the C-terminus of 2 proteins of the Membrane Attack Complex (MAC), C6 and C7. This suggested a possible common role (DiScipio et al., 1999; Ullman and Perkins, 1997; Yu et al., 2000). Factor I can bind and cleave C3b and C4b that exhibit high levels of homology to C5b which is well known to interact with C6 and C7 in the complement system lytic pathway. Recently the identification of a C345C homology region in C5, also present in C3 and

C4, has been implicated in a direct binding interaction with the FIMAC domains of C6 and C7 suggesting that the same domain could play a similar role in the interactions of fI (FIMAC domain) with its natural substrates C3b and C4b (Thai and Ogata, 2003; Thai and Ogata, 2004). The FIMAC contains a total of 10 conserved cysteines that form 5 internal disulphide bridges (C1-C3, C2-C9, C4-C7, C5-C10 and C6-C8) (Lengweiler et al., 1997). At the structural level computational studies have shown structural similarities with the follistatin (FS) domain and have derived an averaged $\beta\beta\beta\beta\alpha\beta$ conformation which has not yet been confirmed experimentally (Ullman and Perkins, 1997). Despite the fact that the residues 38-74 resemble a Kazal-type inhibitor domain (predicted secondary structure $\beta\beta\alpha\beta$), the role of this domain within FIMAC has not been elucidated. The family of multi domain Kazal type inhibitors is a family of multi-domain proteins with high inhibitory potency and a pronounced specificity against distinct target proteases (Schlott et al., 2002). Structurally there are several common features among its members. These include a characteristic cysteine distribution pattern, a typical VCGxD sequence motif and highly homologous three-dimensional structures (Schlott et al., 2002). Although it was believed that it could have the role of a regulator for the SP domain in fI, modelling (Chamberlain et al., 1998) and functional data (Tsiftoglou and Sim, 2004; Tsiftoglou et al., 2005) have shown that this is unlikely. In this most detailed fI model so far, the FIMAC and SP domains were too far apart to interact, while the FIMAC domain is masked by two oligosaccharide sites (one located at N⁵² present in the Kazal-type inhibitor scissile loop) and the two LDLR-A domains hinder possible interaction(s) with the SP domain.

All the identified proteins that contain FIMAC and follistatin (FS) domains are extracellular and participate in protein-protein interactions (Ullman and Perkins, 1997). The FS domain has been recognised in extracellular matrix proteins that modulate cell-matrix interactions (such as the protein osteonectin), induce aggregation of nicotinic acetylcholine receptors (agrin) and in ovaries and the pituitary that bind cytokines. Osteonectin which contains a single follistatin domain is released by platelet degranulation, synthesized by fibroblasts and macrophages at areas of wound repair, and may facilitate deposition or assembly of extracellular matrix proteins. Moreover, it shows strong binding affinity to platelet-derived growth factor (PDGF), albumin, thrombospondin and various collagen types. Follistatin itself binds to the

transforming-growth factor- β -like cytokines activin and inhibin. No protease activity though has been described for proteins that contain the FS domain.

1.2.6.7 The Scavenger Receptor Cysteine Rich (SRCR) domain

The Scavenger Receptor Cysteine Rich (SRCR) domain is an ancient and highly conserved domain that was first identified in the type 1 macrophage scavenger receptor, a trimeric integral membrane protein implicated in atherosclerosis, adhesion and host defence (Hohenester et al., 1999). Each domain is ~100-110 residues long and it is likely to be involved in protein-protein interactions and ligand binding (Aruffo et al., 1997). The fl SRCR domain belongs to the Group A, carries six characteristic highly conserved cysteines which form three internal disulphide bridges (C1-C3, C2-C6 and C4-C5) and is encoded by two exons (Sarrias et al., 2004). Group A members are usually multidomain mosaic proteins that contain a single SRCR domain associated with another functional domain like a protease domain. The domain contains an extensive hydrophobic core, formed by an α -helix, the concave face of a curved β -sheet and a disulphide part of the E-F loop that packs against the α -helix. Structure-based alignments have shown that secondary-structure elements are well conserved within the family.

1.2.6.8 The Low Density Lipoprotein Receptor type A (LDLR-A) domain

The Low Density Lipoprotein Receptor (LDLR) type A (LDLR-A) domains are cysteine-rich domains approximately 40 residues long. This domain was originally identified in the low density lipoprotein cell surface receptor protein (Sudhof et al., 1985), but copies of this type have also been identified in fl, C6, C7, C8, C9 and members of the α_2 M receptor family proteins (Moestrup, 1994; Morley and Walport, 2000). Although it occurs in multiple copies in cell surface receptors, it is found in single or double copies in the complement proteins. It is characterised by three β -sheets each with a 2-4 residue length and the existence of six conserved cysteines that form internal disulphide bridges (apparently C1-C3, C4-C6 and C2-C5) (Ullman et al., 1995). In cell surface receptors the domain is probably responsible for ligand

binding interactions. A cluster of conserved acidic residues located in the C-terminal loop end of the domain (D-C-X-D-G-S-D-E) appear to interact electrostatically with regions rich in basic amino acids. These conserved, negatively charged residues D and E of the domains in the LDLR-A, do not participate in any β -sheets, but are located on an exposed surface loop of the receptor facilitating the binding of apolipoprotein E, a high-affinity ligand of the receptor, through interactions with closely spaced positively charged residues clustered into a surface patch of a long helix (Prevost and Raussens, 2004; Wilson et al., 1991).

1.3 COMPLEMENT FACTOR I

1.3.1 History and functional studies

Factor I was initially purified from serum of guinea pig, rabbit and human at the end of the 1960s (Alper et al., 1972; Lachmann and Muller-Eberhard, 1968; Nelson et al., 1966; Ruddy and Austen, 1969; Tamura and Nelson, 1967). The enzyme activity was primarily linked with effects on cells that bind C3b, the loss of immune adherence, the acquisition of conglutinin-binding and the avoidance of complement mediated lysis. These observations lead to the conclusion that fI, formerly called C3bIna (C3b Inactivator) or KAF (Konglutinogen Activating Factor; the name was given after the cleavage of C3b exposed a binding site on iC3b for the bovine protein conglutinin) is pivotal for the regulation of the C3 convertases by inactivating C3b (Gitlin et al., 1975; Nicol and Lachmann, 1973; Ruddy and Austen, 1969; Ruddy and Austen, 1971) and C4b (Cooper, 1975) in the presence of cofactors like fH (formerly called β 1H) (Pangburn et al., 1977; Weiler et al., 1976; Whaley and Ruddy, 1976a) or C4bp (C4 binding protein) (Fujita et al., 1978). The finding that fI is responsible for maintaining the normal levels of C3 in serum and the investigation of the physiology of the complement system in the first reported case of fI deficiency (Abramson et al., 1971), generated the first evidence for the existence of the alternative pathway amplification loop (Alper et al., 1970a; Alper et al., 1970b; Alper et al., 1972; Lachmann and Nicol, 1973). The infusion of a highly purified fI preparation to a patient with fI deficiency, was found to successfully restore the normal hemolytic and bactericidal activity levels within a few days after treatment, highlighting the crucial role *in vivo* of fI in modulating the alternative pathway activity (Ziegler et al., 1975).

An improved purification protocol for the isolation of fI from serum was described in parallel with some basic work covering aspects of the catalytic properties of fI in 1980 (Crossley and Porter, 1980). Factor H (Weiler et al., 1976; Whaley and Ruddy, 1976a), C4bp (Nagasawa and Stroud, 1980) and CR1 (Fearon, 1980) were identified as cofactors for the cleavage of cell bound C3b by fI. The pattern of degradation of human complement C3b by fI was clearly established in later stages (Davis and Harrison, 1982; Malhotra and Sim, 1984; Sim et al., 1981a). The control of C3b by

factors H and I was further examined in more detail at biophysical and biochemical levels by kinetic and thermodynamic (Pangburn and Muller-Eberhard, 1983) as well as spectroscopic (Isenman, 1983) studies. The role of CR1 as a cofactor for the C3b inactivation by fI was further analysed (Medicus et al., 1983), while an optimized enzymatic assay for measuring fI activity based on the cleavage of C3b in the presence of fH or CR1 was described in the same year (Sim and Sim, 1983). The first monoclonal antibody against fI (MRC-OX21) was raised in 1982 (Hsiung et al., 1982) and was used for the development of a purification protocol for fI from serum (Sim et al., 1993a)

Factor I was shown to be synthesized in three human hepatoma cell lines (Goldberger et al., 1984) and later in human endothelial cells (Julen et al., 1992). Some information on the primary structure of factor I was obtained in 1986 (Yuan et al., 1986), but the primary amino acid sequence and domain organisation of the enzyme was characterised from an analysis of cDNA clones in the following year (Catterall et al., 1987). Further work on the cDNA-derived primary sequence of fI refined the boundaries of the characterised domains (Goldberger et al., 1987) and lead to the mapping of the gene to chromosome locus 4q25 (Shiang et al., 1989). The actual detailed analysis of the *fI* gene including the organisation of exons followed several years later (Vyse et al., 1994a).

Studies using high resolution transmission electron microscopy shed the first light on the native conformation of fI (DiScipio, 1992). It was hypothesized that C3b induced conformational changes in fI during the catalysis event during complex formation with the cofactor (Ekdahl et al., 1990). The failure to detect reactivity of fI with any synthetic substrate had lead to the hypothesis that the enzyme in its native conformation is inactive and that it undergoes internal conformational changes upon complex formation through interactions with the substrate or the cofactors that enable it to cleave the substrate (Kam et al., 1992). Following the first observations about fI structure, more studies using molecular modelling and bioinformatics were carried out (Perkins and Smith, 1993; Perkins et al., 1993b).

In parallel to the biochemical and structural studies, knowledge of fI deficiency increased (Floret et al., 1991; Sim et al., 1993b; Vyse et al., 1994b), leading to the

characterisation of the molecular (DNA sequence) basis of a hereditary complement factor I deficiency (Vyse et al., 1996). The interactions between C3b with fH were investigated in more detail (DiScipio, 1992; Soames et al., 1996), while later it was shown that in the complex formation of C3b, fH and fI there are multiple interactions between the components (Soames and Sim, 1997). The last is the most detailed available study on interactions between the three components. In 1995, the production of a recombinant LDLR-A domain, was the first synthesis of a recombinant, non-catalytic domain, from factor I (Ullman et al., 1995).

The reports of cDNA derived primary sequences of human (Catterall et al., 1987; Goldberger et al., 1987), rat (Schlaf et al., 1999), mouse (Minta et al., 1996), frog (Kunnath-Muglia et al., 1993), chicken (Minta et al., 1996) and shark (Terado et al., 2002) fI have contributed to the exploration of gene structure and organisation issues, the more detailed examination of the biochemical and biophysical properties of fI and the patterns of expression and distribution in various tissues. Cloning work has also revealed the organisation of the human *fI* gene promoter (Minta et al., 1998b), defining elements essential for the activation of the *fI* gene (Paramaswara and Minta, 1999) and indicated that interleukin 6 acts as a major regulator of expression (Minta et al., 1998a; Schlaf et al., 2001a; Schlaf et al., 2001b). The isolation and characterisation of bovine factor I has also been described (Menger and Aston, 2003).

Knowledge that has been acquired in the past years about the enzymic properties of fI, has lead the effort for the production of synthetic chemical inhibitory compounds for the control of this important complement regulator (Fevig et al., 1998; Rupin et al., 1997). This effort would have been supported by the availability of an atomic structure for human fI; to date, there is only a low-resolution model, based on X-ray and neutron low angle scattering (Chamberlain et al., 1998), which is consistent with previous observations about the structure (DiScipio, 1992) and has been useful for understanding the multidomain arrangement in the enzyme.

In recent years, studies focusing on the more detailed characterisation of the catalytic properties of the enzyme as well as in the nature of interactions with its native substrates and cofactors, have revealed that the catalytic domain naturally has a conformation that allows recognition and cleavage of candidate substrates in the

absence of cofactors (Tsiftoglou and Sim, 2004) and that the non-catalytic modules probably have a pivotal role for the interactions of the enzyme with the substrate and the cofactor for the cleavage of C3b or C4b (Tsiftoglou et al., 2005). Recently, it has been shown that mutations in the *fI* gene have been associated with a subgroup of patients with hereditary predisposition to atypical Hemolytic Uremic Syndrome (HUS) (Fremaux-Bacchi et al., 2004).

1.3.2 Primary sequence and modular organisation

The 2.4 kb mRNA from the human *fI* gene encodes a pre-pro-factor I of 583 residues that carries an 18 amino acid residue leader peptide (Catterall et al., 1987). The mature 565 residue protein without the signal peptide consists of two disulphide linked polypeptide chains, the heavy (50 kDa) and the light (38 kDa) chains composed of 318-321 and 244 amino acids, respectively (Catterall et al., 1987) (Fig. 9). The two chains are disulphide linked through a single disulphide bridge. Human fI has a total of six sites of attachment of N-linked oligosaccharides in the Heavy chain at N⁵², N⁸⁵, N¹⁵⁹ and in the Light chain at N⁴⁴⁶, N⁴⁷⁶, N⁵¹⁸, and from amino acid sequencing and deglycosylation studies, it has been shown that all of these are occupied.

A basic characteristic of fI, as in the cases of the other complement serine proteases (with the exception of fD), is its high degree of mosaicity regarding its modular organisation. Starting from its N-terminus (Fig. 9), the heavy chain of fI consists of a FIMAC domain (C²⁵-A⁹⁰) (section 1.2.6.6), an SRCR domain (V⁹⁶-Y¹⁹⁷) (section 1.2.6.7) and two LDLR-A domains (D²⁰²-K²³⁹ and A²⁴⁰-G²⁷⁷) (section 1.2.6.8) (Goldberger et al., 1987). The region F²⁷⁸-A²⁹⁵ corresponds to the D-segment that has been identified in other mammalian, amphibian and fish species (Terado et al., 2002). The light chain of fI contains in its entire length the catalytic subunit domain of the enzyme, the SP domain (I³²²-V⁵⁶⁵) (section 1.2.6.1) (Fig. 9). So far, factor I is the only characterised blood serine protease that has this unique combination of domains and bears the extracellular SRCR domain that has otherwise been found only in membrane proteins (Volanakis, 1998). The SP domain has maintained the majority of the conserved amino acid residues of the chymotrypsin-fold, D¹⁰²⁽⁴¹¹⁾, H⁵⁷⁽³⁶²⁾, S¹⁹⁵⁽⁵⁰⁷⁾,

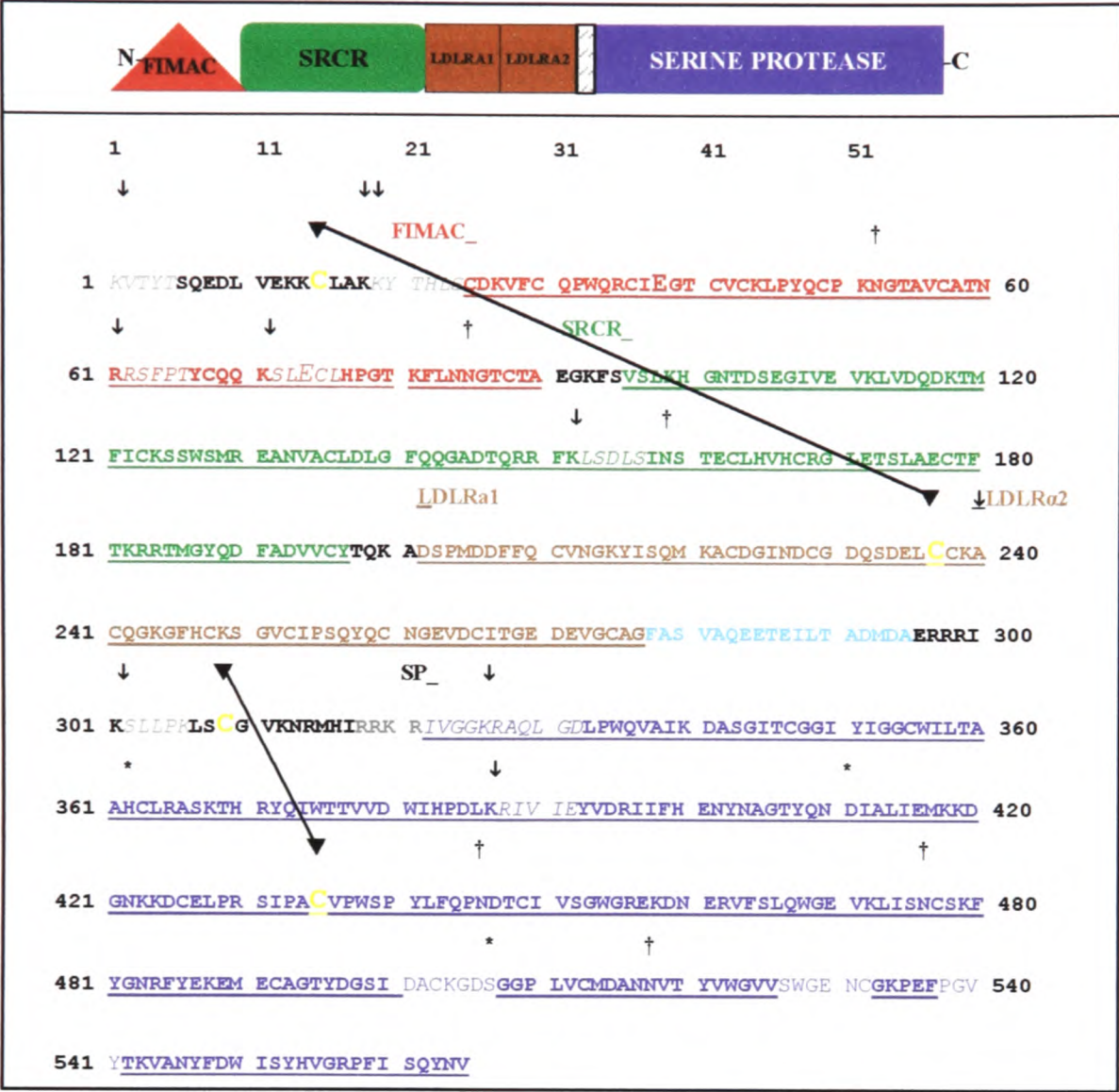


Fig. 9. A schematic representation of human fI and its primary sequence.

fI exhibits a unique linear arrangement of domains; an N-terminal FIMAC (Factor I Membrane Attack Complex) domain (in red), a SRCR (Scavenger Receptor Cysteine Rich) domain (in green) and two LDLR-A (class A Low Density Lipoprotein Receptor) domains (in brown) in the non-catalytic heavy chain. The non-labelled portion represents a connecting segment. In the primary sequence, all domains are illustrated underlined and the start of each domain is indicated by an underscore beside the domain name (e.g. SRCR₋). Non-underlined text in the heavy chain shows non-domain regions. In turquoise the D segment. The signal sequence (18 amino acids) is omitted and residue numbering is from the N-terminus of the mature protein. The R³²¹-I is cleaved post-translationally giving rise to the fI heterodimer. There are in total 40 cysteine residues of which none is unpaired; they all participate in intra- and inter-domain disulphide bridging. The two interdomain disulphide bridges are shown by (▼). The full list of disulphide bridges (mainly deduced by homology) is: Heavy chain: C¹⁵-C²³⁷, C²⁵-C³⁶, C³⁰-C⁷⁵, C⁴¹-C⁵⁷, C⁴³-C⁸⁸, C⁴⁹-C⁶⁸, C¹²³-C¹⁶³, C¹³⁶-C¹⁹⁶, C¹⁶⁸-C¹⁷⁵, C²¹¹-C²²⁹, C²²³-C²³⁸, C²⁴¹-C²⁵³, C²⁴⁸-C²⁶⁶, C²⁶⁰-C²⁷⁵, heavy-light chain: C³⁰⁹-C⁴³⁵ (experimentally determined, see section 4.3.2) and light chain: C³⁴⁷-C³⁶³, C³⁵⁵-C⁵¹³, C⁴²⁶-C⁴⁴⁹, C⁴⁷⁷-C⁴⁹², C⁵⁰³-C⁵³². * shows the residues which (by homology) form the catalytic triad and † shows the six sites of attachment of N-linked oligosaccharides (52, 85, 159, 446, 476 and 518). The non-bold, non-underlined sequences in the Serine Protease (SP) domain (in blue) form the walls of the specificity pocket. The peptide bonds that have been identified by N-terminal sequence analysis as cleavage sites for plasmin (see section 4.3.3) are indicated in italics and ↓ indicates each bond that is cleaved.

D¹⁸⁹⁽⁵⁰¹⁾, S²¹⁴⁽⁵²⁷⁾, W²¹⁵⁽⁵²⁸⁾ and G²¹⁶⁽⁵²⁹⁾ (chymotrypsin numbering (fI numbering in parentheses)). A comparison of the cDNA-derived primary sequence of the fI SP domain with other homologues ones, showed that the fI SP domain is most similar, among complement proteases, with the one of fD (28% identity) (Goldberger et al., 1987). Comparison of the complement protease domains with other structurally characterised homologues, provided evidence that the complement proteases have maintained the chymotrypsin folding (Perkins and Smith, 1993; Perkins et al., 1993a). In the whole molecule there are 40 cysteine residues that all participate in inter- and intra- domain disulphide bridges which contribute to the highly ordered domain and molecular conformations (Fig. 9). The recent discovery that fI is active in the cleavage of synthetic amide substrates in the absence of any cofactor(s), suggested that the SP domain in its resting state has a conformation that accommodates substrate recognition and cleavage (Tsiftoglou and Sim, 2004).

1.3.3 Gene structure

The gene expressing human fI (Accession numbers: cDNA Y00318, Genomic X78594) has been structurally characterised and mapped on the chromosomal locus 4q25 (Catterall et al., 1987; Goldberger et al., 1987), telomeric of the epidermal growth factor gene and centromeric to the gene expressing interleukin-2 (Kolble et al., 1989).

The human gene spans 63kb of DNA and has been shown to comprise 13 exons, the first of which encode the heavy chain and the last five the light chain (Vyse et al., 1994a) (Fig. 10). There is a clear association between the exon structure and the mosaic nature of the enzyme. The first intron has a length of 36 kbp, while the rest vary between 0.19 to 5.9 kbp. The cleavage site between the heavy and the light chains is located within the ninth exon. The first exon is small and encodes the 5' untranslated sequence (29 bp), leader peptide (54 bp) and the first codon of the heavy chain. The second exon encodes the FIMAC domain in contrast to C6 and C7 in which the same domain is encoded by three exons. For the cases of C6 and C7 the organisation of the three exons does not correspond to the boundaries of the domain in C6 and C7. The third and fourth exons of fI encode the SRCR domain. The next two

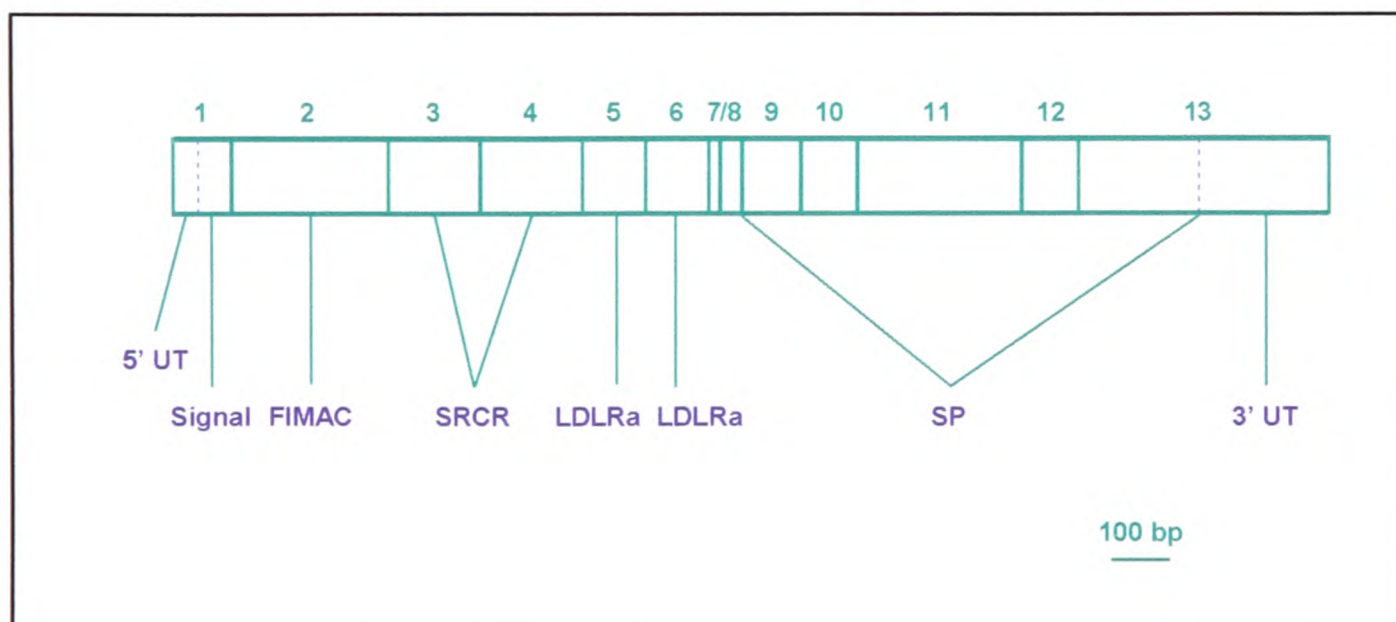


Fig. 10. A representation of the cDNA structure of human f1. Each numbered block represents a single exon with its boundaries. The domains that are encoded by the exons are illustrated below. The vertical dashed lines separate translated from untranslated cDNA regions. Note that the SP domain is encoded by 5 exons in contrast to the other human complement proteases the SP domains of which are encoded by 8 (C2/fB), 6 (MASP-1) or 1 (C1r/C1s/MASP-2/MASP-3).

exons, exons 5 and 6 separated by a very close distance of 142 bp from each other, encode separately the two LDLR-A domains. Exons 7 and 8 are small with lengths of 21 and 36 bp, respectively and are located within a 10 kbp area that separates the *fII* gene into two regions, one that encodes for the non-catalytic heavy chain and one that encodes for the catalytic light chain. The exons 9-13 encode for the serine protease domain in a similar fashion as in trypsin (Craik et al., 1984) and chymotrypsin B (Bell et al., 1984). This is in contrast to fB (Campbell et al., 1984)/C2 (Ishii et al., 1993) and C1r/C1s (Tosi et al., 1989)/MASP-2/MASP-3 (Schwaeble et al., 2002) for which the serine protease domain is encoded by 8 and 1 exons, respectively.

Work by Minta et al. lead to the characterisation of the promoter for human *fII* gene (Minta et al., 1998b). A 4 kbp segment of the 5' flanking region of the gene was cloned and from this segment, a 1474 bp region from the 3'-end was sequenced. It was found to contain a number of transcription consensus sequence elements from which a major and two minor transcription start sites were identified at positions 152, 178 and 198 bp upstream of the translation start site. Although promoter activity was initially located within a 273 bp fragment located at - 112 to + 161 in relation to the major transcription start site, it was made clear that the core promoter activity resides in a 115 bp region located between at - 112 to + 3. This particular region was found to contain an *Inr*-like element overlapping the major cap site, a CTF-NF1 element, two potential CCAAT boxes and an AP-2 element partially overlapping an Sp-1 site. It was concluded that the *fII* gene promoter probably belongs to the TATA-less driven class II promoters whose transcription is regulated by the Sp-1 site. IL-6 cytokine was initially identified as an upregulator of the human *fII* gene (Minta et al., 1998b). Rat *fII* gene expression was found to be upregulated by IL-6, but not IL-1 β , IFN- γ and TNF- α , either alone or in combination (Schlaf et al., 2001a).

1.3.4 Synthesis and post-translational modifications

In mammalian species, such as rat and human, and recently in cartilaginous fish, such as shark, the liver has been shown to be the main site for the biosynthesis of fII (Schlaf et al., 1999; Terado et al., 2002). Expression has also been demonstrated in

endothelial cells (Julen et al., 1992), human primary monocyte cultures (Whaley, 1980), lymphocytes (Lambris et al., 1980) and Raji B cells (Vetvicka et al., 1993).

There has been no circulating zymogen of factor I identified *in vivo* and the mature enzyme circulates in plasma as a heterodimer consisting of a heavy and a light chain that are disulphide linked. This heterodimer is formed intracellularly after an initial removal of an 18 amino acid long leader peptide from the N-terminus of the original polypeptide and further processing within the secretory pathway that includes the proteolytic cleavage of the bond R³²¹-I³²² (Wong et al., 1995) (Fig. 9). This cleavage releases the I³²² that becomes the N-terminus of the light chain in the mature protein. In fI the light chain contains the SP domain and the presence of I at its N-terminus serves a structural role as described earlier in section 1.2.6.1. This type of cleavage is similar to the processing of many other secretory proteins that are synthesized as pro-protein precursor molecules which undergo intracellular proteolytic processing to generate the mature proteins (Misumi et al., 1991). The amino acid sequences in these precursor proteins, commonly used as signals for cleavage by processing endoproteolytic enzymes like Paired Amino acid Cleaving Enzyme (PACE) or furin, are paired basic amino acid residues like K-R (as in the case of fI) or R-R (Hosaka et al., 1991; Parish et al., 1986; Wise et al., 1990). This class of enzymes contains members with serine protease domains homologous to the subtilisin family. Immunocytochemical studies have shown that the membrane attached furin is localised, mainly in the Golgi complex (Misumi et al., 1991). Pro-forms of proteins and enzymes are translocated through the endoplasmic reticulum to the Golgi where they are post-translationally processed to maturation prior to secretion. PACE has been shown *in vitro* to recognize the basic sequence R³¹⁸RKR in human fI and to cleave the bond R³²¹-I³²² (Wong et al., 1995). A zymogenic form of fI has only been described *in vitro* for a mammalian cell expression system transfected to express recombinant human fI. The ordinary transfected cells were secreting predominantly (90%) single-chain fI, probably due to the high level of expression and the saturation of the pro-peptide cleaving capacity of the cells, while co-transfection of the same cells with a construct containing the PACE gene resulted in the secretion of greater than 90% processed (2 chain) recombinant fI (Wong et al., 1995).

On each chain of fI there are three potential sites of attachment of N-linked oligosaccharides located in loop regions (Fig. 17); these sites in the Heavy chain are at N⁵², N⁸⁵, N¹⁵⁹ and in the Light chain at N⁴⁴⁶, N⁴⁷⁶, N⁵¹⁸. The MW difference of approximately 23 kDa (25-27% w/w) that is detected by SDS-PAGE analysis between the observed and the estimated MW, supports the notion that all six sites are occupied (Goldberger et al., 1984). The loop regions where the glycans are attached are probably exposed to the solvent and by covering hydrophobic areas they contribute to the solubility of the molecule. The degree of complex glycosylation is apparent in isoelectric focusing (IEF) which reveals that considerable microheterogeneity of more than 20 isoforms ranging in their isoelectric points from 4.5 to 6.0. This heterogeneity is probably due to natural variation in sialylation as neuraminidase treatment reduces the level of pattern complexity to three major bands with pIs around 7.5 (Kolble et al., 1986). A brief characterisation of the total glycan content of fI showed that the enzyme contains predominantly complex biantennary glycans, 46% of which are disialylated and 26% of which are monosialylated (Ritchie et al., 2002). Recombinant human fI expressed in insect cells exhibited only 55% of the C3(NH₃) cleavage activity of the native enzyme from serum, suggesting that glycans might have some functional participation (Ullman et al., 1998). Since the interactions of fI with fH and C3b are mainly ionic in nature (Soames and Sim, 1997), the presence of neutral oligomannose glycans from the insect cells may weaken these interactions.

1.3.5 Polymorphisms and allelic variation

Polymorphism of the *fI* gene was originally described in the Japanese population and was detected via the application of isoelectric focusing (IEF) on polyacrylamide gels and subsequent western immunoblotting with polyclonal anti-human fI (Nakamura and Abe, 1985). Two common autosomal codominant alleles, IF*A and IF*B and two rare alleles IF*A1 and IF*B1 were identified in the Japanese population (Nakamura et al., 1990; Nakamura et al., 1991; Zhou and Larsen, 1989). Further work showed polymorphism in other Asian populations as well, covering Mongoloid populations, including the Chinese Han population (Zhang et al., 1999), in which fI shows a higher variation than among Caucasoids (Ding et al., 1991; Nishimukai and Tamaki, 1986; Yuasa et al., 1988). The two variant alleles described initially are

probably restricted to Far east Asian populations since groups of Finns, Germans, Senegalese, Turks and Indians were found to be monomorphic for the allele IF*B that is predominant in the Japanese (Kolble et al., 1986). In contrast, homozygous deficiency that implies the occurrence of silent alleles has been only detected in Caucasoids and Negroids, but not in Mongoloid populations (Sim et al., 1993b). In aspects of gene variants, recently (Fremaux-Bacchi et al., 2004) from gene screening of 100 healthy Caucasian controls revealed the presence of four mutations: C1610A (silent mutation, one heterozygous C/A), A1246G (two heterozygous G/A), G1351A (two heterozygous A/G) in exon 11 (Fig. 10) and C1671G (one heterozygous G/C) in exon 13 (Fig. 10). These three mutations cause the amino acid changes R³⁸⁸ to H³⁸⁸, K⁴²³ to R⁴²³ and E⁵³⁰ to N⁵³⁰, respectively.

1.3.6 Evolutionary aspects

The ancient origin of the complement system is nowadays considered widely accepted (Dodds, 2002; Zhu et al., 2005). Work at the molecular evolutionary level earlier had revealed the existence of the complement system not only in vertebrates but also in some deuterostome invertebrates (Smith et al., 1999) and recently in the more primitive protostome lineage (Zhu et al., 2005). Cloning work has led to the identification of complement components from the lectin (section 1.1.1.2) and alternative (section 1.1.1.3) activation pathways in invertebrates (Terado et al., 2002). Functional and molecular cloning analyses have revealed the presence of a primitive complement system that lacks the classical and lytic pathways in invertebrates and cyclostomes that are considered the most primitive extant vertebrates. In contrast bony fish have been found to have both the classical and cytolytic pathways (Terado et al., 2002).

The characterisation of the fl cDNA sequences from various species (section 1.3.1) provided the basis for comparisons of the derived amino acid sequence for evolutionary purposes. Alignment of the deduced amino acid sequences from the isolated cDNAs, revealed that the modular organization has been unchanged since sharks and mammals last shared a common ancestor (Terado et al., 2002). The domains show a high level of conservation with elements like cysteine residues well

conserved. The structural conservation of fI through this broad spectrum of organisms suggests that the basic structure of the enzyme has been maintained largely intact as it facilitates through specific interactions the breakdown of C3b/C3(H₂O)/C4b in the presence of a cofactor (Soames and Sim, 1997). C3 is the central component of the complement system and its control, important for the regulation of the complement system function, must have been important throughout the course of evolution, presumably from its early organisations. For the case of shark, as the fI activity is considered dependent on the presence of a cofactor (section 1.4.2), the presence of at least one of the known cofactors is strongly proposed. The identification of a divergent segment (D segment) present after the second LDLR-A at the C-terminus of the heavy chain in mouse fI, was found intriguing in aspects of evolution and domain conservation (Minta et al., 1996). In aspects of modular conservation, this segment exhibits the largest degree of cross-species variation among the characterised domains. The particular segment is separated into sub regions due to the high level of structural similarity in mammalian and amphibian species. Since the shark fI has a short D segment of 10 amino acids (Human 19, Mouse 35, Xenopus 48) (Terado et al., 2002), it lacks similarity with the mammalian and amphibian fI divergent D-segments. This segment may have evolved independently after the divergence of cartilaginous fish. As the particular region does not have a well-conserved domain structure among species that could be of proposed functional importance, it appears that through evolution exon diversification events may have occurred. Currently, there is no functional data available for the role of the D segment.

Within the framework of a cascade system organisation, like the ones of complement or coagulation, in which enzymes of related structure and function participate, the use of molecular markers found in conserved domains has facilitated the reconstruction of a possible order of enzyme evolution and supported the gathering of new insights into functional linkages among existing cascades (Krem and Cera, 2002; Krem and Di Cera, 2001). Regarding complement, all of the serine proteases contain a serine protease domain that belongs to the chymotrypsin-like clan in which the dominant fold makes use of an S-H-D catalytic triad in which the S is the attacking nucleophile (section 1.2.6.1). Due to the highly conserved nature of the catalytic architecture, evolutionary markers that document changes in the sequences facilitating the catalysis mechanism, like the residues of the catalytic triad, should

provide evidence for the history of an enzyme group. In several protease families, there is a non-random dichotomy as to whether TCN (N for any base) or AGY (Y for C/T) codons encode for serine (Brenner, 1988) either at the active site position 195 (S^{195}) or at the conserved position 214 (S^{214}) that is adjacent to the active site. Apart from the use of these two codons as markers, a third element, that is based on the usage of P or Y for position 225 that is important for the Na^+ regulation of catalysis, can also be used as a marker. The primitiveness of TCN compared to AGY (Diaz-Lazcoz et al., 1995) and of P compared to Y (Krem et al., 2000), have been used to establish the relative ages of the lineages in a number of cascades. The combination of the above markers has lead to the collective formation of two types of configuration: S^{195} : TCN/ S^{214} : TCN/ P^{225} - the most primordial and S^{195} : AGY/ S^{214} : AGY/ Y^{225} - the most modern. Sequence analysis of the human complement components and application of the discussed marker criteria, showed that the fI SP domain exhibits a configuration of S^{195} : TCN/ S^{214} : AGY/ P^{225} also shared by fB/C2 and tPA/uPA (Plasminogen Activator). The particular conformation has only a single codon change, the one for S^{214} , from the most primordial configuration found in fD. In that sense these proteases are ancient and considered older than other proteases like C1r/C1s or Thrombin that show a S^{195} : AGY/ S^{214} : TCN/ Y^{225} conformation and thus must have evolved in later stages. Based mainly on the status of the markers, a proposed diagram illustrating the levels in the evolution of the complement proteases is shown in Fig. 11 (P. Gál, personal communication - unpublished work). Although these analyses require further support from functional studies, especially for the case of MASP-1, they are in good agreement with existing information, about the origins of complement, the evolution, the functions and organisation of its pathways, the structural complexity of the proteases and perhaps about the roles of complement components as an ancient predecessor of the more sophisticated and highly ordered coagulation system.

Regarding the evolution of the serine protease domain, apart from the molecular markers discussed above, the existence of conserved cysteines that participate in core disulphide bridges can also be used as a marker. An unusual characteristic of the fI SP domain is the presence of 11 cysteine residues of which 10 participate in intradomain disulphide bridging, while the remaining $C^{435(122)}$ forms a disulphide bond with C^{309} at the C-terminus of the heavy chain as has been determined experimentally (Tsiftoglou

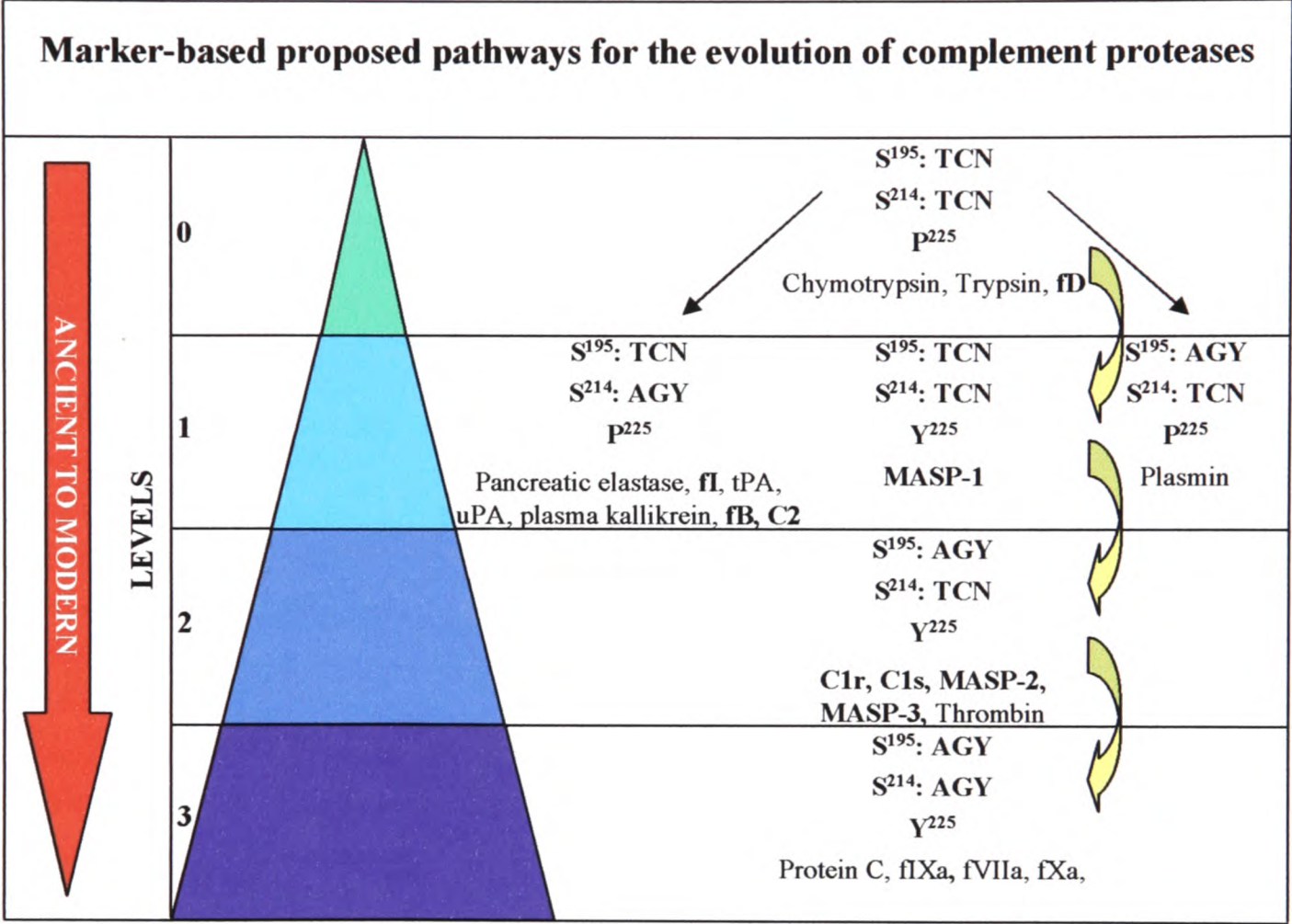


Fig. 11. Diagrammatical representation of the proposed pathways for the evolution of complement proteases. Using collectively three molecular markers for the SP domain, as described in section 1.3.6., the complement proteases were grouped according to the similarity levels among the marker configurations. Other non-complement enzymes were used for reference and comparison purposes. The diagram was constructed on processed information derived from Krem, M. M., and Di Cera, E. (2001). *Molecular markers of serine protease evolution*. Embo J 20, 3036-3045. The evolution of complement proteases is proposed to have occurred in three phases, representing levels 0-2. The proteases of level 0 have overall a simple single domain organisation and exhibit the most primordial marker configuration, while the ones at the subsequent levels show an increased overall domain mosaicity and have more modern marker configurations as diversification during evolution has occurred. Transition from one level to an adjacent one assumes a single marker change. The pathways, indicated by the arrows show the more likely evolutionary transitions. There is now growing evidence from functional studies which support the fact that the transition between levels 1 and 2 has occurred through the evolution of the MASP-1 gene. Based on this diagram it appears that in aspects of evolution the complement system is older than the coagulation system.

et al., 2005) (chymotrypsinogen numbering in parentheses). For the fI SP domain the disulphide bridging pattern, as determined on the basis of homologies, is shown in Table 2. The existence of the disulphide bond C⁴⁴⁹⁽¹³⁶⁾-C⁵¹³⁽²⁰¹⁾ in the complement proteases fD and fI that exhibit two of the oldest configurations of molecular markers discussed above, suggests that these enzymes are with high probability ancient within the frames of serine protease evolution.

1.4 ACTIVITY OF FACTOR I

The activity of factor I is pivotal for the control of the levels of C3 convertases (C4b2a and C3bBb) and for the amplification of the cellular immune response. Factor I has a natural narrow specificity and can cleave two natural substrates; soluble C3b or C4b, or membrane-bound C3b or C4b that are generated on the surface of complement activators after the triggering of the complement response. The proteolysis of these substrates, in bimolecular complexes with a cofactor, structurally disables them from participating in any convertase complex formation on the cell surface of the activator, thus maintaining the balance of the complement system under physiological conditions. In this sense the activity of fI is not limited to one pathway. So far, no natural inhibitors for fI have been identified, while its inhibition artificially using substituted isocoumarins was found to be very low (Kam et al., 1992). The developed boropeptide derivative inhibitor compounds such as DuP 714 (Fevig et al., 1998) and S18326 (Rupin et al., 1997) had low inhibitory activities and were not exclusively selective for fI, but highlighted the interest in the synthesis of inhibitors for fI that could be useful in pharmacotherapy; for example in cases where the depletion of complement could be beneficial like in rheumatoid arthritis or multiple sclerosis (Makrides, 1998).

The proteolysis of the two substrates can occur only in the presence of cofactors like factor H (Fujita and Nussenzweig, 1979), C4bp (Nagasawa and Stroud, 1977), MCP (Seya et al., 1986) or CR1 (Fearon, 1980; Krych-Goldberg and Atkinson, 2001; Malhotra and Sim, 1984). All these fI cofactors have been found to support the cleavage of membrane-bound or soluble C3b or C4b, and soluble methylamine-treated C4 or C3. In physiological conditions fH is specific for C3b/C3(H₂O) (Fujita and Nussenzweig, 1979), while C4bp is specific for C4b/C4(H₂O) (Fujita and Nussenzweig, 1979; Gigli et al., 1979; Seya et al., 1985). The reactions of fI with the substrates and the cofactors are ionic strength and pH dependent; in all the cases the rate of cleavage decreases with the increase in salt concentration. The pH dependence for the cleavage of C3b (Crossley and Porter, 1980) or C4b (Seya et al., 1985) by fI in the presence of fH or C4bp, respectively, shows an optimum below pH 6.0, while for

the cleavages of C3b or C4b when CR1 acts a cofactor the pH optimum is between 7 and 8 (Sim and Sim, 1983).

In non-physiological buffer assay conditions, comparative studies of the potencies of the natural cofactors for the factor I mediated cleavage of C3b and C4b have been carried out. It has been shown that in non-physiological conditions all known complement fI-cofactors have the capacity to interact with both C3b and C4b (Seya et al., 1995) (Table 3). Factor H, that physiologically does not support the cleavage of C4b (soluble or surface-bound), has been reported to support the cleavage of methylamine-treated C4 (C4ma) (Seya et al., 1995) at low ionic strength and acidic pH (50 mM NaCl, pH 6.0). Although weak cofactor activity of fH in the cleavage of C4ma was detected, C4bp expressed sufficient cofactor activity for C4ma over a wide pH range. On the basis of weight, MCP and CR1 have been found to support the cleavage of membrane-bound C4b more efficiently than C4bp. Moreover, CR1 and MCP can act equally effectively on both membrane-bound and fluid phase C4b, while C4bp is more effective on fluid phase C4b. For the cleavage of fluid phase C3b by fI in similar buffer conditions, the order of efficiency on a molar basis had been determined as fH>CR1>>C4bp (Seya et al., 1985). Interestingly, an artificially produced monomeric form of C4bp (mC4bp) has been reported to be a more efficient cofactor for the cleavage of methylamine-treated C3 than the natural multimeric C4bp (heptamer), and equally efficient with MCP in the cleavage of methylamine-treated C4 (Mikata et al., 1998). Unlike natural C4bp, the mC4bp showed more potent C3ma fI-cofactor activity than with C4ma, and was found to behave more like the cofactor MCP rather than C4bp.

1.4.1 Natural substrates

By having evolved features that allow its specific interactions with at least 25 different proteins, C3 is considered as one of the most versatile and multifunctional proteins identified so far. Human C3 is the most abundant complement protein in serum, originally synthesized in the liver as a single polypeptide (pro-C3) (Tack et al., 1979), and processed to form two chains, the α (115 kDa) and the β (75 kDa) that are covalently linked through a disulphide bond (Sahu and Lambris, 2001). The

Table 3. Complement regulators in the support of fI proteolytic activity

Cofactor	Substrate
fH	C3b, C3(H ₂ O) ¹ , C3ma ² and [C4b, C4(H ₂ O) ¹ , C4ma ²]
CR1	C3b, C3(H ₂ O), C3ma and C4b, C4(H ₂ O), C4ma
C4bp	C4b, C4(H ₂ O), C4ma and [C3b, C3(H ₂ O), C3ma]
MCP	C3b, C3(H ₂ O), C3ma and C4b, C4(H ₂ O), C4ma

1. C3(H₂O)/C4(H₂O): C3/C4 with a hydrolyzed thioester.

2. C3ma/C4ma: Methylamine-treated C3/C4.

Both forms 1 and 2 of C3 and C4, in the presence of cofactors, can adopt a conformation that supports their cleavage by fI.

Square brackets indicate substrates for fI only in non-physiological buffer conditions (50 mM NaCl, pH 6.0).

properties of C3 and its homologue C4 are summarised in Table 4. In human C3 from the total number of 27 cysteines, all but one participate in intramolecular disulphide bridges (Dolmer and Sottrup-Jensen, 1993; Huber et al., 1980). The unpaired cysteine C⁹⁸⁸ participates in the post-translational formation of a thioester bond with the γ -amide group of the glutamine Q⁹⁹¹ in the α chain region C⁹⁸⁸-G-E-Q⁹⁹¹-N of C3.

The important identification of the covalent binding of C3 to sheep erythrocytes (Law and Levine, 1977) shed light on early observations made in the 1960s about the binding of C3b to a wide range of unrelated surfaces such as cell membranes, bacterial cell wall components and immune aggregates. Further key experiments established the presence of an internal thioester (Fig. 12); the stoichiometric appearance of a free thiol group when C3 was inactivated after treatment with nitrogen nucleophiles (Janatova et al., 1980), the labelling of the released thiol group with ³H-iodoacetic acid and the localisation of the labelled cysteine in the -C-G-E-Q-sequence by peptide mapping (Tack et al., 1980). The localisation of the labelled cysteine in the particular sequence of the α chain, suggested the formation of a thioester bond between the thiol group of the cysteine and the acyl group of the second glutamyl residue leading to the formation of a 15-member lactone ring. In the same period, it was established that similar thioester bonds in homologous conserved regions were present in C4 (Campbell et al., 1981; Harrison et al., 1981) and α_2 -Macroglobulin (Howard, 1981; Sottrup-Jensen et al., 1981).

The thioester bond is pivotal for the function of C3. Upon activation of C3, the C3a fragment is released from the α chain (sections 1.1.1 & 1.1.1.1), while the remaining macromolecule undergoes a rapid major conformational change that exposes the thioester bond previously hidden in a hydrophobic pocket (Isenman et al., 1981). The exposed thioester of C3b is very labile and short lived with a $t_{1/2}$ of less than 1 s as it can be hydrolysed almost instantaneously by water and other nucleophilic groups, mainly hydroxyl groups or amines (Sim et al., 1981b). Results have supported the notion that although the thioester contributes to the stability of the molecule, it is not the core feature primarily responsible for maintaining the inert conformation of the molecule and that in contrast, it is probably the conformation that primarily protects the thioester from hydrolysis. Once C3 is cleaved to C3b, the transiently exposed thioester bond participates in a transacylation reaction with nucleophilic groups

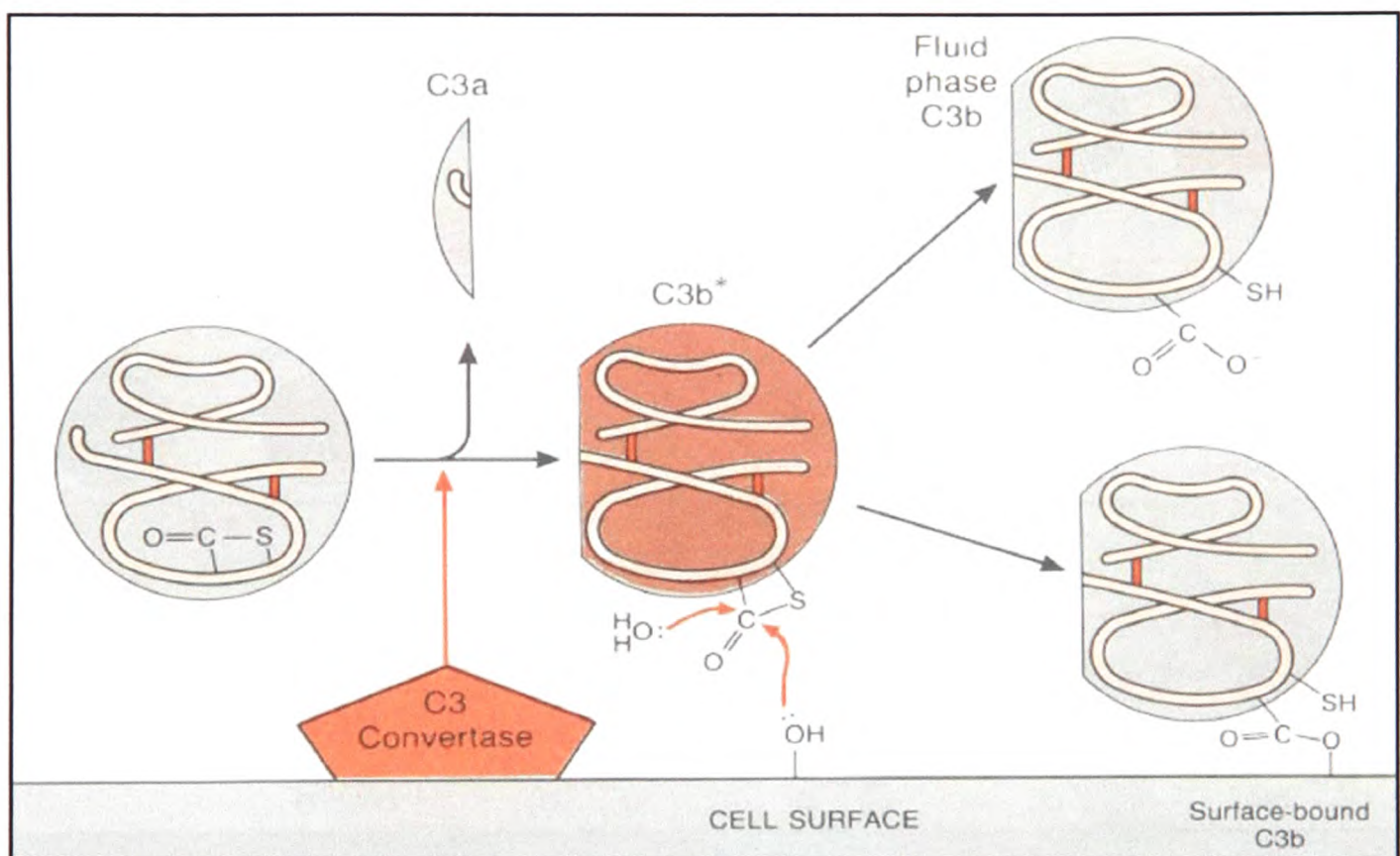


Fig. 12. Scheme representing the activation of C3. In the presence of complement activator surface bound C3 convertase, C3 is split into C3a and C3b. During this proteolysis, the previously protected internal thioester bond of C3 is exposed. The transiently exposed bond can participate through transacylation reactions with nucleophilic groups that can either facilitate the binding of C3 to cell surfaces, or lead to the eventual hydrolysis of C3b in fluid phase by water.

Figure from Law, S. K. A., and Reid, K. B. M. (1995). *Complement*, 2nd Edition (Oxford, IRL Press Ltd.).

Table 4. Properties of human C3 and C4		
	C3	C4
Serum concentration (µg/mL)	1,200	600
MW Apparent ¹	190 kDa	205
MW Calculated ²	184,499 Da	189,326 Da
Subunits (mol. Wt ¹)	α chain (114 kDa) β chain (75 kDa)	α chain (97 kDa) β chain (76 kDa) γ chain (32 kDa)
Amino acids		
Signal peptide	22	19
Mature Protein	1,637	1,717
Carbohydrate (~% w/w)	2.9	7.6
(no. of occupied N-linked sites)	(2, N ⁶³ , N ⁹¹⁷)	(4, N ²⁰⁷ , N ⁸⁴³ , N ¹³⁰⁹ , N ¹³⁷²)
Predominant pattern of glycosylation	α and β chains Oligomannose	α chain Complex type (disialylated biantennary N-glycans) β chain Oligomannose
Theoretical isoelectric point	5.9	6.2
Extinction coefficient (280 nm, 1%, 1 cm)	9.3	9.0
Genetic locus	19p13.3	6p21.3
1. As determined by SDS-PAGE. 2. Based on the primary sequence of the mature protein		

present on cell surfaces, complex carbohydrates or immune complexes with antibodies. C3b molecules that fail to bind to any surface have their thioester rapidly hydrolysed and can thus become susceptible to cleavage by complement factor I in the presence of cofactors.

A number of studies have explored the binding selectivity of C3 and concluded that C3 exhibits various binding patterns. In various simple molecules the binding in most cases to primary hydroxyl groups is preferred over secondary ones both in alcohols and monosaccharides (Law et al., 1984; Sahu et al., 1994). Within this frame it should be noted that C3b does not have the ability to discriminate between self and non-self. The protection of self against undesired effects of complement activation is provided by the complement regulators (section 1.1.2).

The conformational changes that occur through the activation of C3 as well as C4 expose a number of specific, non-covalent binding sites on the surfaces of C3b and C4b that facilitate interactions with soluble and membrane-bound ligands (Sahu and Lambris, 2001). Both C3b and C4b can participate in the formation of C3 and C5 convertases that are crucial for the amplification of the complement activation response (sections 1.1.1 and 1.1.3). Regarding the C3 convertases, the alternative pathway convertase can be stabilised through the interaction of C3b with properdin (section 1.1.2.1). In the limitation of harmful complement activation against self, the specific interactions of C3b and C4b with complement regulators block the assembly of C3 convertase complexes on self cell surfaces. C3b that has bound to complement activating cell surfaces can bind to CR1 on the surface of phagocytic cells, thus operating as an opsonin (section 1.1.2.2) (Ross et al., 1983). Activated C4 can also bind to CR1, while C3b, iC3b and a fragment of iC3b, C3d, can bind to CR2 which is the B-lymphocyte C3d receptor (Ross et al., 1983) (Table 5). Complement Receptors 3 and 4 (CR3 and CR4), that are highly abundant on the surface of phagocytic cells, can specifically recognise only iC3b (Ross et al., 1992), thus making iC3b a much more effective opsonin than C3b. These interactions between C3b and its proteolytically generated fragments contribute to intracellular signalling nowadays believed to be important not only for complement and innate immunity, but also for adaptive immunity (Fearon and Locksley, 1996).

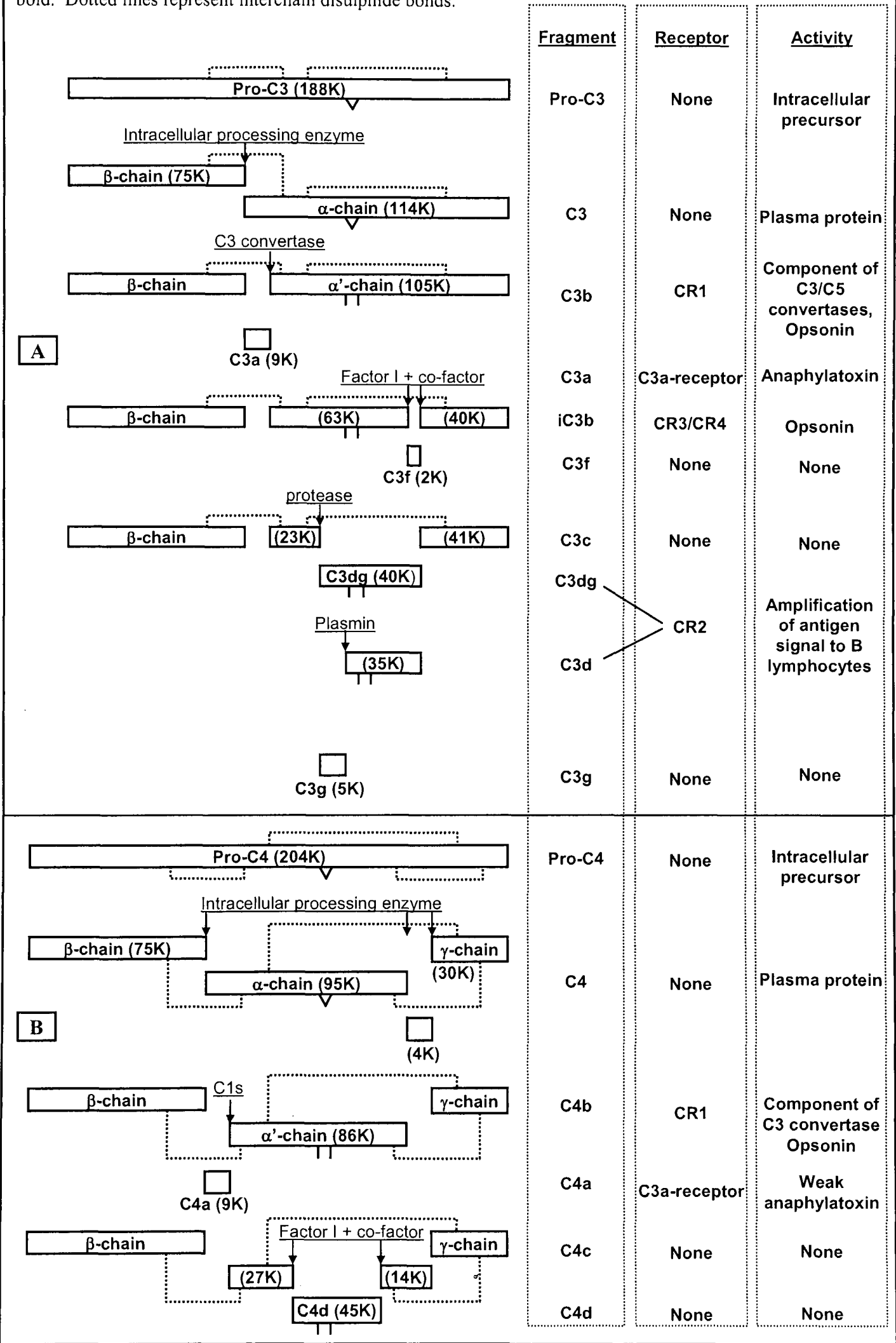
Table 5. Receptors for activated C3 and C4					
Receptors		MW (kDa) ¹	Ligand	Major Role	Localisation ²
Complement Receptor type 1/CD35	CR1*3	160	C3b,	- Cofactor for cleavage of C3b and C4b by Fi - Adherence of C3/C4b-opsinised immune complexes to erythrocytes - Phagocytic uptake - Decay accelerating activity for all types of C3/C5 convertases	L, E, B, G, M
	CR1*1	190	C4b		
	CR1*2	220			
	CR1*4	250			
Complement Receptor type 2/CD21 ³	Form I	139	C3b,	- Regulation of B lymphocyte functions - Epstein-Bar virus receptor	B
	Form II	145	iC3b, C3dg, C3d		
Complement Receptor type 3/CD11b, 18		α chain 165 β chain 95	iC3b	Phagocytosis	G, M, φ
Complement Receptor type 4/CD11c, 18		α chain 150 β chain 95	iC3b	- Phagocytosis - Monocyte migration	G, M, φ
- As determined by SDS-PAGE. - Cell types: E: Erythrocytes, B: B lymphocytes, G: Granulocytes, L: Leukocytes, M: Monocytes, φ: Macrophages. - Toothaker, L. E., Henjes, A. J., and Weis, J. J. (1989). <i>Variability of CR2 gene products is due to alternative exon usage and different CR2 alleles</i>. J Immunol 142, 3668-3675.					

In addition, C3b or C4b can also play an important role in the processing of immune complexes, protecting against the development of immune complex disease (Cochrane and Koffler, 1973; Schifferli et al., 1986). The binding of C3b or C4b to immune complexes (IC) reduces the antibody-antigen(s) interactions, while it contributes to the increased solubility of the complexes in circulation and facilitates their clearance through recognition by cell surface ligands (primarily CR1) on phagocytes or erythrocytes (Czop and Nussenzweig, 1976). Phagocytes can engulf the complexes and destroy them, while erythrocytes (Waxman et al., 1984) can rapidly bind large, preformed immune complexes and transport them to the liver for clearance (Cornacoff et al., 1983). Factor I has been implicated in the release of immune complexes bound to erythrocyte CR1, after it was demonstrated that it can reduce the affinity of ICs binding to erythrocyte CR1 following the cleavage of CR1-bound C3b-IC to iC3b-IC by factor I (Jepsen et al., 1986). The clearance of immune complexes from circulation is important for the avoidance of tissue or organ immune complex mediated injury.

1.4.2 The cleavages of C3b and C4b by factor I

Once generated, both C3b and C4b can be processed rapidly by fI in the presence of a cofactor protein. In a two step proteolysis (Fig. 13), factor I cleaves human C3b in two positions in the COOH-terminal half of the α chain, first between residues R¹²⁸¹ and S¹²⁸² in the site Q¹²⁷⁷-L-P-S-R-S-V-K-I¹²⁸⁵ and then between residues R¹²⁹⁸ and S¹²⁹⁹ in the site Q¹²⁹⁴-L-P-S-R-S-V-K-I¹³⁰² to generate iC3b (inactivated C3b) with the parallel release of C3f (Davis and Harrison, 1982; Sim et al., 1981a) (Table 1). The cleavage produces three disulphide bonded chains with molecular weights of 63 and 41 and 75 kDa (β chain). A third fI cleavage site has also been reported to exist when CR1 or fH serve as a cofactor, between residues R⁹³² and E⁹³³ in the site E⁹²⁸-R-L-G-R-E-G-V-Q⁹³⁶ in iC3b (Ross et al., 1982), although this is possibly an artefact caused by the cleavage of iC3b by other tryptic enzymes in the systems employed. The particular cleavage site is not conserved in mouse fI (Domdey et al., 1982; Fey et al., 1983). iC3b is a fragment quite susceptible to proteolysis by tryptic enzymes and can be further cleaved in the site E⁹²⁸-R-L-G-R-E-G-V-Q⁹³⁶ to generate the two fragments C3c and C3dg. The latter can be further cleaved *in vivo* by plasmin

Fig. 13. Polypeptide structures of C3 and C4. Polypeptide structures of C3 (A) and C4 (B) and their activation products. Inverted triangles represent the internal thioester, which is broken, due to the exposure to nucleophiles, upon conversion of C3 into C3b, and C4 into C4b. The activities they mediate are indicated in bold. Dotted lines represent interchain disulphide bonds.



to C3d and C3g. If C3b is surface bound, the fragments that remain bound on the surface are iC3b, C3dg and C3d. As mentioned in the previous section these fragments are important for mediating a number of responses through specific ligand-receptor interactions. Similarly to C3b, the α chain of C4b is cleaved in a two step proteolysis that yields C4c and C4d (Nagasawa et al., 1980; Press and Gagnon, 1981) (Fig. 13). The two cleavages in human C4b occur between R⁹³⁷ and T⁹³⁸ in the site D⁹³³-H-R-G-R-T-L-E-I⁹⁴¹ and R¹³¹⁷ and N¹³¹⁸ in the site S¹³¹³-S-T-G-R-N-G-F-K¹⁴²¹ (Table 1).

1.4.3 Complement factor I deficiency

Since the participation of C3 is pivotal for the activation of the complement system, both on cell surfaces and in fluid phase, complete absence or lower than normal levels of factor I will unbalance the natural ratios between all forms of C3, affecting complement function. Impaired function often leads to pathogenesis like immune complex diseases and vulnerability to microbial or parasitic infections (Walport, 2001a). The essential humoral immune defect in hereditary fI deficiency is brought about by C3 consumption due to the uncontrolled amplification loop or tickover mechanism in the Alternative activation pathway, continuously converting C3 into C3b (Fig. 1). The limited amount of C3 available for deposition on the surfaces of activators to facilitate opsonisation via complement receptors, appears to be the main determinant of gram-negative preponderance in infections (Vyse et al., 1996). Despite the absence of the capacity of fI to cleave C4b, no abnormalities of the classical pathway (section 1.1.1.1) have been recognised to date, including the ratio C2a/C2 that is used as a detector marker for classical pathway activation. To date, no clear evidence for acquired fI deficiency, such as circulation of autoantibodies to fI, has been reported, with the exception of a nephritic syndrome which has been associated with a non-hereditary case of lowered levels of fI (Forristal et al., 1977).

The first case of fI deficiency to be reported generated the first evidence for the existence of the amplification loop (Abramson et al., 1971). A number of reports published to date describe cases of fI deficiency that have been diagnosed mainly after recurrent episodes of bacterial infections or sepsis (Bonnin et al., 1993; Floret et

al., 1991; Gonzalez-Rubio et al., 2001; Jepsen et al., 1989; Moller Rasmussen et al., 1988; Rasmussen et al., 1986). The infections usually begin in early childhood, while their median age of onset in fI deficiency is 17 months. In clinical specimens from factor I deficient individuals bacteria like *Haemophilus influenzae*, *Corynebacterium diphtheriae*, *Streptococcus pneumoniae*, *Neisseria meningitidis*, *Proteus vulgaris*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* have been cultured or positively identified (Sim et al., 1993b). Susceptibility to viral or mycobacterial infections is not common.

The common major clinical manifestations of fI deficiency are the reduced levels of C3 and fB due to the uncontrolled functioning of the alternative pathway amplification loop. Further secondary immunochemical abnormalities (Barrett and Boyle, 1984; Wahn et al., 1981) might be responsible for decreased levels of fH (Bonnin et al., 1993; Naked et al., 2000) and properdin (Gonzalez-Rubio et al., 2001). In addition, fI deficiency has also been related to “immune complex” type illness (Moller Rasmussen et al., 1988; Solal-Celigny et al., 1982) with enhanced levels of immune complex formation (Gonzalez-Rubio et al., 2001) likely to be due to decreased C3 which in turn reduces opsonisation and clearance. Recently fI deficiency has been shown possibly to be involved in the genetic predisposition to the atypical Hemolytic Uremic Syndrome (Fremaux-Bacchi et al., 2004). In past cases of treatment of fI deficiency with the infusion of normal plasma (Jepsen et al., 1989; Moller Rasmussen et al., 1988; Rasmussen et al., 1990) or purified fI (Ziegler et al., 1975), the normal complement profile was temporarily restored for a short period of time (1-2 weeks) demonstrating that the balance between the amounts of complement components is important for normal function.

The early studies of the hereditary fI deficiency showed that it is consistent with autosomal recessive inheritance. The molecular basis of the deficiency was first described in two British pedigrees (Vyse et al., 1996). In the first pedigree, family 1, two siblings had no detectable circulating fI and no functional alternative pathway. Sequence analysis of their fI cDNA showed that there was a transversion (A to T) at position 1282 which caused an alteration of H⁴⁰⁰ to L⁴⁰⁰. Both siblings from family 1 were homozygous for this transversion, while the same mutation although detected on one of the fI alleles from the proband of family 2, was considered to be rare as it was

not found in 100 randomly-selected individuals. The H⁴⁰⁰ in the serine protease domain is a semi conserved residue and due to its position it could have a structural role, although no role has been assigned yet. Analysis of the expression of the fl H⁴⁰⁰>L⁴⁰⁰ mutant from the isolated primary fibroblasts of family 1, showed that the cells were capable of *in vitro* synthesising and secreting the enzyme at levels similar to the normal fibroblasts. The absence of fl from the serum could be explained on the basis that the particular mutation may reduce the conformational stability of the protein increasing its susceptibility to degradation by proteases. The proband of the second pedigree was found to be a compound heterozygote with one allele having the same mutation as described above and the second allele with a donor splice mutation that resulted in the deletion of the mRNA encoded in the fifth exon for the first LDLR-A domain (Fig. 10).

Another study examined the molecular basis of fl deficiency in a Swiss/German family with no detectable fl in serum (Morley et al., 1998). Analysis of the fl cDNA showed that the patient was a compound heterozygote. The first allele had a C1149T transition that results in a premature stop codon in the centre of the SP domain and thus any synthesized enzyme would lack the serine of the catalytic triad. The second allele had two mutations in exon 8, a G936T resulting in mutation of E²⁸⁵ (at the C-terminus of the heavy chain) to K²⁸⁵ and a G942T introducing a premature stop codon. The enzyme with the latter mutation will lack the SP domain and therefore will not be functional. These two mutations on the same allele were inherited together from the mother and were distinct from the nonsense mutation C1149T.

A similar examination focused on the molecular basis of fl deficiency in siblings from a Brazilian family (Baracho et al., 2003). Sequence analysis revealed that all the cDNA clones from both sisters contained a dinucleotide insertion (AT) between positions 1204 and 1205 within exon 11 that introduces a stop codon 13 bp downstream of the insertion site replacing V³⁷⁹. Both sisters were homozygous for the mutant allele, while their parents were heterozygotes. The synthesized mutant enzyme has a total length of 378 amino acids and physiologically is inactive as the SP domain is absent, thus rendering the enzyme inactive.

A recent investigation examined whether any genetic abnormalities of the fI gene alleles in patients with atypical Hemolytic Uremic Syndrome (aHUS) could be related with the disease state. The investigation was based on 6 patients with familial aHUS and 19 with sporadic aHUS. All had normal fH antigenic levels without any detectable mutations in the fH gene, while six had persistent mild alternative pathway complement consumption along with low C3 levels. From all the patients examined all but 3 had normal fI antigenic levels. At the time of diagnosis patients 1 and 2 had low C3 and factor B levels. Patients 1 and 3 and the father of patient 1 had decreased fI antigenic levels (50% of normal). Genetic analysis in the three patients revealed three different mutations. Patient 1 had the heterozygous nonsense transversion C1366T resulting in the substitution of R⁴⁵⁶ by a stop codon. The particular mutation lies within exon 11 that encodes a region of the SP domain. The father of patient 1 was heterozygote for the same mutation. In patient 3 another heterozygous transversion was detected in exon 13 that encodes a region of the SP domain; G1666A that leads to a stop codon instead of the W⁵²⁸. Patient 2, who had normal fI antigenic levels, and his mother presented a heterozygous nucleotide substitution in exon 13; A1600T that leads to the amino acid change D⁵⁰⁶ to V⁵⁰⁶. All these three mutations detected have not been found in an extended control group (100 healthy individuals).

1.5 COMPLEMENT FACTOR H

Factor H was originally identified by Nilsson and Mueller-Eberhard as a minor contaminant in C3 preparations and was named as β 1H globulin due to its β -electrophoretic mobility in agarose gels (Nilsson and Mueller-Eberhard, 1965). The primary demonstrations of the activities of fH were made in the 70s when fH was demonstrated to support the cleavage of C3b by C3bIna (factor I) (Whaley and Ruddy, 1976a) and accelerate the decay of the alternative complement pathway C3 convertase (Weiler et al., 1976).

1.5.1 Primary sequence and modular organisation

Human factor H consists of 1213 amino acid residues (Ripoche et al., 1988a) and is a single polypeptide chain plasma glycoprotein of 155 kDa (as determined by equilibrium sedimentation centrifugation (Sim and DiScipio, 1982; Whaley and Ruddy, 1976b)) that is present in plasma at concentrations between 110-615 μ g/ml (Pangburn, 2000). fH is a linear, elongated protein (38 x 3.4 nm (Aslam and Perkins, 2001)) that is composed of 20 CCP domains (section 1.2.6.2) (Figs. 3, 14). Studies using transmission electron microscopy showed that fH has a flexible structure that enables it to adopt various conformations (Aslam and Perkins, 2001). Factor H can also form dimers at a range of pH 5-9 at concentrations ranging from 1-11 mg/ml (Perkins et al., 1991). It has a carbohydrate composition of 9.3-18.5% (Jouvin et al., 1984; Sim and DiScipio, 1982) and deglycosylation or desialylation experiments have shown that the glycans do not participate in the binding reaction with C3b (Jouvin et al., 1984). A unique structural feature of fH is the existence of multiple binding sites with diverse specificities for several ligands (Fig. 14). This enables the delivery of several functions pivotal for complement regulation and defence.

1.5.2 Gene structure

Factor H is encoded by a single gene *HF1* that is located on the human chromosome locus 1q32 within the RCA gene cluster (Rodriguez de Cordoba et al., 1999) (section 1.1.2.1) (Fig. 2). The *HF1* gene consists of 23 exons and spans over a

Factor H CCPs	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Binding sites for																				
									C3c				C3c							C3d
➤C3b		*	*						*				*	*	*					*
➤Decay Acceleration Activity	*	*	*	*																
➤Factor I cofactor activity		*	*																	
➤Sialic Acid																			*	*
➤Heparin							*		*											*
➤CRP							*	*	*	*	*									
➤ <i>Borrelia burgdorferi</i> (BbCRASP-1), <i>Streptococcus pyogenes</i> (M/Fba)							*													
➤ <i>Borrelia burgdorferi</i> (OspE)															*	*	*	*	*	*
➤ <i>Candida albicans</i>						*	*												*	*
➤ <i>Neisseria gonorrhoeae</i>															*	*	*	*	*	*
➤ <i>Onchocerca volvulus</i> (mf)								*	*	*	*	*	*	*	*	*	*	*	*	*
➤ <i>Streptococcus pneumoniae</i> (Hic/PspC)								*	*	*	*	*	*	*	*					
➤ <i>Streptococcus pyogenes</i> (Bac)								*	*	*	*	*	*	*	*					
➤ <i>Streptococcus pyogenes</i> (PspC)						*	*	*	*	*			*	*	*					

Fig. 14. Schematic representation of the molecular organisation of human factor H indicating sites of interaction with other complement components, polyanions and microbes. Multiple sites of interactions with C3b have been identified, while CCPs 2-3 are pivotal for the fI cofactor activity of fH. CCPs 19-20 are involved in interactions with sialic acids, while binding sites for heparin have been localised in CCPs 7, 9 and 20. Regarding the interactions with microbes, several sites have been identified. The names of the proteins that are involved in some of the interactions are included in the parentheses.

Data were obtained from 1) Rodriguez de Cordoba, S., Esparza-Gordillo, J., Goicoechea de Jorge, E., Lopez-Trascasa, M., and Sanchez-Corral, P. (2004). *The human complement factor H: functional roles, genetic variations and disease associations*. Mol Immunol 41, 355-367 and 2) Ormsby, R. J. (2005) *Localisation of functional domains within the human complement regulator factor H*, PhD. Thesis, Flinders University.

94 kbp region of genomic DNA (Male et al., 2000). Each exon encodes for a single CCP domain, with the exception of exons 3 and 4 that encode for CCP2. Exon 10 does not contribute to the fH transcript but it is utilized only in the alternative splicing of the *HF1* gene that produces the fH-like 1 protein (Estaller et al., 1991). The FHL-1 protein is composed of seven CCP domains that are identical to the first seven N-terminal CCP domains of fH, while four more remaining amino acids at the C-terminus, the S-F-T-L sequence that is specific for FHL-1, are encoded by exon 10. Factor H and FHL-1 are mainly synthesized in the liver (Schwaeble et al., 1987), while extrahepatic synthesis of both have also been described in peripheral blood cells, lymphocytes, myoblasts, rhabdomyosarcoma cells, fibroblasts, umbilical vein endothelial cells, glomerular mesangial cells, neurons and more (Frieze et al., 1999). It is believed that extrahepatic production (approximately 10% of total production) of fH and FHL-1 provides increased protection of the host cells from complement activation at sites of infection or inflammation. The expression of the fH gene can be regulated by IFN- γ , IL-1, IL-6, TNF- α , LPS, retinoic acid and dexamethasone with variations among the cell lines used, with the IFN- γ and dexamethasone in common (Frieze et al., 1999). Since fH and FHL-1 are both transcribed from the same gene *HF-1* and share the same transcription start site, they both exhibit common patterns of regulation of expression.

1.5.3 Regulation of the alternative pathway of complement activation

The binding of factor H to charged surfaces is important for the alternative complement pathway regulation (Kazatchkine et al., 1979a). The surface-bound C3b can either bind factor B (Bb) or factor H (Soames et al., 1996; Soames and Sim, 1997). The binding of Bb with C3b will lead eventually, by the formation of C3bBb convertase to the production of more C3b, to be deposited on the activator surface. Factor H can control the production of more C3b by blocking the convertase activity by displacing the Bb fragment. This is characterised as the “decay-accelerating activity” of factor H. Similar activity is exhibited by other membrane-bound cofactors such as the CR1 and DAF (section 1.1.2.2). However, on non-activating surfaces, factor H may bind to C3b and to sialylated oligosaccharides, or other polyanions, and the resulting C3b-fH complex is recognised by factor I, which cleaves C3b to generate

inactive iC3b preventing the formation of C3bBb and therefore further C3b deposition.

1.5.3.1 Discrimination between complement alternative pathway activators and non-activators

One of the major roles of fH is to regulate the alternative pathway by blocking the amplification of the C3 convertase on host cells and tissues. Complement activating surfaces have the ability to confer protection (from degradation by fI) on deposited C3b enabling fB to bind and trigger activation. C3b that has bound to non-activating surfaces is not protected as it is cleaved to iC3b in the presence of fH and fI. The initial deposition of C3b to surfaces is not specific on host and non/alterd host cells; however, only for host cells, does fH (and CR1, DAF, MCP) rapidly inactivate C3b and dissociate the C3 convertase.

The capacity of a particular surface to assemble and support the C3 convertase formation determines whether complement activation will be triggered on a cell surface. One of the major parameters that affect the assembly of the convertase is the charge of the surface which depends on the presence of charged groups like sialic acids (typically *N*-acetyl neuraminic acid) and glucosaminoglycans (GAGs). Such groups are widely distributed among human host cells, while activators of the alternative pathway are relatively deficient in sialic acid (Pangburn et al., 1980). Most human cells bear high levels of sialic acids as terminal carbohydrate residues on membrane associated glycoproteins or glycolipids. Cells like endothelial cells and fibroblasts synthesize surface-associated GAGs in the forms of heparan sulphate for example. On the other hand, activator surfaces such as lipopolysaccharides (LPS) usually carry low levels of sialic acids.

The presence of sialic acids on host cell surfaces promotes a high avidity interaction between fH and C3b. The interaction of C3b with fH is weak. If however C3b is bound on a surface adjacent to a suitable charge cluster which will bind fH (weakly), the sum of the 2 types of interaction effectively produces higher avidity binding of fH to C3b. The higher avidity between fH and C3b limits the interaction

between C3b and fB preventing the formation of the convertase. Treatment of sheep erythrocytes *in vitro* with neuraminidase, that removes sialic acids, has converted the cells from non-activators to activators of the complement alternative pathway (Kazatchkine et al., 1979a; Pangburn et al., 1980; Pangburn and Muller-Eberhard, 1978). In contrast, activator surfaces can be converted to non-activators by the addition of heparin; coupling of heparin to zymosan can convert it to a non-activator of the alternative pathway (Kazatchkine et al., 1979b). Regarding the inhibitory activity of polyanions, several parameters like the polyanion molecular weight, the degree of sulfation and the cell/tissue distribution (Parker et al., 1990), can affect the inhibitory activity physiologically or *in vitro* (Maillet et al., 1988).

On the non-activating surface of sheep erythrocytes, fH shows a 5 fold higher apparent avidity for C3b than fB, therefore the binding of C3b to fH is preferred (Kazatchkine et al., 1979a). Attachment of C3b to an activator surface reduced the apparent avidity between C3b and factor H by 3-10 fold in comparison with C3b bound to non-activator or host cell surface (Pangburn et al., 1980). The sialic acid does not have an effect on the binding of fB. The affinity of fB for deposited C3b remains constant whether C3b is bound to zymosan (yeast cells walls), sheep erythrocytes or disialylated sheep erythrocytes (Kazatchkine et al., 1979a).

Structurally with regards to the host cell recognition process, it has been determined that from the three regions of fH that contain C3b binding sites, CCPs 1-4, 6-10 and 16-20 (Fig. 14), the removal of region 6-10, abolishes the binding of fH to an activator surface (Sharma and Pangburn, 1996). It was then proposed that all three sites are essential for high avidity binding with C3b on a non-activating surface, while the site 6-10 is used on activating surfaces for a low affinity interaction. Removal of the region 16-20 located at the C-terminus of fH, showed that it reduces dramatically the binding of fH to non-activating surfaces (by 90%) highlighting its importance for host recognition. The particular region is nowadays known to contain the two CCP domains 19 and 20 that support the binding of sialic acids and heparin (CCP 20).

Since pathogenic microorganisms may be usually potent activators of the complement response, many pathogens have evolved mechanisms for protection from complement activation. Pathogens can produce sialic acid or express proteins on their

surface that can bind fH and FHL-1 proteins and exploit them to protect their cell surfaces from complement activation. The pathogens have been described to bind fH through various binding sites (Fig. 14). Bacteria such as *Escherichia coli*, group B streptococci and group B meningococci produce sialic acid capsules that can bind fH mimicking host cells (Joiner, 1988), while some *Neisseria* species can incorporate sialic acid into surface expressed lipopolysaccharides (Ram et al., 1999). A large number of microbes and parasites that express proteins that can bind to fH have been identified (reviewed in Rodriguez de Cordoba et al., 2004) and include: *Borrelia burgdorferi*, *Candida albicans*, *Echinococcus granulosus*, HIV, *Neisseria gonorrhoeae*, *Onchocerca volvulus*, *Streptococcus pneumoniae* and *Yersinia enterocolitica*. The multicellular parasite *Echinococcus granulosus* (hydatid cyst disease) concentrates host fH in the walls of the cyst formed by the parasite (Diaz et al., 1997).

1.5.3.2 Decay Accelerating Activity

Following the first stage of AP activation, fH can also compete with the formed C3 convertase, the C3bBb complex. fH exhibits a “Decay Accelerating Activity” (DAA) by having the capacity to dissociate the formed convertase molecules through the ability to compete for binding to C3b (Fig. 15). In the absence of properdin (which stabilises the complex), the C3bBb convertase has a very short $t_{1/2}$ of approximately 90 seconds at 37 °C, while in the presence of properdin the $t_{1/2}$ is approximately 5-10 minutes (Fearon et al., 1973). Later it was demonstrated that fH can enhance the physiological decay of the properdin stabilised C3 convertase (Weiler et al., 1976). The stabilization of C3bBb by activated properdin minimizes intrinsic decay, while it also protects in the bimolecular complex C3b from the fI-mediated cleavage. fH restores control of the system through the decay-dissociation of the complex and the subsequent exposure of the released C3b to fI. The fI cofactor activity of fH prevents irreversibly the regeneration of the convertase at the same site. The decay of the convertase is accompanied by release of Bb which is unable to re-bind. The interactions of fH with C3b are specific as fH cannot accelerate the decay of the CP C3 convertase which contains C4b. In addition, factor H has also been found to interact with the properdin AP C5 convertase complex, C3b₂BbP (Fischer and

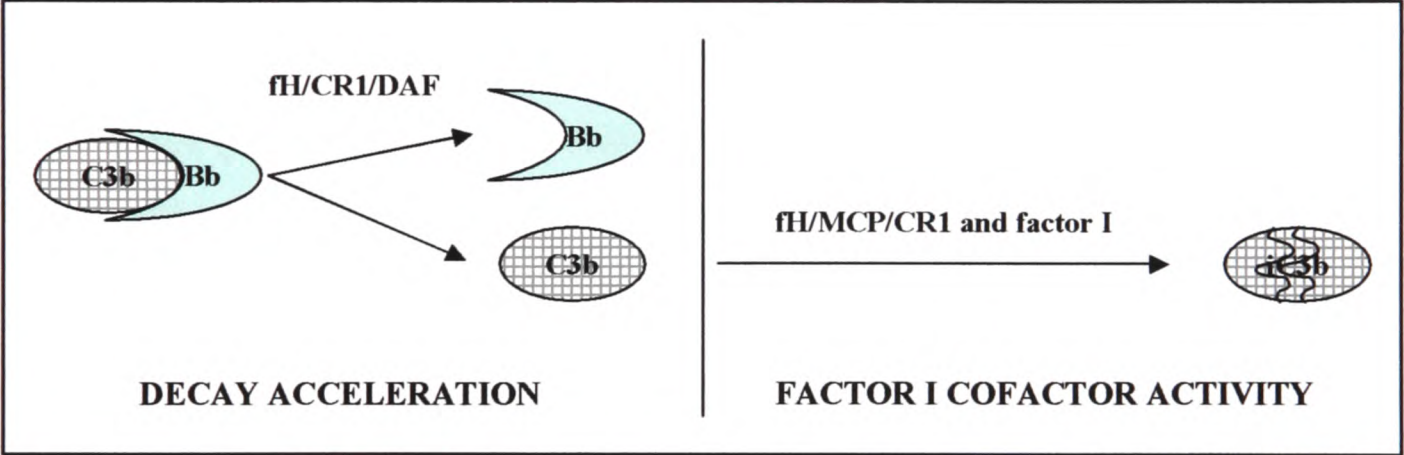


Fig. 15. Decay acceleration and cofactor activities of factor H. Other proteins structurally related to fH, but expressed on cell surfaces, have similar activities to fH (e.g. CR1/CD35, DAF/CD55, MCP/CD46).

Kazatchkine, 1983), as well as with the CP C5 convertase of the classical pathway C4b2a3b (Isenman et al., 1980).

1.5.4 Interactions between factor H, factor I and C3b

The physiological role of fI is considered in context with the molecular interactions with the cofactors and the substrates. For the understanding of the mechanisms that support the particular interactions it has been necessary to consider the enzyme as a molecular entity which interacts with the other components through specific sites in a tri-molecular complex. The nature of the molecular basis for the physiological role of human factor I has been investigated mainly through studies of the interactions of the enzyme with human C3b and fH. A number of studies focused on these particular interactions using spectroscopic, biophysical, thermodynamic and enzymological approaches (DiScipio, 1992; Isenman, 1983; Pangburn and Muller-Eberhard, 1983), while others focused on the identification of sites of interaction among the participating components.

The early analyses using circular dichroism studies indicated that when fH and C3b associate in free solution, no major changes occur in the secondary structure of C3b or fH (Discipio and Hugli, 1982). Using spectroscopic and enzymological studies to study the C3b-fH-fI complex, it was shown that in physiological conditions, upon conversion of fluid phase C3b to iC3b by fI in the presence of fH, C3b undergoes major conformational changes as detected by the decrease in the surface hydrophobicity of the molecule, with the first cleavage in the α' chain of C3b probably being the rate-determining step for the induction of conformational changes (Isenman, 1983). These changes diminish the interactions with fH and fI, probably due to the loss of at least one high affinity binding site, enabling their release from the ternary complex. It was shown through kinetic analyses that for fI the true substrate is the C3b-fH bimolecular complex, since fH is the limiting factor for supporting the cleavage of C3b. Moreover, fI was found to show very tight binding to its C3b-fH substrate (assuming a K_a : 10^7 M^{-1} for the C3b-fH interaction) with a calculated K_m : $2.5 \times 10^{-8} \text{ M}$, but also to exhibit a low rate of catalytic efficiency (Isenman, 1983).

Further studies which investigated the kinetic interactions in more detail, showed that in physiological conditions the action of fI on its bimolecular substrate, fluid-phase C3b-fH which is in equilibrium between free C3b and fH, follows Michaelis-Menten kinetics (Pangburn and Muller-Eberhard, 1983). Kinetic data suggested that C3b-fH formation is a fast reaction and that under physiological conditions, at given concentrations of C3b and fH, the initial rate of the cleavage reaction was found to be directly proportional to the concentration of fI. The cleavage reaction product iC3b was found not to have an inhibitory effect in agreement with the fact that neither fH nor fI binds significantly to iC3b (Pangburn and Muller-Eberhard, 1978). Although at physiological conditions the affinity (K_a) of fH for fluid phase C3b at 37 ° C was found to be constant ($1.6 \times 10^6 \text{ M}^{-1}$ assuming a 1:1 stoichiometry of C3b:fH (Pangburn and Muller-Eberhard, 1983)), the avidities of fH to surface-bound C3b can show heterogeneity (K_a in the range of 7×10^5 to $3 \times 10^{-7} \text{ M}^{-1}$ (DiScipio, 1992) depending on ionic strengths in fluid phase and quality of surface states).

The interaction of fI with zymosan bound-C3b (in the absence of fH) at physiological conditions, (at 0 ° C), was found to be weak ($7 \times 10^4 \text{ M}^{-1}$) and ionic strength dependent ($5.7 \times 10^5 \text{ M}^{-1}$ at 0 M NaCl) (DiScipio, 1992). fH was found to support the binding of fI to C3b. The observation that the binding of fH to C3b augments the affinity of C3b for fI (Soames and Sim, 1997), supports the earlier proposal that fH evokes conformational changes in C3b supporting an optimum binding of fI to the C3b-fH complex: a conformational change imparted in C3b by fH was proposed by Isenman (Isenman, 1983). It was then suggested that fH binds directly to C3b and similarly fI binds tightly to the C3b-fH complex; Only molecules of C3b that are not occupied by fH would exhibit low binding affinities for fI (DiScipio, 1992).

The most recent studies of C3b-fH-fI interactions explored these findings further and provided the first evidence for the direct interaction between factors H and I in agreement with the fact that fH enhances the binding of fI to C3b (Soames and Sim, 1997). The studies based on direct binding assays confirmed the earlier results and established that fH and fI interact directly with one another. It was proposed that the fI-fH and fH-C3b interactions may stabilise the weak fI-C3b interaction. A model was proposed for the C3b-fH-fI interactions in which the binding of fH to C3b causes a

conformational change in C3b: fI binds to both C3b and fH in the complex. The cleavage of C3b causes conformational changes that cause the release of fH due to a decrease in the stability of the enzyme-product complex which prevents product inhibition. The studies of Soames and Sim (1997) also explored the unusual pH optima for the catalysis. All the interactions between the three components exhibited similar pH-dependence: Maximal binding was observed at low pH values between 4.0 and 5.5; a sharp decrease was observed between pH 6.0 and 7.0, followed by a small decrease between pH values 7.0 and 8.5. These results were similar to the pH dependence of the catalysis of C3b to iC3b in the presence of fH and fI (Sim and Sim, 1983).

1.5.5 Sites of interaction between factor H, factor I and C3b

1.5.5.1 Binding sites on C3b

In order to understand the molecular interactions between C3b and fH a number of binding sites have been identified on both proteins. As the interaction of C3b with fI is very weak, interest has been shown mainly in the sites of association between fH and C3b; this is a complex and multi-point interaction.

Studies using synthetic peptides, fragments of C3 and antibodies, including anti-idiotypic antibodies, focused more on the C3 α chain, while the β chain was less investigated. The early work identified a fH binding site within the C3d fragment region between residues 1187-1249 (Lambris et al., 1988) (Figs. 13, 16). Briefly, various synthetic peptides corresponding to the 1187-1249 region of C3 were produced and examined for their patterns of differential binding to fH as well as their effect on the fI-cofactor activity of fH. The level of the binding of the synthetic peptides to fH was measured by the binding of fH to the microtitre-plate-immobilised C3 fragments and the detection was carried out using either anti-fH polyclonal antibodies or ^{125}I -fH. A second region in the amino-terminal region of C3b α' chain has been found to be pivotal for the interaction with fH. Following previous studies that a synthetic peptide corresponding to residues 749-789 of human C3 can inhibit the binding of fB and fH to C3b (Ganu and Muller-Eberhard, 1985), synthetic

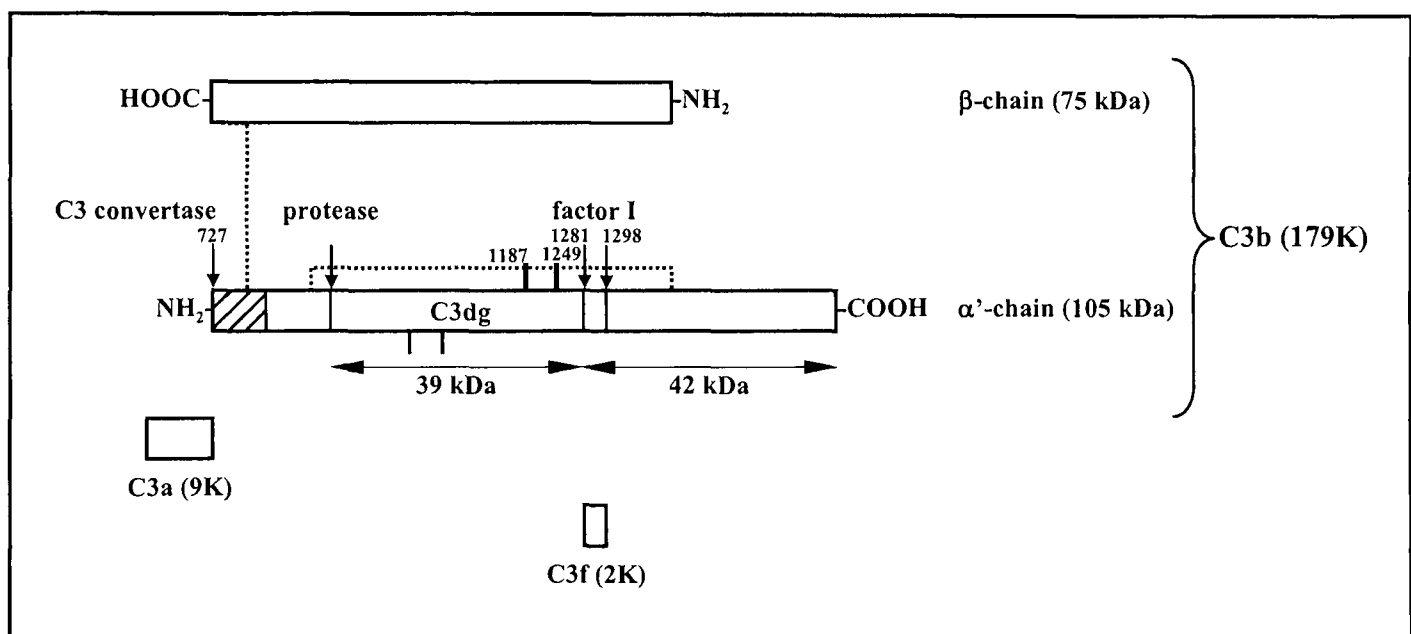


Fig. 16. Schematic representation of human C3b. The figure illustrates the regions that have been shown to contain sites of ligand binding for the interactions with cofactors and other complement components. The area 727-768 is represented in the shaded box. The dotted lines represent the two interchain disulphide bonds; the two open vertical lines below the C3dg region the broken thioester bond; the vertical arrows sites of protease cleavage, including the ones for C3 convertase(s) and factor I. The sites of interaction with fH are discussed in section 1.5.5.1.

peptides representing stretches of amino acid residues of the region 727-768 of C3 and polyclonal antibodies raised against these peptides had been also found to inhibit the binding of CR1, fH and fB to C3b (Becherer et al., 1992) (Fig. 16). In the same report, an anti-C3c monoclonal Ab, which recognizes a neo-antigenic epitope expressed upon cleavage of C3 to C3b was also found to inhibit the binding of CR1, fH and fB to erythrocyte-bound C3b. Further work was carried out by the construction of deletion mutants or chimeras of human C3 with the corresponding segments from cobra venom factor (CVF), *Xenopus* or trout C3 for the understanding of the role of the region 727-768. CVF is a homologue of C3b, but the convertase formed with CVF, CVFBb, is not controlled by fH and fI. It was found that the conversion of the deletion mutant C3 Δ 727-768 to iC3b by factor I in the presence of fH or CR1 was significantly reduced in contrast to the case of MCP, while all of the chimeras, except C3 Δ 727-768, were capable of forming the fluid-phase alternative pathway C3 convertase and all reacted with properdin (Lambris et al., 1996). This indicates, that some or all of the C3 residues that constitute one of the CR1 or fH binding sites are located within the region 727-768 and that the MCP binding sites in C3b differ from those for fH and CR1.

Collectively, it has been proposed that the various cofactors (CR1, fH and MCP) bind to C3 via multiple sites of which only some are common and that for efficient binding, more than one site may be required. In addition, site-directed mutagenesis studies had also indicated that residues ⁷³⁰DE and ⁷³⁶EE of the sequence DEDIIEE within the region 727-768 appear to be involved in the interactions with fH, CR1, CR3 and fB (Taniguchi-Sidle and Isenman, 1994) (Fig. 16). In addition, the production of engineered mutants of human C3 showed that deletion of the region 759-766 (downstream of the DEDIIEE sequence) or an insertion of 6-amino acid residues between residues 1200-1201 (within the C3d region 1187-1249 shown to contain sites of interaction with fH) did not affect the cleavage of C3b by fI in the presence of fH (Ogata et al., 1998).

There is evidence for two more sites on C3 for fH binding. Work by Nilsson and Nilsson (Nilsson and Nilsson, 1987) showed that rabbit anti-human C3 antibodies that bound to Sepharose coupled Anti-anti-fH IgGs (anti-H idiotypic abs) were shown to bind the 42 kDa fragment of C3c (C-terminal of α chain) (Figs. 13, 16). In addition,

mAbs raised against the β -chain of human C3, blocked fH binding, suggesting the existence of at least one binding site for fH on the β -chain (Worner et al., 1989). The binding of fH to the β chain of C3(NH₃), the structural equivalent of C3b, was confirmed in later studies (Soames and Sim, 1997).

Although the sites of interaction of fI with C3b have not been well explored due to the weakness of the interaction, in more recent studies it was shown by ligand blotting that factor I can bind to both the α and β chains of C3(NH₃) (Soames and Sim, 1997) (Fig. 16). Apart from the weak interaction of fI with the cleavage sites on the α chain through the SP domain, fI also interacts with the β chain probably via the fI heavy chain, non-catalytic domains which are believed to be involved in ligand binding. Recent functional work (Tsiftoglou et al., 2005) presented in this thesis indicated that the proteolytic properties of fI rely strongly on the heavy chain region that appears to facilitate interactions in the C3b-fH-fI complex, at least through the direct interaction with fH (Soames and Sim, 1997).

In order to illustrate the importance of these sites on C3b individually or collectively in the interactions with the cofactors or the enzyme, further detailed work is required. Structural data as well as detailed site-directed mutagenesis work would provide a good insight.

1.5.5.2 Binding sites on fH

The first region of fH identified to host a C3b binding site was the 38 kDa N-terminal region (Alsenz et al., 1984; Sim and DiScipio, 1982). Partial proteolysis of native human fH with trypsin was used to generate two fragments, a 38 and an approximately 120 kDa that were purified to homogeneity and examined for their binding to C3b and for their cofactor activities. Only the 38 kDa fragment was found to be active. This particular fragment was able to bind directly only to fluid phase C3b and showed fI cofactor activity, similar to fH on a molar basis. The 120 kDa fragment had no fI cofactor activity supporting the fact that the site responsible for the cofactor activity was located in the 38 kDa region. N-terminal amino acid sequencing of the fragments showed that the 38 kDa fragment is composed of amino acids 1-323 which

correspond to CCPs 1-6 (Ripoche et al., 1988b) (Fig. 14). Using a computational approach based on the comparative analysis of multiple alignments of the human, mouse and bovine fH sequences with known 3D structures of CCPs (human fH CCPs 5, 15 and 16), it was suggested that CCPs 3-4 interact directly with C3b, while CCPs 2-5 support the orientation of CCPs 3 and 4 for the interaction (Soames et al., 1996). Further mutagenesis work carried out on the expression of deletion mutants of human fH defined and established the functional site within CCP domains 1-4 and showed that the CCP 1-3 unit is sufficient for cofactor activity, but for full activity the SCR 1-4 is required (Gordon et al., 1995). Although the site for the fI-cofactor activity of fH was localised, further work was carried out to characterise the location of other sites that participate in other interactions with C3b.

More recent deletion mutant work showed that more sites of such interaction exist. The findings are summarised in Fig. 14. It is currently suggested from the work of Sharma and Pangburn (Sharma and Pangburn, 1996), Jokiranta (Jokiranta, 2000) and Ormsby (Ormsby, 2004), that there are C3b/C3c binding sites in CCP 9, CCPs 12-14 and a C3b/C3d binding site in CCP 20.

1.6 CURRENT MODEL OF STRUCTURE FOR FACTOR I

Factor I was first studied structurally using transmission electron microscopy (DiScipio, 1992) and then by X-ray and neutron low angle scattering (LAS) (Perkins et al., 1993b). The early observations reported that the enzyme naturally has a bilobal conformation of $130 \pm 14 \text{ \AA}$ (13 nm) in length with the two bulbous parts, representing the Heavy and the Light chains, to have maximal diameters of $54 \pm 8 \text{ \AA}$ and $49 \pm 9 \text{ \AA}$, respectively. The shape and observed dimensions were compatible with the two chain structure and the sizes of the two chains as characterised by SDS-PAGE and sequencing results, respectively. The modelling studies using the X-ray and neutron scattering methodology showed that the overall length was between 12.8-15 nm and also that fI in physiological concentrations is monomeric in solution with an $A_{280}^{1\%, 1 \text{ cm}}$ of 12.3-14.1 (Perkins et al., 1993b). The identity of the serine protease fold of the light chain was additionally confirmed through amino acid sequence comparisons with other serine protease enzymes (like chymotrypsin, trypsin and elastase) whose crystal structures had been characterised in detail (Perkins and Smith, 1993).

The most detailed model of fI to date is based on solution neutron and X-ray scattering and homology modelling (Chamberlain et al., 1998) examining serum derived fI (native) and insect expressed fI (recombinant) (Ullman et al., 1998) (Fig. 17). This model proposes fI as a V-shaped molecule with the first three N-terminal domains of the heavy chain forming a compact triangular arrangement, where the first and third domain are linked by the disulphide bond formed between C^{15} and C^{237} that were earlier thought to be unpaired. It is now known that all the cysteines (40 in total) in human fI, all participate in inter and intra-domain molecular disulphide bridging (Fig. 9). From all the cysteines only the two pairs C^{15} and C^{237} and C^{309} and C^{435} form inter-domain disulphide bridges. Together with the SP domain, this defined two globular entities that would form a bilobal structure in factor I as originally observed. This is in contrast to other multidomain serine proteases of blood plasma coagulation, like VIIa and IXa, which exhibit extended arrangements.

The multiple sequence alignments for the four domain types in fI indicated consistency with the scattering analyses, and showed that the FIMAC domain is basically charged, the two LDLR-A domains are acidic and that the SRCR and SP domains have close to neutral charges. The opposite charges on the FIMAC and the LDLR-A domains are believed to support the domain arrangement by creating electrostatic attraction. In parallel with the known information about the interactions between C3(NH₃), fH and fI (discussed in section 1.5.4), the identification of the two globular entities in the fI bilobal structure, proposed that the triangular lobe may interact with the cofactor, while the SP lobe is capable of interacting with the substrate. In these interactions, the regions surrounding the catalytic triad of the SP domain and most of the SRCR and LDLR-A1 domains in the triangular region are mainly predicted to participate. In addition, the fitting of N-linked glycans to the central location of the interlobal region of the fI model (as shown in Fig. 8 of (Chamberlain et al., 1998), suggested that on one hand the glycans may be able to affect the interactions with the substrates or/and cofactors by regulating the protein surfaces that are left exposed for participating in protein-protein interactions, and on the other they may have the ability to participate in the ionic interactions directly.

1.7 MAJOR CONTRIBUTIONS FROM THE MRC IMMUNOCHEMISTRY UNIT TO RESEARCH ON HUMAN FACTOR I

1. First monoclonal antibody against fI

Purification of human C3b-inactivator by monoclonal antibody affinity chromatography.

L. Hsiung, M. R. Brandon, A. N. Barclay, E. Sim & R. R. Porter.

Biochem. J. (1982) 203, 293-298.

2. First report of complete amino acid sequence

Characterisation of the Primary Amino Acid Sequence of Human Complement Control Protein Factor I from an analysis of cDNA clones.

C. F. Catterall, A. Lyons, R. B. Sim, A. J. Day & T. J. R. Harris.

Biochem. J. (1987) 242, 849-856.

3. In vitro biosynthesis of complement Factor I by human endothelial cells.

N. Julen, H. Dauchel, C. Lemerrier, R. B. Sim, M. Fontaine & J. Ripoche.

Eur. J. Immunol. (1992) 22, 213-217.

4. First report of complete amino acid sequence for species other than human

Characterisation of *Xenopus laevis* complement factor I structure-conservation of modular structure except for an unusual insert not present in human factor I.

L. M. Kunnath-Muglia, G. C. Chang, R. B. Sim, A. J. Day & R. A. B Ezekowitz.

Molec. Immunol. (1993) 30, 1249-1256.

5. Best available methodology for isolation of fI and cofactors

Complement factor I and its cofactors in control of the complement system convertase enzymes.

R. B. Sim, A. J. Day, B. E. Moffatt & M. Fonatine.

Methods Enzymol. (1993) 223, 13-35.

6. First-low resolution structure

Molecular modelling of the domain structure of factor I of human complement by X-ray and neutron scattering.

S. J. Perkins, K. F. Smith & R. B. Sim.

Biochem. J. (1993) 295, 101-108.

7. First synthesis of recombinant domain

Beta-sheet secondary structure of an LDL receptor domain from complement factor I by consensus structure predictions and spectroscopy.

C. G. Ullman, P. I. Harris, K. F. Smith, R. B. Sim, V. C. Emery & S. J. Perkins.

FEBS Lett. (1995) 371, 199-203

8. The most detailed available study on interactions between C3b-fH-fI

The interactions between human complement factor H, factor I and C3b.

C. J. Soames & R. B. Sim.

Biochem. J. (1997) 326, 553-561.

9. Complement factor I, R. B. Sim

Handbook of proteolytic enzymes (eds. A. J. Barrett, N. D. Rawlings & J. F. Woessner)

pp 140-144

Academic Press, London, 1998.

10. First synthesis of full-length recombinant human fI in non-mammalian cells

Human complement factor I: its expression and secretion by insect cells and its immunological and structural

characterisation.

C. G. Ullman, D. Chamberlain, A. Ansari, V. C. Emery, P. I. Haris, R. B. Sim & S. J. Perkins
Molec. Immunol. (1998) 35, 503-512

11. First report of synthetic substrates

Human complement factor I does not require cofactors for cleavage of synthetic substrates

S. A. Tsiftoglou & R. B. Sim

J. Immunol. (2004) 173, 367-375

12. Heavy chain of f1 pivotal for the conversion of C3b to iC3b

The Catalytically Active Serine Protease Domain of Human Complement Factor I

S. A. Tsiftoglou, A. C. Willis, P. Li, X. Chen, D. A. Mitchell, Z. Rao & R. B. Sim.

Biochemistry (2005) 44, 6239-6249.

1.8 AIMS OF THE THESIS

Work on factor I has been carried out for many years in the MRC Immunochemistry Unit (section 1.7). Although several aspects of the structure and function of this key complement regulator were known prior to the start of the experimental work described in this thesis, key information regarding its enzymic activity and specificity properties was not available. Towards obtaining a complete picture at the molecular level, of how the structure and conformation determines the function of the enzyme (binding and enzymatic) the following areas were considered of high importance to focus on:

- 1) To understand whether the enzyme circulates physiologically in an active form, or, as has been proposed for fD, whether contact with the substrate/cofactor is required to induce an enzymatically active conformation.
- 2) To investigate in detail the enzymatic specificities of the serine protease domain using synthetic substrates and examine the specificities of potential inhibitor compounds.
- 3) To explore the role of the heavy chain in the interactions with the substrate and the cofactor and investigate how it affects the natural enzymatic proteolytic properties of fI.
- 4) To investigate the means of obtaining an atomic structure.

Chapter Two

CHAPTER 2: GENERAL MATERIALS AND METHODS

2.1 SOURCES OF REAGENTS AND MATERIALS

The following reagents were purchased from **Sigma** [St. Louis, MO, USA]: barium chloride, ferric chloride, mercuric chloride, nickel chloride, bovine serum albumin (BSA), 1,4-dithiothreitol (DTT), 2-[4-(2-hydroxyethyl-1-piperazine)] ethanesulphonic acid (HEPES), 1,3,4,6-tetrachloro-3 α ,6 α -diphenylglycoluril (Iodogen), polyethylene glycol (PEG 3,350 molecular weight), Coomassie brilliant blue R, ammonium persulphate, Fast 5-Bromo-4-Chloro-3-Indolyl Phosphate/Nitro Blue Tetrazolium (BCIP/NBT) alkaline phosphatase substrate tablets, N, N, N', N'-tetramethylethylenediamine (TEMED), Tween 20. Human plasmin was bought from **Kabi Diagnostica** [Stockholm, Sweden]. Cobalt chloride was purchased from **Twinstar chemicals Ltd.** [Middlesex, UK]. Sequencing Grade Endoproteinase Glu-C (V8 protease) was obtained from **Promega** [Madison, WI, USA]. Ammonium hydrogen carbonate, chromium potassium sulphate, ethylene diamine tetra acetic acid (EDTA), sodium phosphate, manganese chloride, tris (hydroxymethyl) aminoethane (Tris) and zinc chloride were obtained from **BDH Laboratory Supplies** [Poole, UK]. PBS tablets were purchased from **Oxoid Limited** [Basingstoke, Hampshire, UK]. Calcium chloride, glycine, magnesium chloride and sodium chloride were bought from **Riedel-de Haën** [Seelze, Germany]. Acrylamide/Bis-acrylamide (30% (w/v) acrylamide:0.8 % (w/v) Bis-acrylamide, 37.5:1) and sodium dodecyl sulphate (SDS) (20% w/v) stock solutions from **National Diagnostics** [Atlanta, GA, USA]. Spectro/Por 6 dialysis tubing was from **Medical Industries Inc.** [Los Angeles, CA, USA]. Microfluor white plates were from **Thermo Electron Informatics** [Franklin, MA, USA]. Pooled human plasma was bought from **HD Supplies** [Aylesbury, UK]. Sodium ¹²⁵Iodide and all the chromatographic materials were purchased from **Amersham Biosciences UK Limited** [Chalfont St Giles, Bucks, UK].

The protease inhibitors ϵ -amino caproic acid (ϵ ACA), antipain, aprotinin, benzamidine, bestatin, chymostatin, leupeptin, pepstatin A, 1,10-phenanthroline, phenylmethyl sulphonyl fluoride (PMSF) and soybean trypsin inhibitor (SBTI) were

bought from **Sigma**. The inhibitors [4-(2-Aminoethyl)benzenesulfonylfluoride HCl] (Pefabloc-SC), Na-tosylglycyl-3-DL-amidinophenylalanine methyl ester (Pefabloc Xa) and Na-(2-Naphthylsulfonylglycyl)-4-amidino-(D,L)-phenylalanine piperidide acetate (NAPAP) (Pefabloc TH) were purchased from **Pentapharm Ltd.** [Basle, Switzerland]. Z-D-Phe-Pro-methoxypropylboroglycinepinanediol Ester (Boro MPG) was bought from **Calbiochem** [EMD Biosciences, Inc., San Diego, CA, USA], lima bean trypsin inhibitor type IIL (LBTI) from **Fluka** [Buchs, Switzerland] and Suramin from **Bayer AG** [Leverkusen, Germany]. Hirudin was obtained from **Accurate Chemical and Scientific Corp.** [Westbury, NY, USA]. The Aminomethylcoumarin (AMC) synthetic substrates were purchased from **Calbiochem**, **ICN Biochemicals** [Ohio, USA], **American Diagnostica Inc.** [Greenwich, CT, USA] and **Bachem** (Budendorf, Switzerland). Pre-cast 10 % or 4-12% NuPAGE Bis-Tris gels and PVDF membranes were bought from **Invitrogen** [Carlsbad, CA, USA]. StrataClean Resin was obtained from **Stratagene** [La Jolla, CA, USA].

2.2 PROTEIN CHARACTERISATION

2.2.1 Estimation of protein concentration

Protein concentration in samples was measured by reading the optical density at 280_{nm} and calculated according to the Beer-Lambert Law:

$$OD_{280nm} = \epsilon_{280nm} \cdot c \cdot L \quad (1) \quad \rightarrow \quad c = OD_{280nm} / \epsilon_{280nm} \cdot L \quad (2)$$

where OD_{280nm} is the optical density of the sample at 280nm, ϵ is the known extinction coefficient of the protein at 280nm (OD units $M^{-1} cm^{-1}$), and the L is the length of the light path. By convention, extinction coefficient can be expressed in ϵ [280nm, 0.1% (0.1 g/100 ml), 1 cm], which is the ϵ_{280nm} of a protein at 1 mg/ml in a 1 cm cell. For the determination of concentrations of solutions of known composition formula 2 has been used. For protein mixtures, ϵ (280nm, 0.1%, 1 cm) of 1 was used (Pace et al., 1995), while for the components human C3, fH, C1-Inh and IgG the ϵ_{280nm} values of 1.0, 1.4, 0.4 and 1.4 were used. For fI the theoretical ϵ (280nm, 0.1%, 1 cm) value of 1.5, calculated for the mature enzyme using the ProtoParam software [<http://au.expasy.org/tools/protparam.html>] was used for all applications. In physiological concentrations however, fI has experimentally been shown to be monomeric in solution with an $A_{280}^{1\%, 1\text{ cm}}$ of 12.3-14.1 (Perkins et al., 1993). Similarly, for the fI SP domain the theoretical ϵ (280nm, 0.1%, 1 cm) value of 2.7 calculated for the light chain of the enzyme was used for all applications (Chapters 4 & 5).

2.2.2 Sodium dodecyl sulphate polyacrylamide gel electrophoresis and autoradiography

Proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by (Laemmli, 1970). The resolving gel, composed of various percentages (w/v) of acrylamide/Bis-acrylamide (containing 30% (w/v) acrylamide:0.8 % (w/v) Bis-acrylamide) (typically 7.5-10%), 375 mM Tris-HCl, pH 8.8, and 0.025% (w/v) SDS, was polymerised with 0.05% (w/v)

ammonium persulphate and 0.05% (v/v) N, N, N', N'-tetramethylethylenediamine (TEMED). It was overlaid with the stacking gel, which contained 5% (w/v) acrylamide/ bis stock preparation, 125 mM Tris-HCl, pH 6.8, 0.025% (w/v) SDS, 0.05% (w/v) ammonium persulphate and 0.05% (v/v) TEMED. SDS-PAGE analysis of samples was carried out separately under non-reduced and reduced conditions. This approach permits analysis of samples containing proteins with disulphide bridges. The sample preparation, SDS-PAGE sample buffer composition and Coomassie blue staining are as described by (Fairbanks et al., 1971). The composition of sample buffer was 0.2 M Tris-HCl, 8 M Urea, 2% (w/v) SDS, 2 mM EDTA, pH 8.0 with 0.001% (w/v) Bromophenol blue. For analysis under reduced conditions, the sample buffer was made 40 mM DTT and for non-reduced samples, 20 mM iodoacetamide (IAM). Electrophoresis was performed at 100-200 V in 25 mM Tris, 0.2 M glycine, 0.1% (w/v) SDS, pH 8.2, using 10 x 14 cm large format or 7 x 9 cm minigel format **Bio-Rad** [Watford, UK].

For some protein analysis steps, such as N-terminal sequencing, pre-cast 10% NuPAGE Bis-Tris gels [Invitrogen] were used. The particular gel system provides separation over a wider molecular weight range and supports the visualization of low molecular weight fragments, at a minimum of 2.5 kDa, which could not be observed using the Laemmli buffer system. The samples were prepared using the NuPAGE sample buffer (4X) and analysed by electrophoresis (constant voltage of 200V) using MES buffer (50mM 2-(N-morpholino) ethane sulphonic acid, 50mM Tris-Base, 3.4 mM SDS, 1 mM EDTA, pH 7.3) according to the manufacturer's instructions.

Dilute protein samples were concentrated on Strataclean resin prior to SDS-PAGE analysis. Briefly, 7 µl Strataclean resin suspension was added to a variable volume of sample and after brief vortexing the mixture was left to stand for 10 min at room temperature (RT). The beads were recovered by centrifugation (8,000 g, 5 min, RT), treated with 10 µl SDS-PAGE sample buffer as above, and the whole bead suspension in sample buffer was loaded onto the gel.

Proteins on gels were visualised by staining with Coomassie Brilliant Blue R (Fairbanks et al., 1971) or by silver staining using the Bio-Rad silver staining kit (**Bio-Rad**) according to the manufacturer's instructions. Radiolabelled proteins were

detected by developing autoradiographs from heat dried (under vacuum) gels. The dried gels containing the ^{125}I -labelled samples were exposed to X-ray film (Fuji RX) from **Fuji Photo Film UK Ltd.** [London, UK] at $-70\text{ }^{\circ}\text{C}$ (usually for 16 hrs.) in autoradiography cassettes with intensifying screens. Films were developed using the Xograph imaging system Compact X4 model from **Xograph Imaging Systems Ltd.** [Gloucestershire, UK.].

2.2.3 Western blotting

For a western blotting experiment, after SDS-PAGE, proteins were transferred from the unfixed, unstained gel to Immobilon-P PVDF (Polyvinylidene fluoride) membrane by **Millipore** [Watford, UK] using a Fastblot B 43 semi-dry horizontal blotter [**Whatman Biometra**; Biometra GmbH; Goettingen, Germany] using a continuous buffer system as described by Towbin et al. (Towbin et al., 1979). Prior to blotting, the gel and the membrane were soaked in the continuous transfer buffer of 48 mM Tris, 39 mM Glycine, 0.037% (w/v) SDS and 20 % (v/v) methanol. For the blotting a constant current of 0.8 mA/cm^2 was applied for 74 min. After the transfer was completed, the gels was stained with Coomassie Blue to ascertain transfer efficacy, the membrane was blocked at $4\text{ }^{\circ}\text{C}$ using as a blocking buffer PBS, 0.5 mM EDTA, pH 7.4 with 5% (w/v) skimmed milk powder and 0.1% (w/v) Tween 20. The membrane was washed thoroughly three times, 10 min. for every wash, using PBS, 0.5 mM EDTA, 0.1% (w/v) Tween 20, pH 7.4 and then incubated with the primary antibody diluted in the same buffer for 1 h at RT with shaking.

After washing extensively, as above, the membrane was incubated with the secondary antibody conjugated to alkaline phosphatase, diluted with PBS, 0.5 mM EDTA, 0.1% (w/v) Tween 20, pH 7.4, for 1 h at RT with shaking. After washing again as above target proteins were detected by incubating the membrane in Fast BCIP/NBT (5-Bromo-4-Chloro-3-Indolyl Phosphate/Nitro Blue Tetrazolium) (1 tablet in 10 ml of H_2O) [**Sigma**] for 10-15 min. The target protein(s) were visualised by the appearance of specific dark-purple bands on the membrane. Alternatively, for secondary antibodies that were conjugated to horse-radish-peroxidase, the ECL chemiluminescence detection kit [**Amersham**] was used according to the manufacturer's instructions. Briefly, equal

volumes of ECL reagents were mixed and incubated with the membrane for 1 minute. The membrane was wrapped in cling film and transferred into an autoradiography cassette with intensifying screens. Autoradiography was carried out using the same methodology described in section 2.2.2 with an X-ray film exposed to the membrane for 0.5 to 3min prior to film development.

2.3 PROTEIN PURIFICATION

All purifications were carried out at 4 °C unless otherwise stated.

2.3.1 Human C3

Human C3 purification was carried out as described by Dodds with minor modifications (Dodds, 1993). Briefly, fresh citrated plasma (20 ml) was made 1 mM Pefabloc-SC and 10 µg/ml SBTI, then precipitated with 50 mM barium chloride (by addition of 1 ml of 1 M BaCl₂) for 1 h on ice for the removal of prothrombin which in ion-exchange chromatography elutes close to C3 and C4. Centrifugation followed (4°C, 30 min, 10,000 rpm, JA-20 rotor, **Beckman Coulter (UK)** [Buckinghamshire, UK] and the recovered supernatant was made 1 mM Pefabloc-SC and 5 mM EDTA, pH 7.4. The plasma was precipitated with 5% (w/v) PEG (3,350 MW) for 30 min on ice, by the addition of 15% (w/v) PEG in Buffer A (20 mM Tris, 50 mM ε-ACA, 5 mM EDTA, pH 7.4). The supernatant was recovered as above. A Q-Sepharose Fast Flow column (16/10, 16 mm diam x 10 cm) connected to an ÄKTA FPLC system (**Amersham Biosciences UK Limited**) was equilibrated in 95% Buffer A, 5% Buffer B (20 mM Tris, 50 mM εACA, 5 mM EDTA, 1M NaCl, pH 7.4), and the supernatant was applied to it. The unbound proteins were washed out with 95% buffer A: 5% buffer B, then bound proteins were eluted with a 200 ml linear salt gradient from 5% to 50% buffer B (50 mM to 500 mM NaCl).

C3 eluted from the Q-Sepharose column in the second peak (Fig. 18, top), in the 200-250 mM NaCl range. After SDS-PAGE analysis fractions that contained C3 were pooled together (Fig. 18). The pooled C3 was diluted with half its volume of water and made 1mM Pefabloc-SC. The dilute C3 pool was loaded (<4 mg C3 per run) on a Mono-Q column (HR 5/5, 5 mm diam x 5 cm) pre-equilibrated with 90% Buffer A and 10% Buffer B on the FPLC system. The column was eluted with a 20 ml linear gradient from 10 to 300mM NaCl. C3 eluted as a single peak, in the 250 mM NaCl range, and was observed to be >90% pure by SDS-PAGE analysis (Fig. 19). It was also estimated to be >90 % 'live' (i.e., its thiol ester was intact) by the limited extent of cleavage by factor I in the presence of fH; factor I can only cleave, in the presence

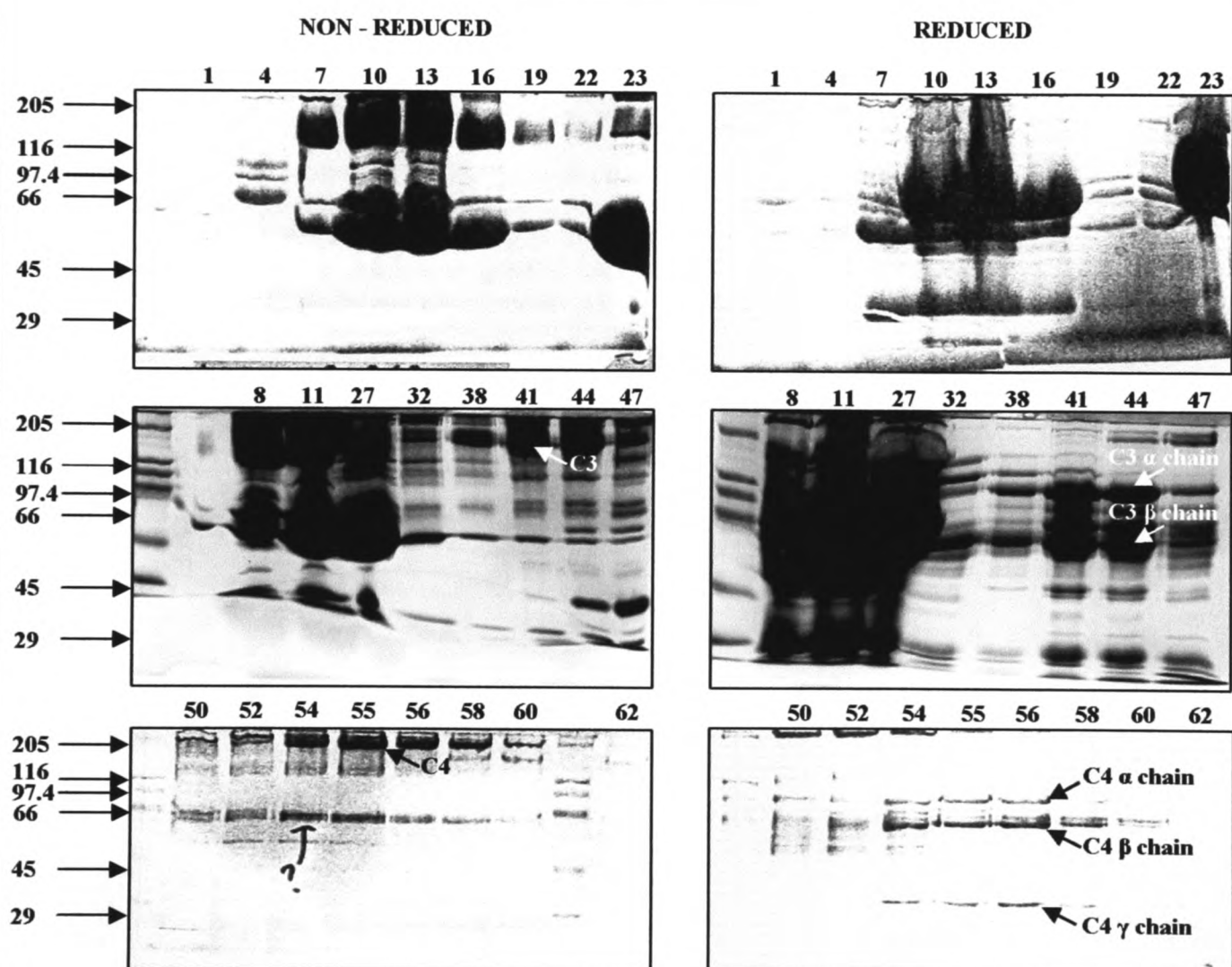
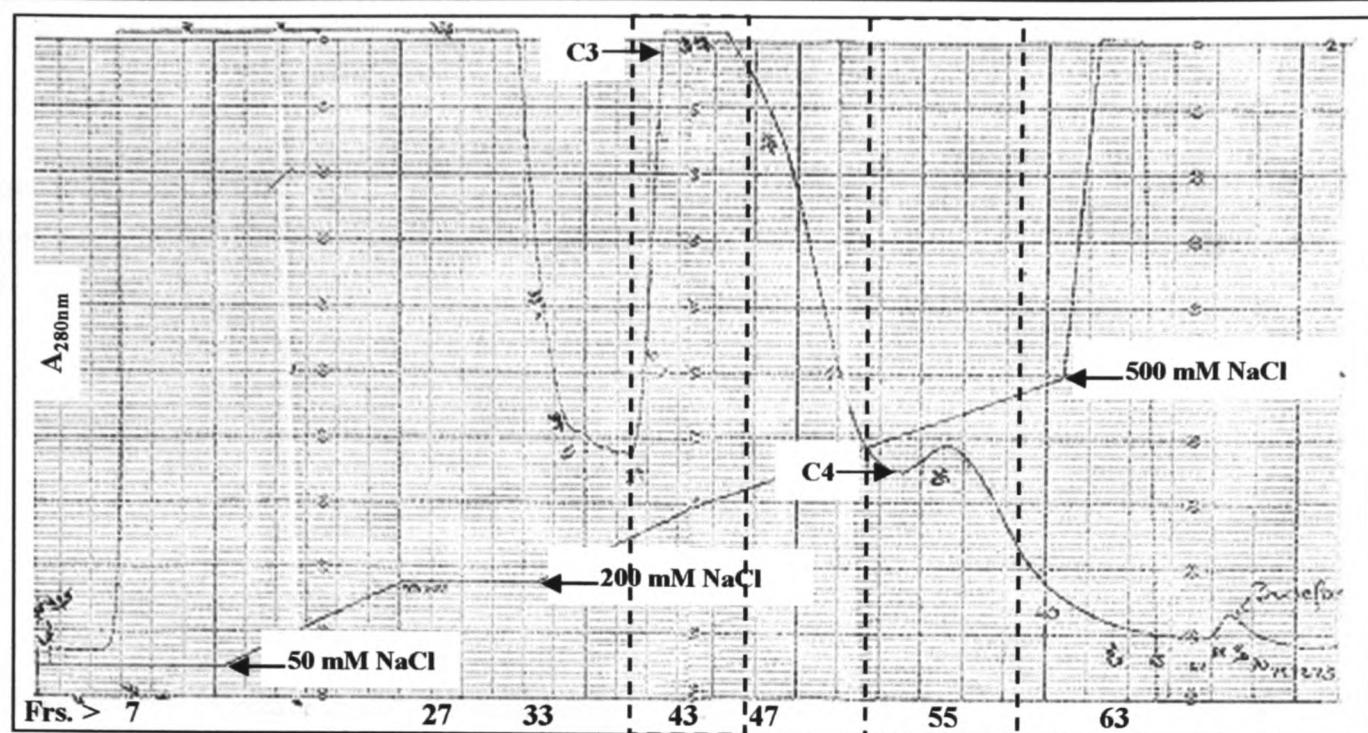


Fig. 18. SDS-PAGE analysis of C3 purification (I). 20 ml of plasma was precipitated with 5% (w/v) PEG (3,350 M.W.) for 30 min on ice, by the addition of 15% (w/v) PEG in Buffer A and the recovered supernatant was applied on a Q-Sepharose Fast Flow column (16/10, 16 mm diam x 10 cm) that was equilibrated in 95% Buffer A (20 mM Tris, 50 mM ϵ -ACA, 5 mM EDTA, pH 7.4), 5% Buffer B (20 mM Tris, 50 mM ϵ -ACA, 5 mM EDTA, 1 M NaCl, pH 7.4) and connected to an FPLC system. The elution was based on a 200 ml linear salt gradient from 5% to 50% buffer B (50 mM to 500 mM NaCl). C3 eluted from the Q-Sepharose column in the second peak, in the 200-250 mM NaCl range. After SDS-PAGE analysis fractions that contained C3 were pooled. The dashed lines in the chromatogram indicate the elution regions for C3 and C4.

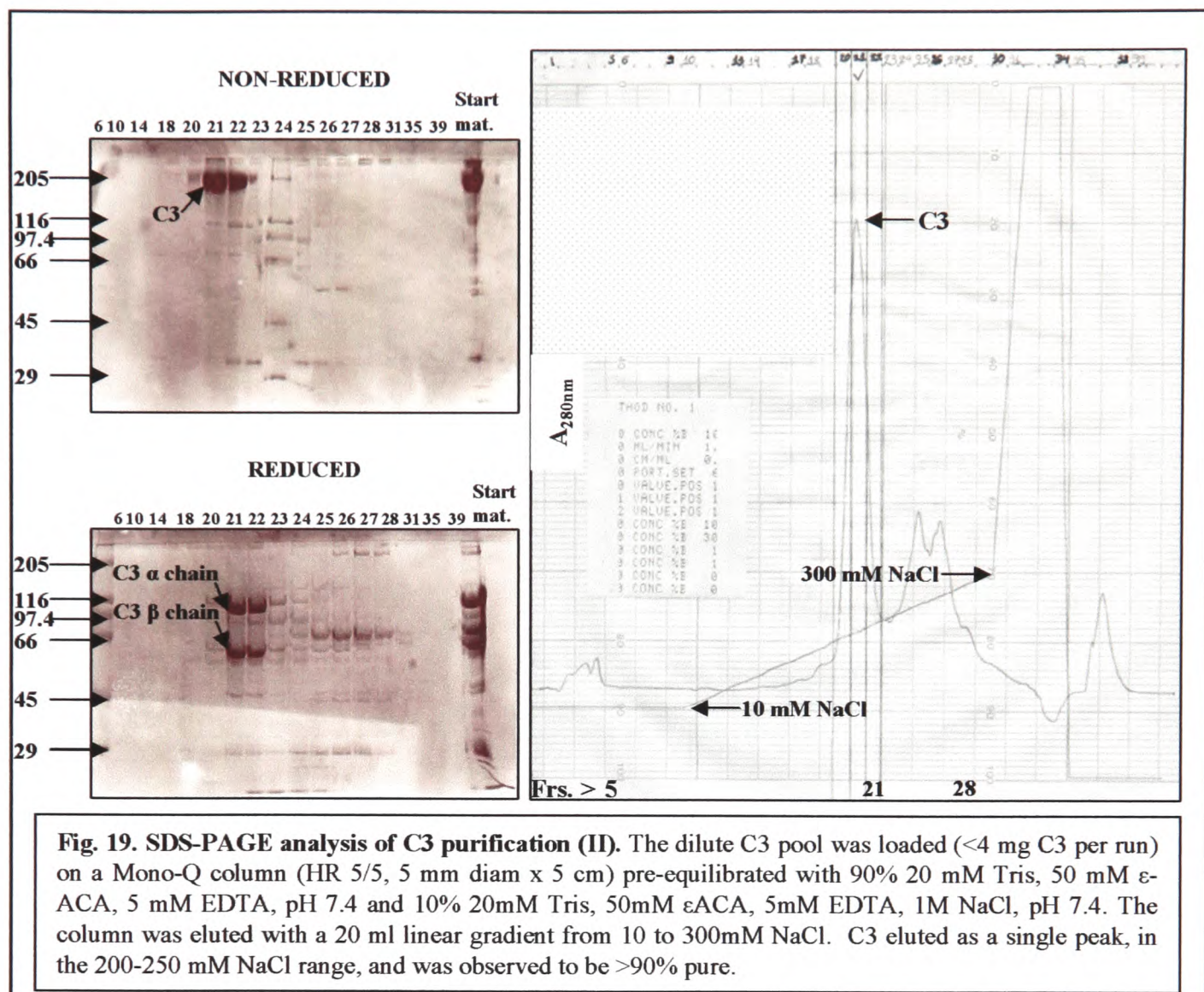


Fig. 19. SDS-PAGE analysis of C3 purification (II). The dilute C3 pool was loaded (<4 mg C3 per run) on a Mono-Q column (HR 5/5, 5 mm diam x 5 cm) pre-equilibrated with 90% 20 mM Tris, 50 mM ϵ -ACA, 5 mM EDTA, pH 7.4 and 10% 20mM Tris, 50mM ϵ ACA, 5mM EDTA, 1M NaCl, pH 7.4. The column was eluted with a 20 ml linear gradient from 10 to 300mM NaCl. C3 eluted as a single peak, in the 200-250 mM NaCl range, and was observed to be >90% pure.

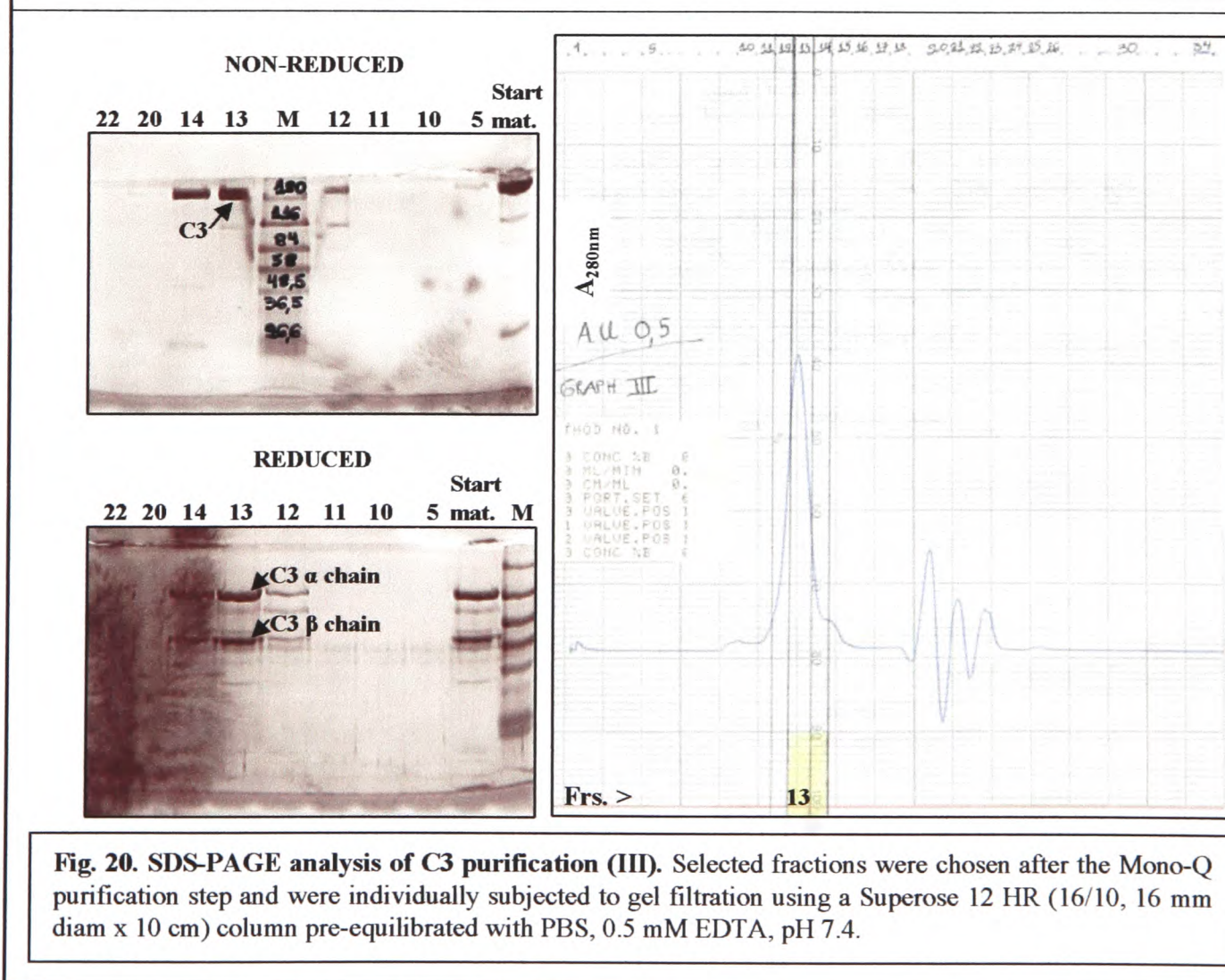


Fig. 20. SDS-PAGE analysis of C3 purification (III). Selected fractions were chosen after the Mono-Q purification step and were individually subjected to gel filtration using a Superose 12 HR (16/10, 16 mm diam x 10 cm) column pre-equilibrated with PBS, 0.5 mM EDTA, pH 7.4.

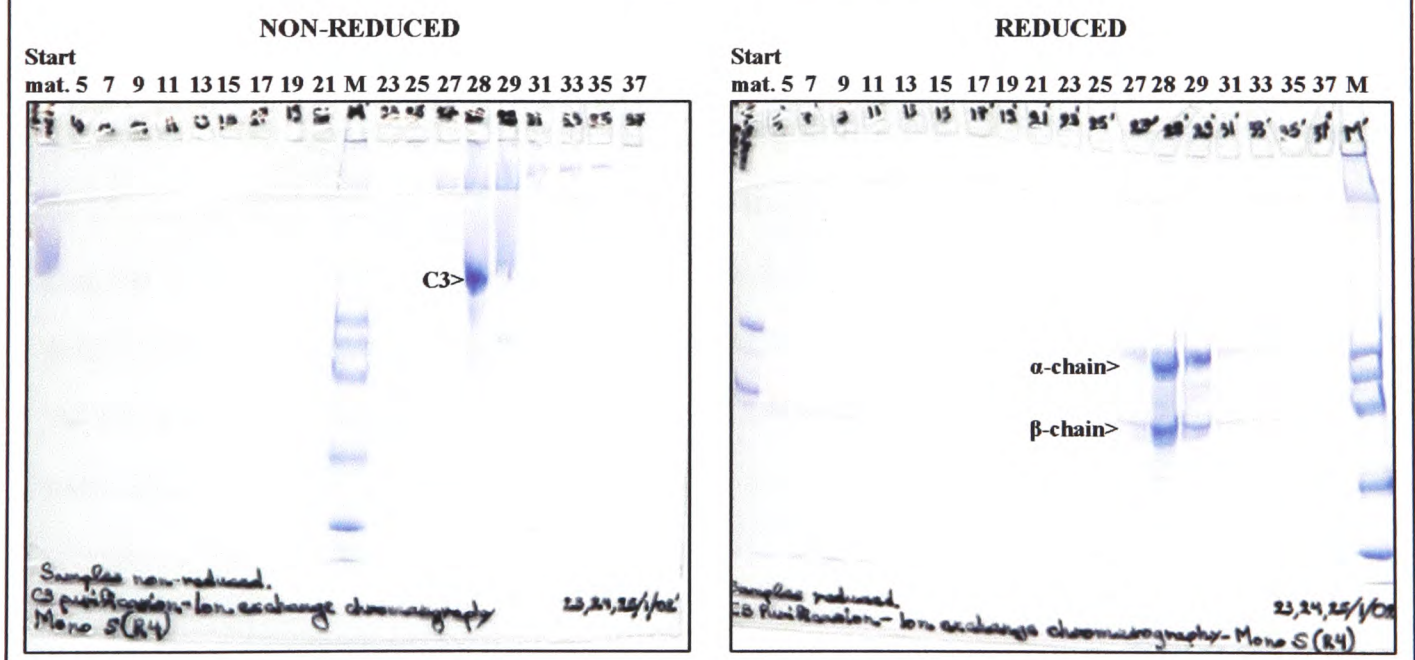
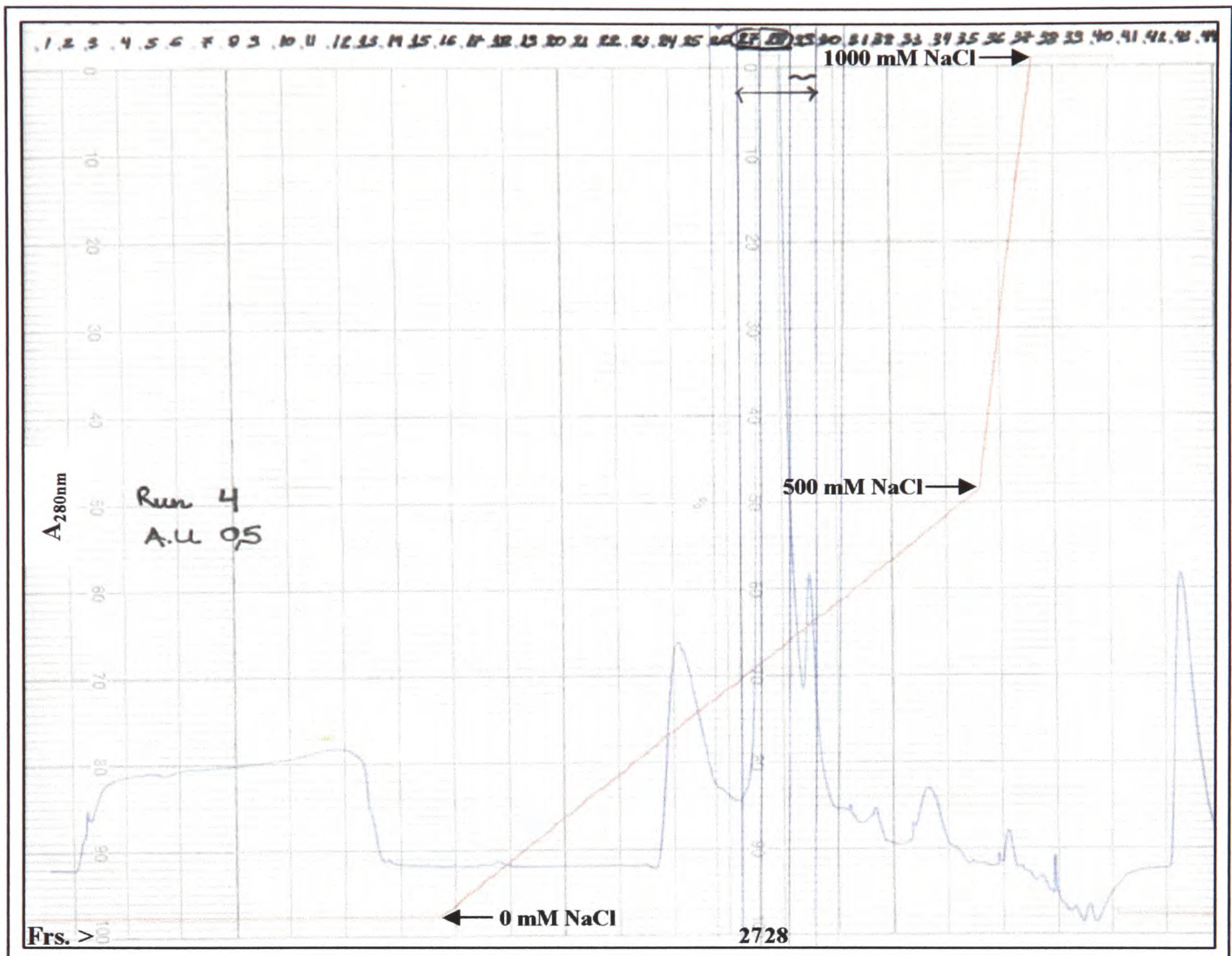


Fig 21. SDS-PAGE analysis of C3 purification (IV). The purest C3 protein from all gel filtration runs was pooled, dialysed against 20 mM MES, 5 mM EDTA, pH 6.0 buffer and was loaded (<1 mg C3 per run) on a Mono-S column (HR 5/5, 5 mm diam x 5 cm) pre-equilibrated with the same buffer. The column was eluted with a 20 ml linear salt gradient from 0% to 50% 20 mM MES, 5 mM EDTA, 1 M NaCl, pH 6.0 (0 mM to 500 mM NaCl). C3 eluted as a single peak, in the 250 mM NaCl range, and was observed to be >98% pure.

of a cofactor, forms of C3 in which the thioester has been cleaved (Parkes et al., 1981; Soames and Sim, 1997) (section 1.4). Selected fractions were subjected to gel filtration using a Superose 12 HR (16/10, 16 mm diam x 10 cm) column pre-equilibrated with PBS, 0.5 mM EDTA-pH 7.4 (Fig. 20). The purest C3 protein from all gel filtration runs was pooled together, dialysed against 20 mM MES, 5 mM EDTA, pH 6.0 buffer and was loaded (<1 mg C3 per run) on a Mono-S column (HR 5/5, 5 mm diam x 5 cm) pre-equilibrated with the same buffer (Fig. 21). The column was eluted with a 20 ml linear gradient from 0 to 500mM NaCl. C3 eluted as a single peak, in the 250 mM NaCl range, and was observed to be >98% pure. The desired fractions were pooled and then dialysed against 20 mM HEPES, 140 mM NaCl, pH 7.4, frozen in liquid nitrogen in 200 µl aliquots and kept at -80 °C for storage.

2.3.1.1 Production of C3 with cleaved thioester (C3(NH₃))

C3 with a cleaved thioester like C3(H₂O), C3u (Haemolytically inactive C3 with intact alpha- and beta-chains), C3(NH₃) or “dead” C3 is a substrate for fI (section 1.4). Treatment of C3 with a small nitrogen nucleophile, such as methylamine and ammonia, results in haemolytically inactive C3 (Muller-Eberhard, 1961) as a consequence of nucleophilic attack of its internal thiol ester bond (Parkes et al., 1981). The thiol ester bond in C3 was cleaved using ammonia as the nucleophile, according to (Soames and Sim, 1997) with modifications. C3 purified to homogeneity (0.2-0.8 mg/ml final concentration) was incubated with a final concentration of 0.2 M ammonium hydrogen carbonate for 90 min. at 37 °C, ensuring that the final pH ≤ 8.0. At the end of the incubation, the reactions were dialysed against 20 mM HEPES, 140 mM NaCl, pH 7.4 for the removal of excess ammonium hydrogen carbonate flash frozen in liquid nitrogen and stored at -20 °C for use as a fI substrate.

2.3.2 Human factor I

Human factor I was isolated from plasma using the protocol described by Sim (Sim et al., 1993) with certain modifications. One of the purifications carried out is described in detail as follows.

2.3.2.1 Filtration

One litre of outdated pooled plasma was thawed overnight at 12 ° C and then transferred to 4 ° C. The plasma was made 5 mM EDTA by the addition of 25 ml of sodium EDTA, pH 7.4 from a 0.2 M stock solution and filtered through 4 layers of folded muslin cloth, in order to trap any lumps of clotted material. The use of glassware in this stage for the manipulation of plasma was avoided as glass can increase the rate of clotting of plasma *in vitro*. For the purification of fI to be used for enzymology studies, the use of the serine protease inhibitor PEFABLOC-SC as described in the original protocol was also avoided. The plasma was further filtered, once through a 3 MM chromatography paper from **Whatman plc** [Brentford, Middlesex, UK] and then through a glass column (7 cm diam.) containing a 1.5 cm bed of packed Sepharose 4B pre-equilibrated with 50 mM NaH₂PO₄, 150 mM NaCl, 15 mM EDTA, pH 7.4. These two final filtration steps supported the removal of any lipid and fibrin strings that can block the affinity columns employed in the subsequent steps of the purification.

2.3.2.2 Removal of Plasminogen/Plasmin by affinity chromatography

The filtered EDTA-plasma was passed down a column of Lysine-Sepharose (5.2 cm diam x 8.5 cm) pre-equilibrated with 100 mM sodium phosphate, 150 mM NaCl, 15 mM EDTA, pH 7.4. This step ensured the removal of plasminogen/plasmin (Chibber et al., 1974; Deutsch and Mertz, 1970) that can bind weakly to immunoglobulin columns. The column was regenerated by washing with 200mM ε-aminocaproic acid in 100mM sodium phosphate, 150mM NaCl, 15mM EDTA, pH 7.4 and re-equilibrated in the same buffer.

2.3.2.3 Removal of unwanted binding proteins by a guard column

A non-immune IgG-Sepharose column [(2 cm diam x 8.5 cm), rabbit non-immune IgG coupled to CNBr-activated Sepharose 4B to a concentration of 10 mg of IgG/ml of packed resin] was used to absorb proteins which bind to Sepharose or to immunoglobulins. The plasminogen/plasmin depleted plasma was passed through this

guard column, pre-equilibrated with 25mM Tris-HCl, 140mM NaCl, 0.5mM EDTA, pH 7.4 (running buffer). This step is crucial and ensures the removal of IgG binding proteins like rheumatoid factors, C1q and fibronectin. After passage of plasma through the lysine-Sepharose and guard columns the volume of the starting material was 1,300 ml.

2.3.2.4 Isolation of factor I by affinity chromatography

The processed plasma was split into two equal volume batches (650 ml each) and each batch was passed separately through a 65 ml MRC OX21-Sepharose column. This strategy was employed in order to increase the yield of the purification based on earlier observations of the saturation limits of the particular column. For this column the running buffer was 25mM Tris-HCl, 140mM NaCl, 0.5mM EDTA, pH 7.4. The plasma was passed down the column at a constant rate of 0.5 ml/min using a peristaltic pump and at the completion of each run, the column was washed extensively with running buffer for the complete removal of unbound proteins until the OD₂₈₀ of the run-through was ≤ 0.030 . Elution of the column with 130 ml (2 Column Volumes, CV) 3M MgCl₂, pH 6.8 followed each wash and the eluate was collected in 1.5 ml fractions. Re-equilibration with 4 column volumes (CV) of running buffer followed each run. The OD₂₈₀ of each fraction was measured and the elution profile composed is shown in Fig. 22. The total final volume of the flow-through plasma of both batches was 1,520 ml.

The possibility of exploiting the usage of an FPLC system for increasing the reproducibility and speed of the affinity step was also investigated by packing the same volume (65 ml) of MRC-OX21-Sepharose into an XK 26/20 column [Amersham Biosciences UK Limited]. The column was connected to an ÄKTA Prime FPLC system (Amersham Biosciences UK Limited) and was run using the same buffer conditions as for the manual run. The use of a crude start material like filtered plasma, flowing under constant valve pressure, introduced several running difficulties (mainly the high back-pressure problems due to the accumulation of lipoprotein material within the column) that prolonged the time required for this step and lead to anomalies in the running conditions. Such anomalies introduced impurities

in the collected eluate and therefore in terms of practical usage and efficiency, this strategy was not pursued further.

Based on the elution profiles of the two batches, as judged from the OD₂₈₀ readings, the fractions corresponding to the elution range of fI were all pooled together (batch 1: frs 19-46 (42 ml) (Fig. 22) and batch 2: frs 11-41 (46.5 ml), total volume 88.5 ml). The material was extensively dialysed against 10mM NaCl till $\text{MgCl}_2 \leq 10 \text{ mM}$ and then against PBS, 0.5 mM EDTA, pH 7.2. Once the dialysis steps were completed, the material was subjected to filtration through a 0.45 μm nylon membrane filter [**Whatman**] for the removal of any fine precipitate. The OD₂₈₀ of the filtered preparation (147 mL) was measured (0.269) for reference before the preparation was concentrated under N₂ (section 2.4.1). The preparation was concentrated until the OD₂₈₀ was 0.902 and the volume 37 ml. This corresponded to 22.25 mg of protein. The fI preparation was then passed manually through a 1 ml HiTrap Protein G HP column [**Amersham Biosciences UK Limited**] for the removal of IgG that is often present in the purification. After the treatment 38 ml of the run-through were collected with an OD_{280nm} of 0.663. In aspects of total protein content, that pool contained approximately 16.79 mg indicating losses of 5.46 mg (24.5%) from the pre-Protein G stage. The 5.46 mg that were removed corresponded to IgG, while the 16.79 mg of protein content was approximately equal to 48% of the theoretical total fI in the 1,000 ml of the plasma start material.

Size exclusion/gel filtration was then carried out using a Superdex 200 16/60 (16 mm diam x 16 cm column) connected to an ÄKTA Purifier system [**Amersham Biosciences UK Limited**]. Prior to the separation, the fI preparation was concentrated on an Amicon Ultra 10,000 MWCO ultracentrifugal filter device (by **Millipore** [Billerica, MA, USA] according to the manufacturer instructions from 37 ml to 10 ml. The column was pre-equilibrated using 25 mM HEPES, 145 mM NaCl, 0.5mM EDTA, pH 7.4 as the running buffer and then the preparation was loaded; 2 ml for each run, with 5 runs in total. 2 ml fractions were collected. The separation was reproducible and the chromatogram from the first run is shown in Fig. 23. The SDS-PAGE analysis confirmed that the separation was successful and that the material contained in fractions C4-C10 (Fig. 23) of each run was homogeneous fI (Fig. 24). Fractions C4-C10 from all the five runs were pooled together (pool 1) for further

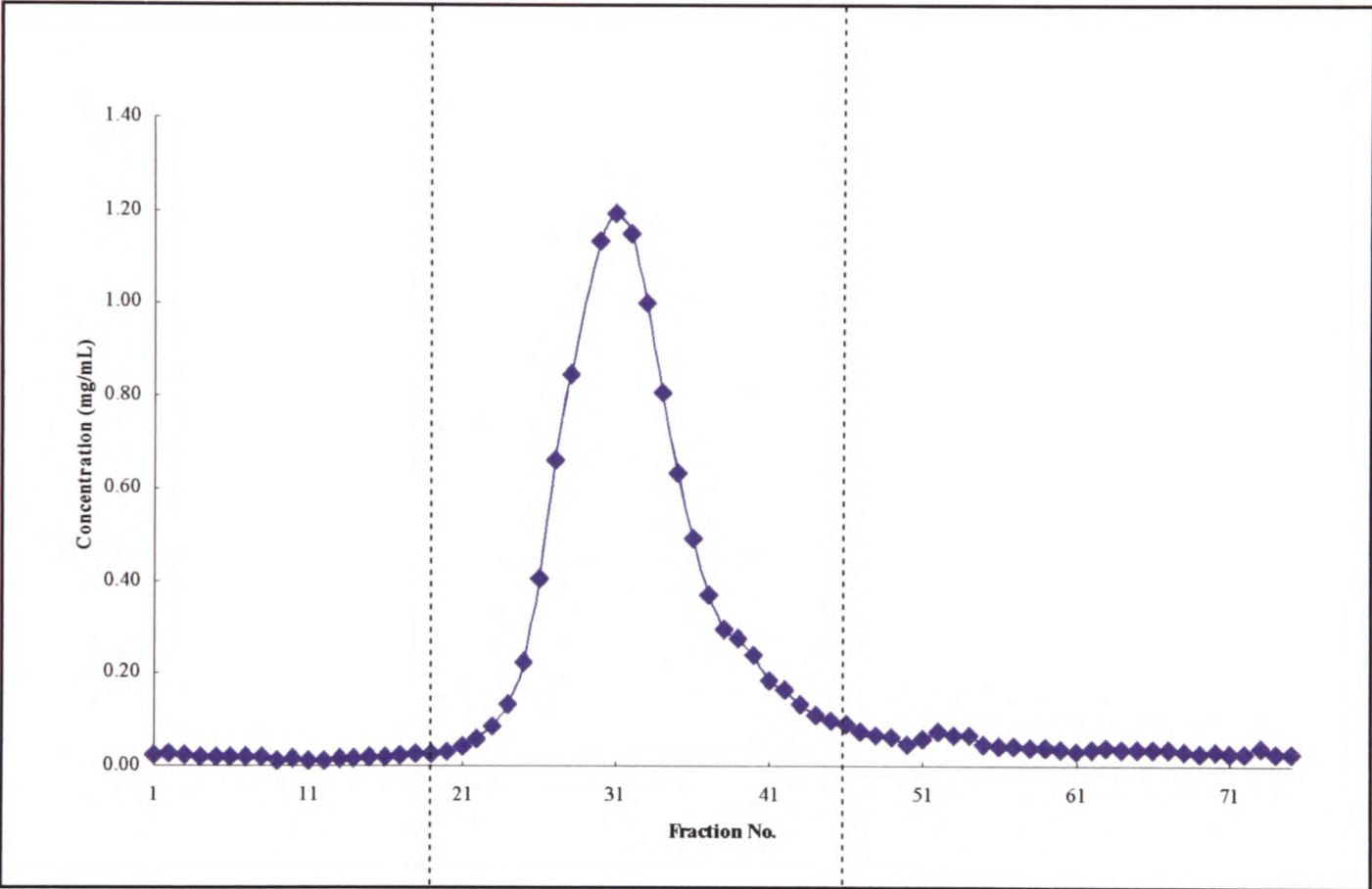


Fig. 22. Isolation of human fI by affinity chromatography. The elution of the MRC-OX21-Sepharose column with 3M MgCl₂, pH 6.8 was examined by measurements of the concentration of all the eluate fractions collected for batches 1 and 2. The measurements were recorded, processed and plotted for both batches; both batches were found to have similar elution profiles. The plot corresponds to the elution profile of batch 1. Fractions 19-46 were pooled for further processing. The dashed lines indicate the region in the elution profile that corresponds to the material pooled.

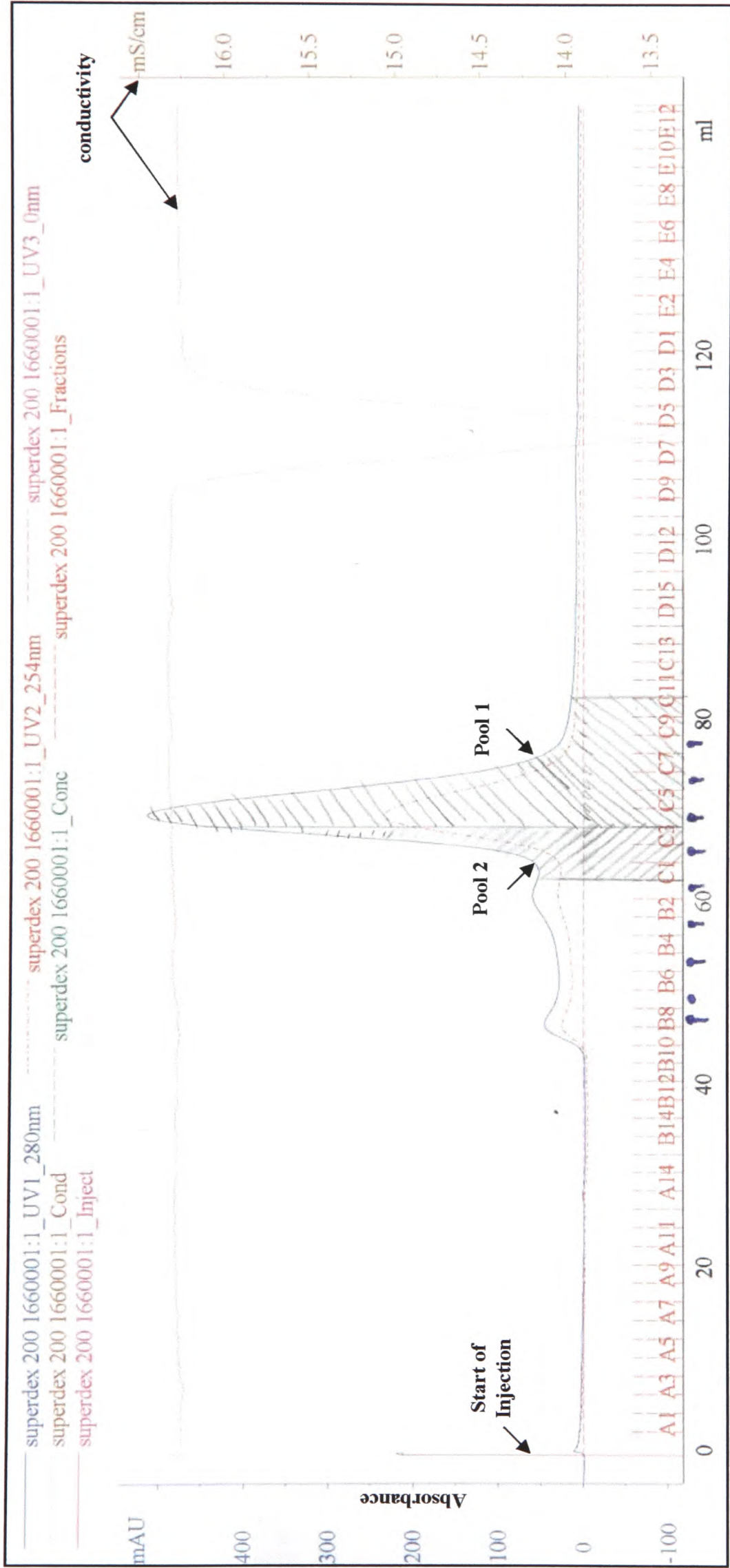


Fig. 23. Size exclusion chromatography for the purification of human complement factor I. 2 ml of 1.635 mg/ml of hfl were loaded for each run; the subsequent SDS-PAGE analysis following the first run showed that fractions C4-C10 contained homogeneous fl. Fractions C4-C10 from all the five runs were pooled together (pool 1) as material of high purity, while fractions C1-C3 were pooled separately (Pool 2). By the completion of all five runs Pool 1 contained 8.04 mg of fl at the concentration of 0.115 mg/ml, while Pool 2 contained 2.65 mg of fl at the concentration of 0.083 mg/ml. The peak areas shaded with grey stripes correspond to the material contained in the two pools. The blue and red traces correspond to absorbance readings at the wavelengths of 280 and 254 nm, respectively.

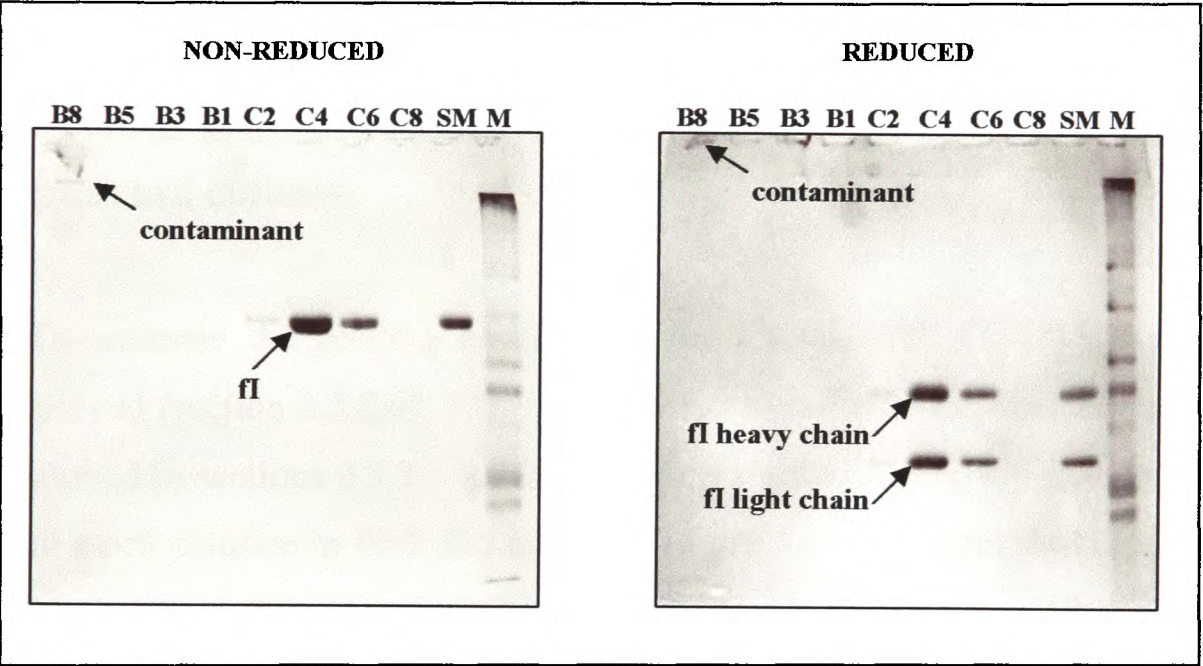


Fig. 24. SDS-PAGE analysis. Analysis following the final size exclusion clean-up chromatography step shown in Fig. 23; 30 μ l from each selected fraction was analysed under non-reduced and reduced conditions. SM represents the start material. All the material eluted within the fractions C4-C10 was determined to be homogeneous and was therefore pooled for further studies.

functional and structural studies, while fractions C1-C3 that contained less pure fI were pooled together for applications in which the purity of the fI used is not crucial (Pool 2). Pool 1 was calculated to contain 8.04 mg of fI at a concentration of 0.115 mg/ml, while Pool 2 contained 2.65 mg of fI at a concentration of 0.083 mg/ml. The final yield of 10.69 mg of fI indicated a loss of 6.10 mg (total protein) (36.3%) from the post-Protein G stage. The final yield of purified enzyme corresponded to 30.5% of the approximate 35 mg of fI estimated to be present in 1 L of human plasma. The stage by stage progression of the fI purification is shown in Table 6. The material from both pools was separated in aliquots of 500 µl, flash-frozen in liquid nitrogen and stored at -20 °C.

2.3.2.5 Estimation of the binding capacity of the MRC-OX21-Sepharose column

To estimate the binding capacity of the 65 ml MRC-OX21-Sepharose column employed (section 2.3.2.4), 1,000 ml of plasma start material was treated similarly as described in sections 2.3.2.1-2.3.2.3 and then approximately 428,400 dpm of ¹²⁵I-fI (4 µl of stock solution in PBS, 0.5 mM EDTA, pH 7.4 with a specific activity of 107,100 dpm (disintegrations per minute)/µl/50 ng) was added as a tracer. The measurement of radioactivity in two equal volume batches (650 ml each) that were processed through the MRC-OX21-Sepharose column (as in section 2.3.2.4), showed that the total radioactivity present in the run-through material was 108,500 dpm, corresponding to 25% of the amount of tracer added prior to the affinity step. This indicated that the total capture of ¹²⁵I-fI on the column was 319,000 dpm corresponding to 75% of the amount of the tracer. Based on this percentage it was assumed that 75% of the total fI contained in plasma, would have been captured on the column upon the completion of the two runs for both batches. Based on the usual estimate of average fI concentration in plasma (35 µg/ml) (Table 1), the MRC OX21 column can bind approximately 13 mg of fI.

2.3.3 Human factor H

Table 6. Progression of the fI affinity purification	
Start material: 1,000 ml of human plasma-It should contain approximately 35 mg of fI ¹	
Stage	Total protein ²
Post MRC-OX21 [Dialysed & filtered eluate]	22.25 mg
Post-Protein G	16.79 mg [↓] ³ (Difference: 5.46 mg/24.5%)
Post-Size exclusion	10.69 mg [↓] (Difference: 6.10 mg/36.3%)
Final yield of enzyme	30.5% of the approximate 35 mg of fI in 1 L of human plasma
1. Based on the average concentration of fI in human plasma (35 µg/µl) (Table 1). 2. For each stage the yield value corresponds to the protein remaining in solution by the end of the particular stage. 3. The [↓] sign marks a reduction in the protein content in solution from the total amount at the end of the previous stage; the values in parentheses indicate the amount of protein loss and its % in relation to the previous yield.	

Human factor H was purified from serum using the method of Sim et al. (Sim et al., 1993) using a similar approach as for the purification of fl.

2.3.4 Human C1-Inhibitor

Human C1-Inh. was purified from serum using the protocols by Sim and Reboul (Sim and Reboul, 1981) and Pilatte et al. (Pilatte et al., 1989) and was kindly donated by Dr. M. Kojima (MRC Immunochemistry Unit, Dept. Biochemistry, University of Oxford).

2.3.5 Immunoglobulin G (IgG)

Immunoglobulin G (IgG) was prepared at RT from rabbit antiserum using a 1 ml HiTrap Protein G HP column [**Amersham Biosciences UK Limited**] according to the manufacturer's instructions at RT. The column was initially washed with 5 ml 0.1 M Glycine-HCl, pH 2.2 and then equilibrated with 10 ml of PBS, 0.5 mM EDTA, pH 7.4. Typically, 0.5-1.0 ml of antiserum was diluted to 5 ml with PBS, 0.5 mM EDTA, pH 7.4, filtered through a 0.45 µm Millex HV filter Unit [**Millipore**] and applied to the column. The column was subsequently washed with 10 ml of the same buffer, until the OD_{280nm} of the flow-through was ≤ 0.050 , and then IgG was eluted with 0.1 M Glycine-HCl, pH 2.2 and collected in 1 ml fractions. To each fraction 200 µl of 1 M Tris-HCl, pH 9.0 was added immediately to neutralise the eluate. The quality of the eluate fractions was assessed by SDS-PAGE (Fig. 25). Selected fractions of high-purity IgG were pooled and dialysed against PBS, 0.5 mM EDTA, pH 7.4, flash frozen in liquid nitrogen and stored at -20 °C.

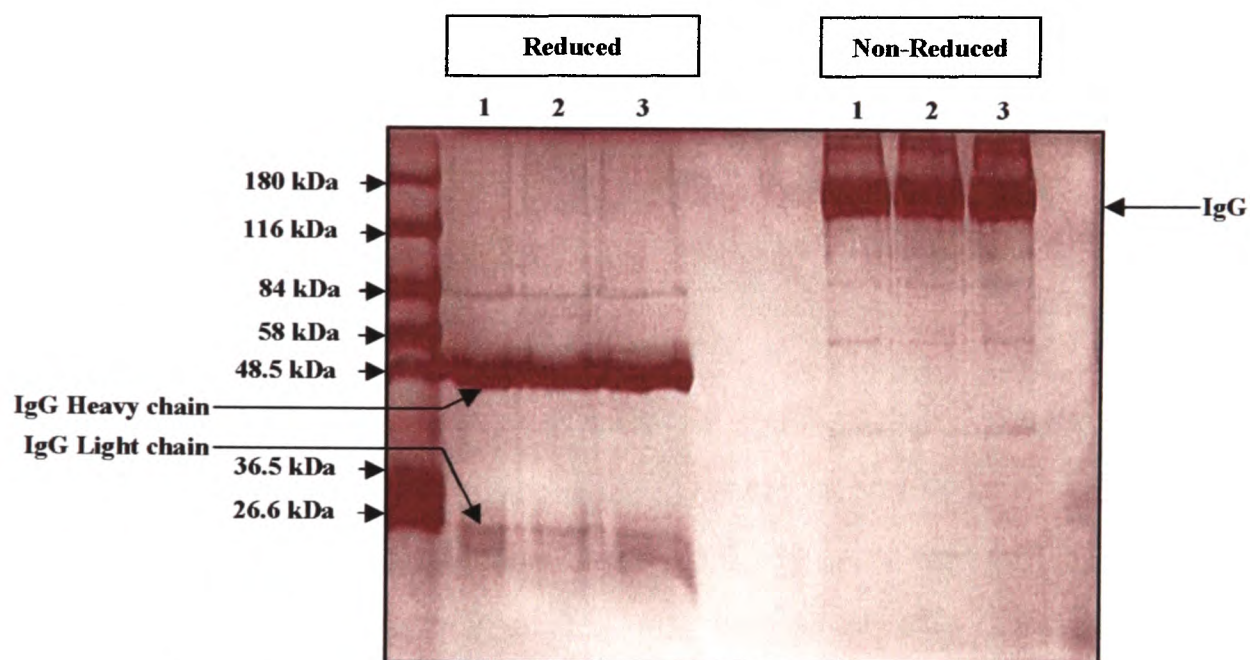


Fig. 25. IgG purification. Polyclonal Immunoglobulin G (IgG) was purified from 0.5 ml of three different rabbit antiserum preparations (1-3) as described in section 2.3.5. From each eluate, the three fractions with the highest concentration were pooled together and subjected to SDS-PAGE analysis both under reduced and non-reduced conditions. For the SDS-10% PAGE analysis, 10 μ l from each pool were analysed per track (Pool preparation 1: 0.757 μ g/ μ l, Pool preparation 2: 0.797 μ g/ μ l and Pool preparation 3: 1.158 μ g/ μ l). Under reduced conditions the IgG runs as two major products, an intense band with an approximate MW of 50 kDa, corresponding to the dominant heavy chain and one faint with an approximate MW of 25 kDa which corresponds to the light chain. As among a pool of polyclonal antiserum the light chain within the IgG population is variable, the band corresponding to the light chain appears as multiple species of similar molecular weights. Under non-reduced conditions all IgG molecules appear as a single band of approximate 150 kDa.

2.4 PROTEIN MANIPULATIONS AND MODIFICATIONS

2.4.1 Protein concentration

Large volumes of protein solutions (> 75 ml) were concentrated under N₂ in a 400 ml stirred cell (Amicon by **Millipore** [Billerica, MA, USA] using a YM10 membrane (Amicon) [10,000 MWCO] under a constant pressure of 2 Atm. For smaller volumes and some special applications the Microcon, Amicon ultra (**Millipore**) and the Vivaspın by **Vivascience AG** [Hannover, Germany] ultracentrifugal filter concentrators [10,000 MWCO] were employed.

2.4.2 Radioiodination

C3(NH₃) and other proteins were labelled with ¹²⁵I using Iodogen (1,3,4,6-tetrachloro-3 α ,6 α -diphenylglycoluril) [**Sigma**] as the oxidising agent (Fraker and Speck, 1978). Conical centrifuge tubes (1.5 ml) were coated with 200 μ l Iodogen (20 μ g/ml in chloroform) and excess Iodogen washed off with 0.5 ml 20 mM HEPES, 140 mM NaCl, 5 mM CaCl₂, pH 7.4. A protein sample (50 μ g in 50-250 μ l of PBS or HBS buffer) was transferred to the Iodogen-coated tube and incubated with 5 μ l Na¹²⁵I (0.5 μ Ci) and 2 μ l of 1M K₂HPO₄/KH₂PO₄ pH 7.4 for 10 min on ice. Non-protein incorporated ¹²⁵I was removed by gel filtration on a PD-10 Sephadex G-25 gel filtration column [**Amersham**] presaturated with BSA (2ml of 5 mg/ml in PBS, 0.5 mM EDTA, pH 7.4). Radiolabelled protein fractions were pooled and stored at 4 °C. The specific radioactivity of the labelled protein was finally expressed in dpm (disintegrations per min)/ μ g units [1 μ Ci= 2.22 x 10⁶ dpm] as determined by measurements taken on a Mini-Assay type 6-20 manual γ counter [**Mini Instruments**, Burnham-on-Crouch, Essex, UK] which counts with 70% efficiency. All the measurements recorded in cpm (counts per minute) units and then converted to dpm units using the formula cpm x 100/70=dpm.

Chapter Three

CHAPTER 3: ENZYMATIC ACTIVITY OF INTACT HUMAN COMPLEMENT FACTOR I

3.1 INTRODUCTION

Although the biochemical properties of human fI had been studied in detail, mainly through the interactions with the substrates and cofactors (sections 1.4, 1.5.4 and 1.5.5), at the start of this work little was known about how the structure of the enzyme determines its natural molecular conformation and how this conformation supports the catalytic mechanism. A key aspect that was examined was whether fI naturally circulates in a catalytically active form; that is whether in the non-zymogen form of the mature enzyme, the catalytic subunit has a naturally active conformation that supports substrate recognition and cleavage.

Among the human complement proteases the serine protease domain of fI is most similar to fD (28% amino acid sequence identity). For both fD and fI it had been shown that both cleave their natural substrates in the presence of cofactors, and that their rates of inhibition by substituted isocoumarins are very low (Kam et al., 1992). The very low esterolytic activity of fD (Kam et al., 1987), was later proposed, through structural studies, probably to be due to a reversible substrate-induced conformational change within the area of the active site that is believed to be required for optimal alignment of the catalytic triad (Jing et al., 1999; Xu et al., 2001). As fI is known to require a cofactor for the cleavage of a natural substrate, it was believed that fI has a similar mechanism of function. This hypothesis was further supported through evidence by Ekdahl and colleagues (Ekdahl et al., 1990) which indicated a C3b-induced realignment of the catalytic site of fI on the basis of reactivity of fI with the serine protease inhibitor DFP. The fI mediated cleavage of C3b, in the presence of cofactor was completely inhibited by DFP when fI and C3b were incubated together with DFP prior to the addition of fH. For fI such a reversible substrate-induced conformational change might be required for the optimal alignment of the catalytic triad and could explain the catalytic properties of the enzyme known at the time.

In addition, it was also not clear whether a Kazal-like inhibitor module, present within the FIMAC domain of the heavy chain (section 1.2.6.6) (Chamberlain et al., 1998), has a control effect on the catalytic subunit. That is whether the Kazal-domain naturally inhibits the fI SP domain and upon complex formation with the substrate and the cofactor the inhibition effect stops. It could be that both or one of the above, the presence of the Kazal-like type inhibitor domain in the heavy chain and a reversible substrate-induced conformational change mechanism in the active site, are important for the catalytic mechanism of fI.

To examine the above possibilities and facilitate a number of measurements on the enzyme activity of fI, the amidolytic activity of fI against fifteen fluorogenic AMC-derivative substrates was initially investigated. Kam and colleagues (Kam et al., 1992) had examined 50 peptide thiobenzoyl esters, but found that none was cleaved by fI. The AMC (7-amino-4-methylcoumarin) derivatives provide greater sensitivity in substrate assays than their thiobenzyl ester counterparts, and have been used in studies involving highly selective and low activity serine proteases, such as blood coagulation factors IXa (McRae et al., 1981), VIIa (Kam et al., 1990) and the complement proteases fD, C2 and fB (Kam et al., 1987). Prior to this work there has been no data describing any synthetic substrates for fI. All previous studies on the enzymatic activity of fI were carried out by examining the proteolytic activity of fI against a natural substrate, C3b or C4b, in the presence of a suitable cofactor like fH or CR1 for the case of C3b (Sim and Sim, 1983) or C4bp for the case of C4b (Fujita and Nussenzweig, 1979).

The identification of synthetic substrates cleaved by fI in the absence of cofactors was novel and led to the development of an amidolytic assay which facilitated a number of functional biochemical studies on fI, supported the examination of the effects of inhibitor compounds on fI and formed the basis for the understanding in more detail of the mode of interactions between the enzyme, the cofactor(s) and the substrate(s).

3.2 MATERIALS AND METHODS

3.2.1 Cleavage of ^{125}I -C3(NH₃) by factor I. A proteolytic assay for factor I

The procedure is based on details given by Sim and Sim (1983). Fig. 26 shows the quality of the C3(NH₃), fH and fI preparations used for the studies. Using as reaction buffer 10mM potassium phosphate, 0.5mM EDTA, 0.1 % Tween 20, pH 6.2 (Buffer A) made 50 µg/ml with SBTI in 100 µl reaction volume, approximately 17,500 dpm of ^{125}I -C3(NH₃) (7.5 ng) was mixed with 62 ng of fH and variable amounts of fI starting from 40 ng. For controls ^{125}I -C3(NH₃) only, ^{125}I -C3(NH₃) with fH, ^{125}I -C3(NH₃) with fI and ^{125}I -C3(NH₃) with fH and fI were prepared. The final concentrations of the individual components in the reaction volume of 100 µl were 0.405 nM (0.075 ng/µl) for ^{125}I -C3(NH₃) [1 µM of C3(NH₃) corresponds to 185 ng C3(NH₃)/µl solution], 4 nM (0.62 ng/µl) for fH [1 µM of fH corresponds to 155 ng fH /µl solution] and 4.55 nM (0.40 ng/µl) for fI (start concentration) [1 µM of fH corresponds to 88 ng fI /µl solution]. All mixtures were incubated for 1 h at 37 °C and the reactions were stopped by the addition of 50 µl of sample buffer made 40 mM with DTT. The mixtures were analysed by SDS-8.5%PAGE. Each gel was dried and the results obtained by autoradiography (Fig. 27). ^{125}I -C3(NH₃) is seen as a two-band pattern, the highly labelled 114 kDa α chain and the β chain of 75 kDa. On incubation with fI and fH, the α chain is cleaved into three fragments, one (~70 kDa) running just above the β chain (on the autoradiograph it appears to co-run with the β chain), another of 41 kDa and a small fragment (C3f, 2 kDa) (Fig. 13) which is not visible on the gel. The rate of cleavage of the α chain is proportional to the proteolytic activity of fI. Similarly to the proteolytic assay based on ^{125}I -C3(NH₃), for a number of experiments a similar assay was set up using non-radioiodinated C3(NH₃). An example is shown in Fig. 28. Small adjustments to the concentrations of the components and the final reaction volumes were carried out where necessary in various series of assays described in this thesis. The exact details of each series are described at the relevant sections.

3.2.2 Effect of inhibitors on factor I proteolytic activity

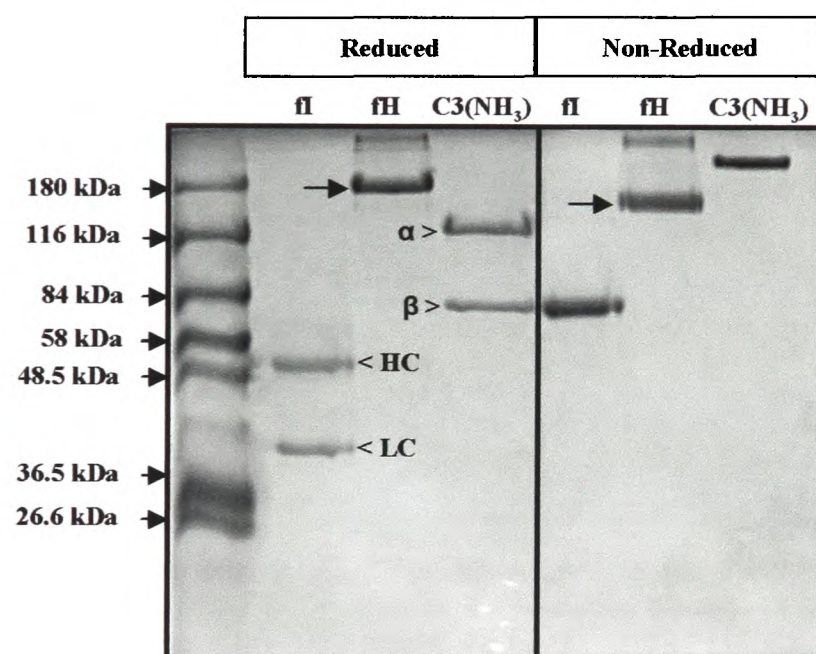


Fig. 26. SDS-PAGE analysis. Complement components factor I (fI), factor H (fH) and C3(NH₃) purified as described in Chapter 2 were analysed on a SDS-10% PAGE gel under reduced and non-reduced conditions with Coomassie Blue staining. Under reduced conditions the heavily glycosylated (20-25% w/w) fI appears as two bands of which the first corresponds to the ~50 kDa heavy chain (HC) and the ~ 38 kDa light chain (LC) which is the serine protease domain. Similarly, C3(NH₃) appears as two bands corresponding to the alpha (α) and beta (β) chains of the molecule. fH appears as a single band under both conditions, while fI and C3(NH₃) under non-reduced conditions appear as single bands. Some aggregated fH is visible in both conditions.

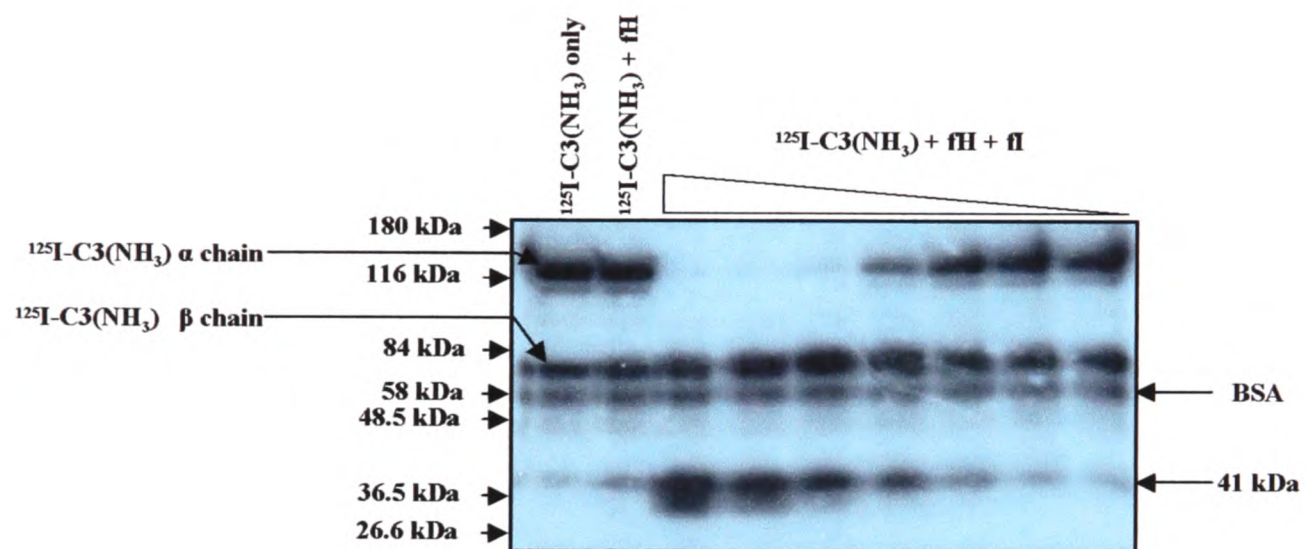


Fig. 27. Assay of proteolytic activity of fI (I). The autoradiograph of an SDS-8.5% PAGE gel shows the cleavage of $^{125}\text{I-C3(NH}_3\text{)}$ by a series of 2-fold serial dilutions of fI in the presence of fH. $^{125}\text{I-C3(NH}_3\text{)}$ is seen as a two-band pattern, the highly labelled 114 kDa α chain and the β chain of 75 kDa. fI can cleave the substrate $^{125}\text{I-C3(NH}_3\text{)}$ only in the presence of fH. On incubation with fI and fH, the α chain is cleaved into three fragments, one (~ 70 kDa) running just above the β chain (on the autoradiograph it appears to co-run with the β chain), another of 41 kDa and a small fragment (C3f, 2 kDa) which is not visible on the autoradiograph. The rate of cleavage of the α chain is proportional to the proteolytic activity of fI. Using 10 mM potassium phosphate, 0.5 mM EDTA, pH 6.2, 50 $\mu\text{g/ml}$ SBTI as reaction buffer, a series of digestion mixtures were prepared using for every mixture 17,500 dpm per gel track (7.5 ng) of $^{125}\text{I-C3(NH}_3\text{)}$, 62 ng of fH and variable amounts of fI through a 2-fold dilution beginning with 40 ng of fI. The arrow indicates the gradually decreasing concentration of fI. The final reaction volume for all mixtures was 100 μl . The reaction was left to proceed for 1h at 37 $^\circ\text{C}$ and then stopped by addition of SDS-PAGE sample buffer made 20 mM with DTT. The band running below the β chain of $^{125}\text{I-C3(NH}_3\text{)}$ is BSA which contaminated the preparation during the iodination (section 2.4.2).

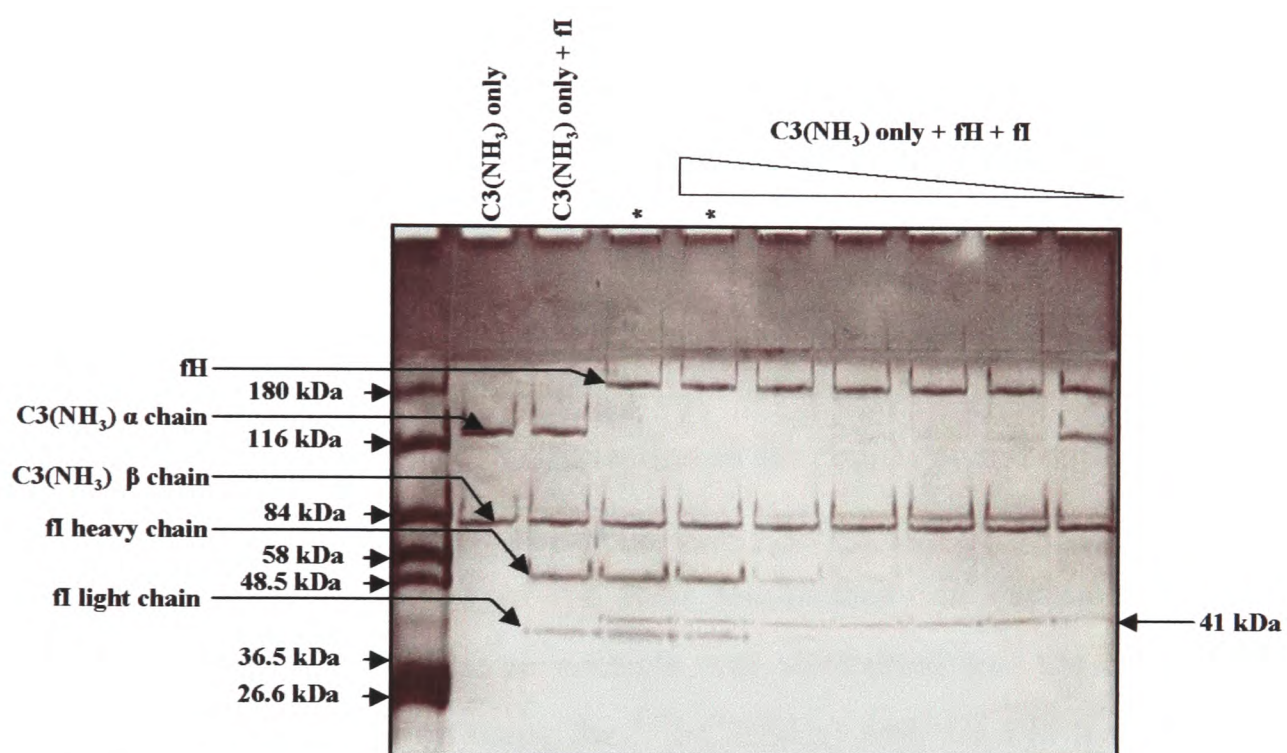


Fig. 28. Assay for the proteolytic activity of factor I (II). The SDS-8.5% PAGE gel shows the cleavage of C3(NH₃) by a series of 2-fold serial dilutions of fI in the presence of fH. C3(NH₃) is seen as a two-band pattern, the 116 kDa α chain and the β chain of 75 kDa. fI can cleave the substrate C3(NH₃) only in the presence of fH. On incubation with fI and fH, the α chain is cleaved into three fragments, one (~70 kDa) running just above the β chain (on the autoradiograph it appears to co-run with the β chain), another of 41 kDa and a small fragment (C3f, 2 kDa) which is not visible on the gel. The rate of cleavage of the α chain is proportional to the proteolytic activity of fI. Using 10 mM potassium phosphate, 0.5 mM EDTA, pH 6.2 as reaction buffer, a series of digestion mixtures were prepared using for every mixture 2.3 μ g of C3(NH₃), 1.23 μ g of fH and variable amounts of fI through a 2-fold dilution beginning with 1.56 μ g (*). The arrow indicates the gradually decreasing concentration of fI. All reaction components had been dialysed against the same buffer. The final reaction volume for all mixtures was 50 μ l. The reaction was left to proceed for 1 h at 37 ° C and then stopped by addition of SDS-PAGE sample buffer made 20 mM with DTT. The bands on the gel were visualised with Coomassie Blue staining as described in section 2.2.2

Various compounds were tested as inhibitors in the proteolytic assay. Each compound was prepared from a stock solution using buffer A for all dilutions. Each compound was tested in the reaction mixture at a final concentration as listed in detail in Tables 10 and 11. fI (9.7 ng) was pre-incubated with the test compound at 37 ° C for 1 h. prior to the addition of fH (31 ng). Incubation was continued for 1 h at 37 ° C, then ^{125}I -C3(NH₃) (17,500 dpm) was added, and the incubation at 37 ° C continued for 40 minutes. Final reaction volume was 100 µl. The final concentrations of the individual components in the reaction volume of 100 µl were 0.405 nM (0.075 ng/µl) for ^{125}I -C3(NH₃), 2 nM (0.31 ng/µl) for fH and 1.1 nM (0.097 ng/µl) for fI. Samples were analysed by SDS-PAGE as described above, to measure the extent of cleavage. The positive control reaction of ^{125}I -C3(NH₃) with fH and fI did not reach complete cleavage during 40 min. and thus the data set could be used for comparison studies. The data were collected by the dissection of the dried gel and the measurement of the radioactivity of the cleavage products. The radioactivity in the 41 kDa product was expressed as a percentage of the total radioactivity in each gel track. Averaged background from the negative controls was subtracted and the data obtained were expressed as % inhibition using the ^{125}I -C3(NH₃) with fH and fI control as standard (0% inhibition).

The majority of the compounds that were tested in the proteolytic assay at pH 6.2 were also tested at the same final concentrations in the same assay using the physiological pH of 7.4. That was considered necessary in order to enable direct comparisons between selected compounds of which the inhibitory properties may vary with pH. Using 10 mM potassium phosphate, 0.5 mM EDTA, 0.1 mM Tween 20, pH 7.4 as reaction buffer, fI (57 ng) was pre-incubated with the test-compound at 37 ° C for 1 h prior to the addition of fH (370 ng). Incubation was then continued for 1 h at 37 ° C, then 5 µg of C3(NH₃) were added and incubation was continued again at 37 ° C for 12.5 minutes, so that the reaction in the positive control C3(NH₃)+fH+fI will not reach completion. The final reaction volume was 40 µl. The final concentrations of the individual components in the reaction volume of 40 µl were 0.68 µM (125 ng/µl) for C3(NH₃), 0.06 µM (9.25 ng/µl) for fH and 0.016 µM (1.43 ng/µl) for fI. The samples were analysed under reduced condition by SDS-8.5% PAGE and Coomassie Blue staining.

3.2.3 Synthetic substrates for factor I

The following substrates were tested for cleavage by fI: R-AMC, GR-AMC, GGR-AMC, LGR-AMC, FGR-AMC, IEGR-AMC, FR-AMC, PFR-AMC, FSR-AMC, GPR-AMC, VPR-AMC, DPR-AMC, VLK-AMC, AAPV-AMC and LLR-AMC. Each substrate, 50 μ M in 100 μ l 25 mM Bicine, 0.5 mM EDTA, pH 8.25 (buffer B) with or without 146 mM NaCl (final concentration) was added to 3.52 μ g fI in 100 μ l of the same buffer in a Microfluor white plate. The final reaction volume was 200 μ l. The amidolytic activity of fI was measured using a microtitre plate reader (Fluoroskan, Thermo Life Sciences, Basingstoke, UK) by excitation at 355 nm and continuous monitoring of emission at 460 nm for 1 h or more at 37 °C. Activity was expressed as the change in emission (Δ OD) per minute at a linear portion of the emission curve [initial rate]. The activity was finally expressed as pmoles AMC released/min/ μ g enzyme using the formula $(x/290.21)*1000$ (calculated from complete substrate turnover) to convert the calculated Δ OD/min/ μ g (x).

Series of 2-fold serial dilutions of fI were assayed using FGR-AMC at 25 μ M to determine a standard fI concentration that would yield a readily measurable level of activity. The standard final concentration of fI used for most assays was 0.2 μ M (17.6 μ g/ml) unless otherwise stated. For selected substrates the K_m and V_{max} values were obtained for both fI and human thrombin, tested under identical conditions, using the Lineweaver-Burk plot method.

3.2.4 Effects of pH, salt strength and divalent cations on factor I amidolytic activity

The optimal pH for the AMC substrate cleavage was determined by the level of activity of fI on FGR-AMC substrate in a multiple buffer system. fI in 50 μ l of 1 mM Tris-HCl, 25 mM NaCl, pH 7.4 was added to 100 μ l of 20 mM acetic acid, 20 mM MES, 20 mM HEPES, 20 mM Bicine, 100 mM NaCl with a pH range of 6.0 to 10.0 and 50 μ l of FGR-AMC in water was added to give a final substrate concentration of 25 μ M with 1 μ M (88 μ g/ml) fI in a final reaction volume of 200 μ l.

To examine the effect of the salt strength on fI, FGR-AMC (final concentration 25 μ M) was added to fI (0.2 μ M final concentration, 17.6 μ g/ml) in buffer B (25 mM Bicine, 0.5 mM EDTA, pH 8.25) with NaCl concentrations ranging from 0-1000 mM.

The effects of Zn^{++} , Mg^{++} , Co^{++} , Ca^{++} , Mn^{++} , Fe^{+++} , Ni^{++} , Hg^{++} , Cu^{++} , Ba^{++} and Cr^{++} ions on fI activity were also explored. Each metal ion was tested individually at a final concentration of 1mM on fI (final concentration of 0.2 μ M (17.6 μ g/ml)) in 25 mM Bicine, pH 8.25. The fI and metal salts were incubated for 1 hour at 37 ° C prior to the addition of the DPR-AMC substrate (25 μ M final concentration).

3.2.5 Effect of inhibitors on factor I amidolytic activity

Inhibitors covering a wide spectrum of proteases were examined in the amidolytic assay. Compounds at various concentrations were pre-incubated for 1 h at 37 ° C with fI (final concentration 0.2 μ M, (17.6 μ g/ml)) in buffer B prior to the assay of cleavage of FGR-AMC (25 μ M final concentration) in the same buffer with 146 mM NaCl final concentration. The concentration of each compound used was chosen on the basis of solubility and mode of action of the compound. Compounds that were found to cause significant inhibition were explored further in separate dose-dependent measurements under identical conditions to those used for the initial screening. Selected compounds were also tested at the same final concentrations for their inhibitory properties on the same assay, using as reaction buffer 25 mM Bicine, 0.5 mM EDTA, 0.1% Tween 20, pH 7.4. This was done for comparison of the inhibitory properties of selected compounds in both the proteolytic and amidolytic activities under similar buffer conditions.

3.2.6 Effects of C3(NH₃) and fH on factor I amidolytic activity

FI at a final concentration of 0.1 μ M (8.8 μ g/ml) was incubated alone or in various combinations with up to 5-fold molar excess of fH, C3(NH₃) or BSA (a negative control) in 150 μ l of 25 mM Bicine, 0.5 mM EDTA, 152 mM NaCl, pH 8.25 for 1 hour at 37 ° C, prior to the addition of the DPR-AMC substrate (25 μ M final

concentration) in 50 μl of the same buffer. When all the components C3(NH₃), fH and fI were tested together, fI was added just prior to the addition of the substrate. fH, C3(NH₃) and BSA were also tested in the absence of fI. Activity at 37 ° C was monitored as described above.

3.2.7 Heat stability of factor I

The heat stability of fI activity was tested in both proteolytic and amidolytic assays. Primarily a series of fI aliquots of 40 ng in 75 μl of buffer A (section 3.2.1) were preheated at temperatures from 37 to 91 ° C for 30 minutes and tested for proteolytic activity at 37 ° C. The assay was stopped before complete cleavage of ¹²⁵I-C3(NH₃) and the autoradiograph produced was used for the measurement of the degree of cleavage of the α chain of ¹²⁵I-C3(NH₃) for each sample using computer software (TotalLab, Nonlinear Dynamics Ltd., Newcastle-upon-Tyne, UK).

FI aliquots of 3.52 μg in 50 μl of buffer B (section 3.2.3) were preheated at temperatures from 37 to 77 ° C for 30 minutes prior to assay for the cleavage of the VPR-AMC substrate (25 μM final concentration). The final concentration of fI was 0.2 μM (17.6 $\mu\text{g}/\text{ml}$) and of NaCl 146 mM. For each set of tests the level of activity in each sample was expressed as % total activity lost compared to the 37 ° C control.

3.2.8 Calorimetric studies on factor I-Differential Scanning Calorimetry (DSC)

Calorimetric studies on human factor I were carried out with the kind assistance of Mrs. Barbara M. Vegh (Institute of Enzymology, Hungarian Academy of Sciences, Budapest, Hungary). Calorimetric measurements were performed on a VP-DSC [MicroCal, Northampton, MA, USA] differential scanning calorimeter. Denaturation curves were recorded between 20 and 80 ° C in N₂ under a constant pressure of 2.5 Atm using a scanning rate of 1 ° C/minute. Two types of measurements were carried out. The first examined the denaturation of fI from 20 to 80 ° C by heating up the sample twice from 20 to 80 ° C, with an intermediate slow (1 ° C) cooling step of cooling the sample down to 20 ° C. The second examined the denaturation of fI by

heating the sample from 20 to 50 ° C, then cooling the sample down to 20 ° C and heating up again from 20 to 80 ° C. All the enzyme samples were measured in a cuvette of 0.5 ml at the concentration of 0.31 mg/ml. The samples were in PBS, 0.5 mM EDTA, pH 7.4 and the same buffer was used for reference measurements. All measurements were done in triplicate and the heat capacities were calculated according to Privalov (1979).

3.3 RESULTS

3.3.1 Factor I activity on peptidyl-AMC substrates

The enzyme specificity of fI was explored, by examining its catalytic activity using the panel of fifteen peptidyl-AMC substrates listed in Table 7. A summary of the results obtained via spectrofluorimetry is shown in Fig. 29. The greatest release of the AMC fluorophor was observed using the D-P-R derivative. Detectable enzyme activity was also observed using V-P-R, G-P-R, L-L-R and F-G-R. The D-P-R-AMC derivative is typically supplied as a substrate for use in determining activity of thrombin and trypsin. The V-P-R and G-P-R derivatives are routinely used to assay thrombin activity, and the F-G-R derivative is used to detect the activity of tissue plasminogen activator (tPA). In the fI assays cleavage was also seen with V-L-K, P-F-R, F-R, R, G-G-R, F-S-R and I-E-G-R substrates, although at lower levels. There was no detectable cleavage of L-G-R, A-A-P-V and G-R derivatives. All the substrates tested, with the exception of V-L-K-AMC and A-A-P-V-AMC, had arginine at their P₁ position. V-L-K-AMC was cleaved at a comparatively low rate and A-A-P-V-AMC was not cleaved at all. Within this series of fI assays, there is a clear selectivity for arginine at P₁ position and a strong preference for proline at the P₂ position. Only five substrates were cleaved at a significant rate, and these had at the P₃ position benzoylated D, V, G, L and F in order of decreasing rate (Table 7). The characterised cleavage sites in the natural substrates of fI are P-S-R, L-L-R in C3b and T-G-R, R-G-R in C4b. Of these “natural” sequences, only L-L-R was tested, and its AMC derivative was cleaved by fI. A third site in iC3b, L-G-R, is controversially stated to be cleaved by fI (section 1.4.2). However, L-G-R-AMC was not cleaved by fI.

The K_m and V_{max} values for fI were calculated for three selected synthetic substrates: D-P-R, V-P-R, F-G-R (Fig. 30), and are compared with the values obtained for human thrombin (Table 8). The K_m values calculated for fI and thrombin with D-P-R and V-P-R derivatives are very similar. The K_m of thrombin for the F-G-R-AMC substrate is 5 fold lower than for fI.

Table 7. Summary of fluorogenic substrates examined for cleavage by human factor I.

Substrates ¹	Substrate for	Activity (fmoles AMC released/min./μg enzyme) ²
Boc-Asp(OBzl)-Pro-Arg-AMC ^{3, 4} (DPR-AMC)	Thrombin and Trypsin	500
N-alpha-t-Boc-Val-Pro-Arg-AMC ⁵ (VPR-AMC)	Thrombin	350
Z-Gly-Pro-Arg-AMC ⁴ (GPR-AMC)	Thrombin	250
Z-Leu-Leu-Arg-AMC ⁴ (LLR-AMC)	-	165
Methylsulfonyl-D-Phe-Gly-Arg-AMC ⁶ (FGR-AMC)	tPA	120
N-alpha-t-Boc-Val-Leu-Lys-AMC ⁵ (VLK-AMC)	Plasmin	30
Pro-Phe-Arg-AMC ⁷ (PFR-AMC)	Pancreatic and Plasma Kallikreins	30
Boc-Phe-Ser-Arg-AMC ⁴ (FSR-AMC)	Factor XIa	30
Boc-Ile-Glu-Gly-Arg-AMC ⁴ (IEGR-AMC)	Factors Xa, IX	20
Z-Gly-Gly-Arg-AMC ⁴ (GGR-AMC)	Urokinase, tPA, Trypsin and Thrombin	20
Z-Phe-Arg-AMC ⁷ (FR-AMC)	Plasma and Glandular Kallikreins	10
Bz-Arg-AMC ⁴ (R-AMC)	Trypsin and Papain	10
N-alpha-t-Boc-Leu-Gly-Arg-AMC ⁵ (LGR-AMC)	Horseshoe Crab Clotting Enzyme	0
MeOSuc-Ala-Ala-Pro-Val-AMC ⁷ (AAPV-AMC)	Elastase	0
H-Gly-Arg-AMC ⁴ (GR-AMC)	Cathepsin C	0

1. All substrates were used at 25 μM final concentration and factor I at 0.2 μM.

2. The values presented correspond to the mean of 2 determinations. Reproducibility was ± 5-10 %.

3. AMC: 7-Amino-4-methylcoumarin, Boc: t-Butyloxycarbonyl, OBZ l: benzyl, MeOSuc: methoxysuccinyl, Z: carboxybenzyloxy, Ac: acetyl, Bz: benzoyl.

4. Bachem

5. ICN Biochemicals

6. American Diagnostica

7. Calbiochem

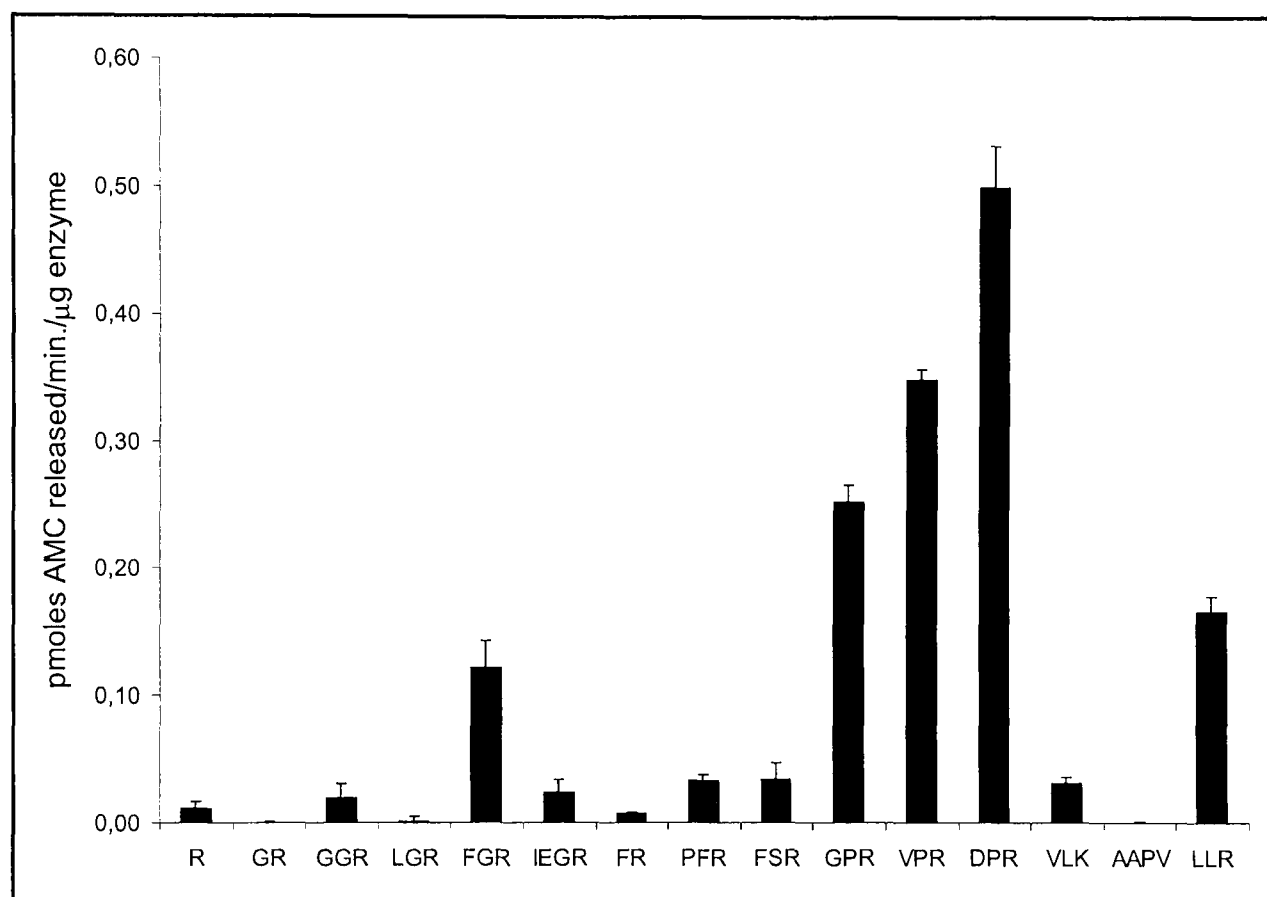


Fig. 29. Cleavage of peptidyl-AMC substrates by human fI. Fifteen peptidyl AMC substrates (25 μ M final concentration) in 50 μ l 25 mM Bicine, 146 mM, 0.5 mM EDTA, pH 8.25 were added to 3.52 μ g of fI in 150 μ l of the same buffer in white Microfluor plate wells and incubated for 1 h at 37 ° C. Cleavage was monitored continuously by measuring emission at 460 nm and the level of activity is shown as pmoles of AMC released/min./ μ g enzyme. Human thrombin (0.01 μ g) was used as a calibration standard since it releases all AMC present within 1 h. Substrates are as defined in table 7.

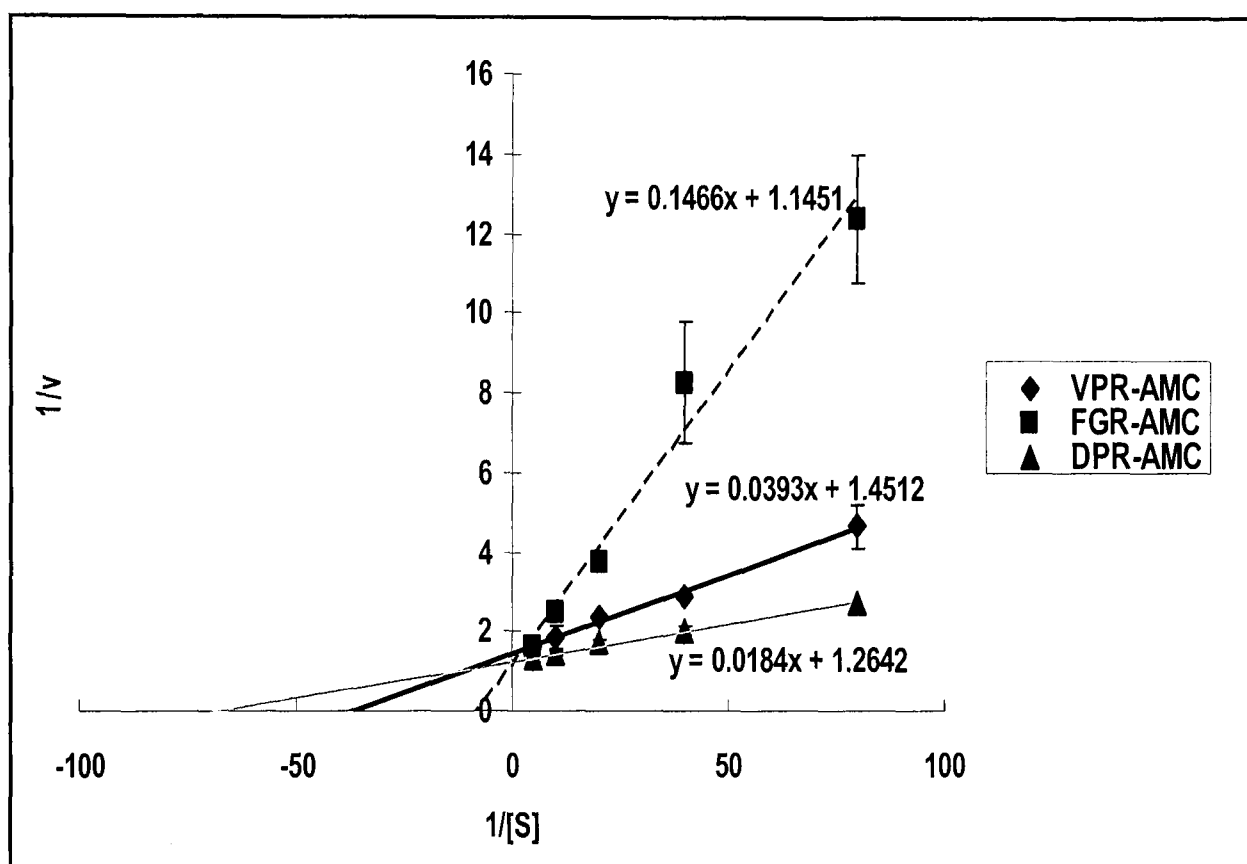


Fig. 30. Calculation of K_m and V_{max} values for selected substrates cleaved by fl. The values were calculated using the Lineweaver-Burk plot method. Each calculation was based on a mathematical equation derived for the best fit trendline for each set of data corresponding to a particular substrate. The concentration of the substrate $[s]$ is in mM and the reaction velocity v has been measured in pmoles AMC released/min/ μ g enzyme.

Table 8. Kinetic constants for the hydrolysis of selected substrates by factor I and Thrombin.

Substrates	K_m (μM)		V_{max} (pmoles AMC released/min./ μg enzyme)	
	factor I	Thrombin	factor I	Thrombin
D-P-R-AMC	14.6	19.9	0.79	49,751
V-P-R-AMC	27	9.6	0.69	32,051
F-G-R-AMC	128	25	0.87	3,940

The $V_{\max}$ values calculated for thrombin are however, much higher than those for fI. For the D-P-R and the V-P-R substrates the $V_{\max}$ values of thrombin are 6.3×10^4 and 4.6×10^4 -fold higher respectively than for fI. For the F-G-R-AMC substrate the $V_{\max}$ value for thrombin is 4.5×10^3 -fold higher than for fI. Thus, relative to thrombin, fI has similar affinity for these substrates, but much lower catalytic efficiency.

3.3.2 Investigation of effects of fH and C3(NH₃) on factor I amidolytic activity

To explore whether fH or C3(NH₃) affect the amidolytic activity of fI, it was decided to test the levels of the amidolytic activity of fI against the D-P-R-AMC substrate in the presence of either or both fH and C3(NH₃). As shown in Fig. 31, excess fH, C3(NH₃) or a mixture of both, have no effect on the amidolytic activity of fI. In addition, BSA used as a negative control, also has no effect and these proteins have no intrinsic (or contaminating) amidolytic activity. For cleavage of the natural substrates C3b and C4b by fI, it has been hypothesized that protein-protein interactions between fI, fH and substrate are required to induce formation of fI into a functionally active state. However, fH and C3(NH₃) clearly do not influence the amidolytic activity of fI in relation to small peptide-AMC substrates. This suggests that one of the roles of fH in the fI-mediated cleavage of C3b is to determine the substrate orientation. The amidolytic activity assay is suitable for the detection and quantification of fI activity independent of cofactor proteins.

3.3.3 Effects of pH, salt strength and divalent ions on factor I amidolytic activity

To determine the optimal conditions in which fI exhibits the highest level of amidolytic activity, a series of assays were carried out over a range of pH values, and also NaCl concentrations. In the range of pH values examined, pH 6-10, the activity appeared to rise steadily from pH 6.0 reaching a peak at 8.25 after which there was a gradual decrease (Fig. 32). The bell shaped curve centred around pH 8.0 is characteristic of many serine proteases.

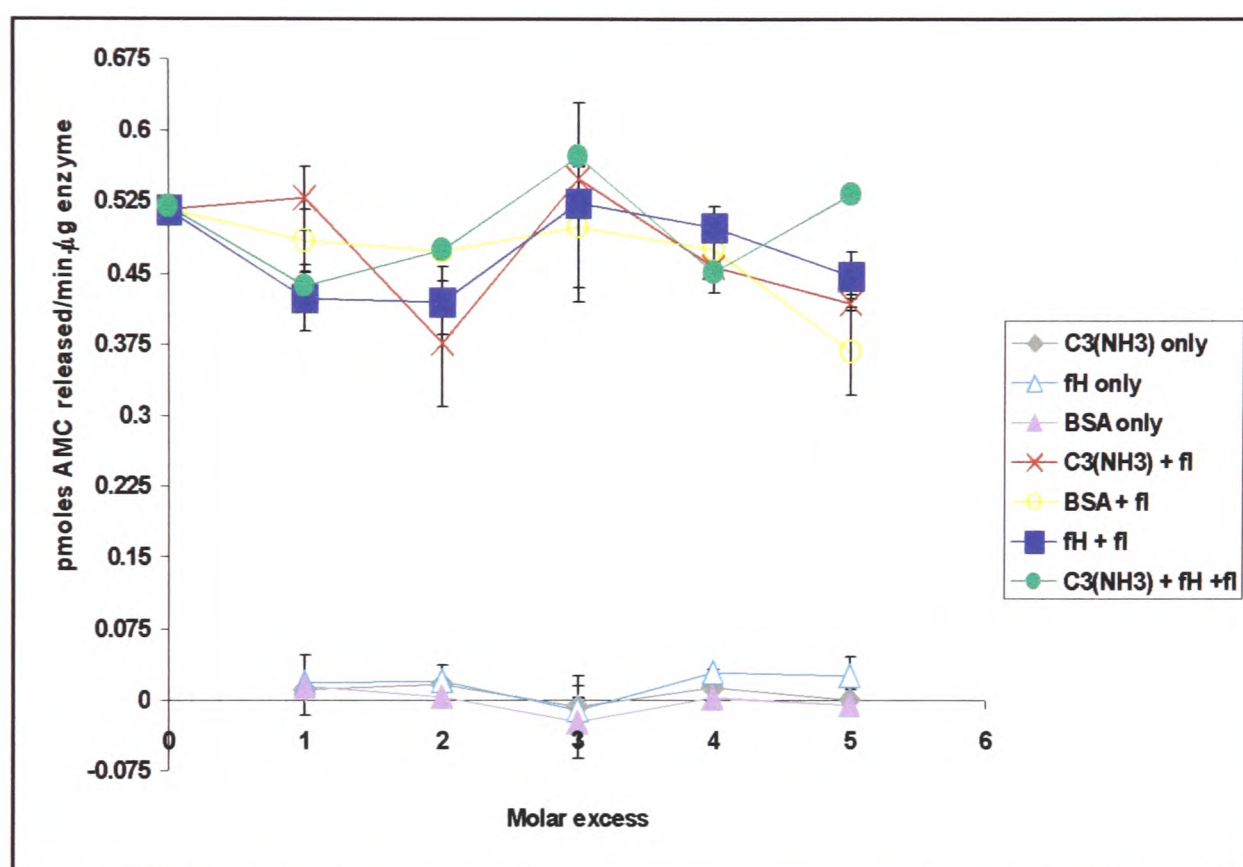


Fig. 31. Investigation of possible effects of fH and C3(NH₃) on the amidolytic activity of fI. fI at a final concentration of 0.1 μ M (8.8 μ g/ml) was incubated alone ("0 molar excess"-positive control) or in various combinations with fH, C3(NH₃) or BSA (control) of up to 5 times molar excess over fI, in 150 μ l of 25 mM Bicine, 0.5 mM EDTA, 152 mM NaCl, pH 8.25 for 1 hour at 37 ° C, prior to the addition of the DPR-AMC substrate (25 μ M final concentration) in 50 μ l of the same buffer. fH, C3(NH₃) and BSA were also tested in the absence of fI (fH only, C3(NH₃) only and BSA only). Activity was monitored as described earlier. Background readings (buffer only control) were subtracted. The error bars correspond to standard deviation values derived from measurements at independent experiments carried out in triplicate under identical conditions.

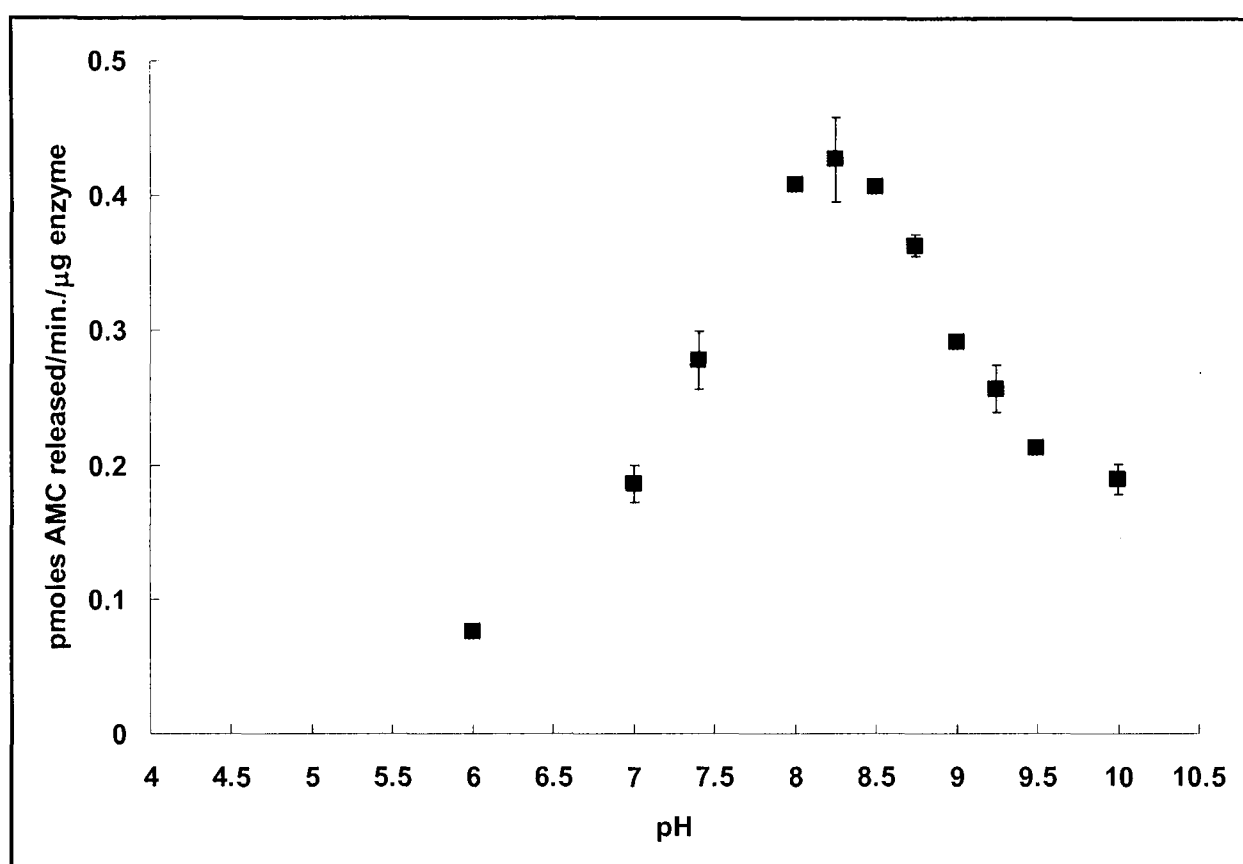


Fig. 32. Effect of pH on FGR-AMC cleavage by fl. FGR-AMC (50 μ l of 100 μ M) in water was added to 50 μ l of fl in 1mM Tris, 25 mM NaCl, 0.5mM EDTA, (1 μ M-88 μ g/ml final concentration) and 100 μ l of 20 mM acetic acid, 20 mM MES, 20 mM HEPES, 20 mM Bicine, 100 mM NaCl with a pH range between 6.0 and 10.0. Cleavage in each sample was monitored and expressed as activity as described in the methods section.

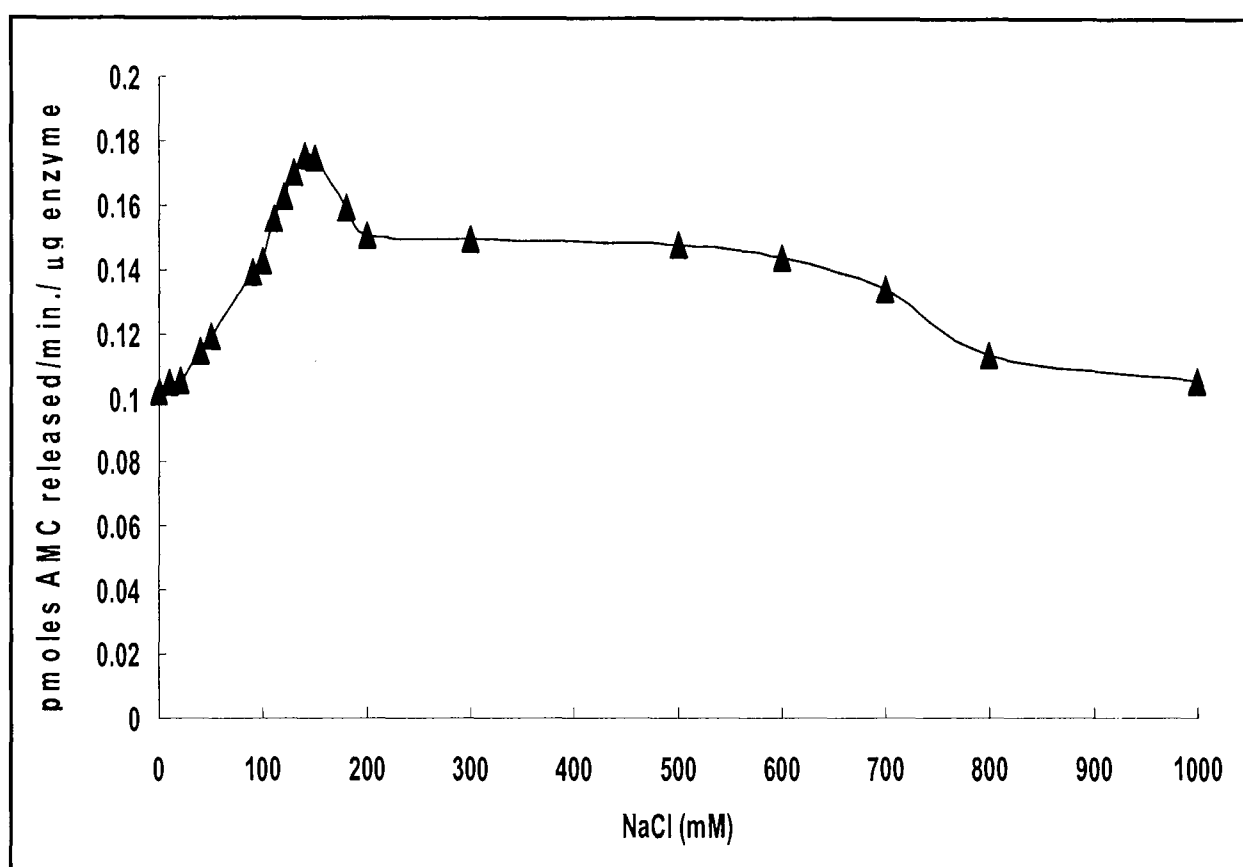


Fig. 33. Effect of salt strength on FGR-AMC cleavage by fl. FGR-AMC in water (50 μ l of 100 μ M) final concentration) was added to 50 μ l of fl in 1mM Tris, 0.5mM EDTA, (0.2 μ M-17.6 μ g/ml final concentration) and 100 μ l of 50 mM Bicine, 1.0 mM EDTA, pH 8.25 with a final NaCl concentration ranging between 0-1000 mM. Cleavage in each sample was monitored and expressed as activity as described in the methods section.

Table 9. Summary of effects of divalent metal ions on amidolytic activity of fl¹.

Metal ion ²	% Inhibition ³ (pH 8.25)
No effect	
Zn ⁺⁺ , Mg ⁺⁺ , Co ⁺⁺ , Ca ⁺⁺ , Mn ⁺⁺ , Ba ⁺⁺ , Ni ⁺⁺	0-9
Inhibitory effect	
Cu ⁺⁺	23
Hg ⁺⁺	26
Cr ⁺⁺	54
Fe ⁺⁺⁺	59

1. The effects of metal ions were examined in the amidolytic assay using FGR-AMC as the substrate.
2. All ions were used at 1 mM final concentration and factor I at 0.2 μM. 2. The salt source for each ion is listed in section 2.1.
3. The values presented correspond to the mean of 2 determinations. Reproducibility was ± 5-10 %.

The activity of fI showed a moderate increase with the increase of NaCl concentration between 0 and 145 mM reaching a maximum in the region of physiological salt strength (Fig. 33). At higher salt concentrations the activity showed a small decrease reaching a plateau from 200-600 mM and then a further small gradual decrease up to 1 M. The effect of salt is much more limited compared to that of pH. There is less than a 2-fold difference in amidolytic activity between optimum and very low or very high salt strength, while an approximately 6-fold difference in activity exists between pH 6.0 and pH 8.25.

In addition, the effect of divalent metal ions was also tested in the amidolytic assay (Table 9). Of all the 11 metal ions examined, consisting of 10 bivalent and 1 trivalent species, Zn^{++} , Mg^{++} , Co^{++} , Ca^{++} , Mn^{++} , Ba^{++} and Ni^{++} were found to have no significant effect on the activity of fI. In contrast, Cu^{++} , Hg^{++} , Cr^{++} and Fe^{+++} were found to have an inhibitory effect, with Cr^{++} and Fe^{+++} causing almost 60 % loss of activity at pH 8.25. The others led to a decrease in activity ranging from 0-30 % loss. From the selection of metal ions tested, Zn^{++} , Cr^{++} and Fe^{+++} were also tested in the proteolytic assay using C3(NH₃) as substrate, both at pH 7.4 and 8.25 (Table 10). Zn^{++} , which did not inhibit the activity of fI in the amidolytic assay, did inhibit the breakdown of ¹²⁵I-C3(NH₃) at concentrations of 20 or 100 µM as previously reported by Crossley and Porter (Crossley and Porter, 1980). This is consistent with Zn^{++} binding to fH, as reported by Day and Sim (Day and Sim, 1986), but not binding to fI. However, Cr^{++} and Fe^{+++} inhibit fI directly both the proteolytic and the amidolytic assays at all pH values examined. The inhibitory effect of these two ions has not been tested before in the proteolytic assay. Crossley and Porter (Crossley and Porter, 1980) had previously reported that Mg^{++} , Mn^{++} , Ca^{++} , Co^{++} and Ni^{++} do not affect fI cleavage of C3(NH₃) in the presence of fH.

3.3.4 Effect of inhibitors on factor I activity

To test the effect of inhibitors on fI, both the proteolytic and the amidolytic assays were used, the latter more extensively. In all cases an excess of inhibitor was incubated with fI for 1 hour at 37 °C prior to the addition of the substrate. Inhibitors, both natural and synthetic, were first tested in the amidolytic assay (Table 11).

Table 10. Summary of effects of selected inhibitors on the proteolytic activity of fl.

Full Name	Targets	Final concentration	% Inhibition ¹ (pH 6.2) ³	% Inhibition ² (pH 7.4) ³
Leupeptin	Serine & Cysteine proteases	10 µM	29	30
Pefabloc SC	Serine proteases	0.25 mM	100	100
Suramin	Serine proteases	1 mM	90	100
Benzamidine	Serine proteases	20 mM	0	0
Z-D-Phe-Pro-methoxypropylboroglycinepinanediol Ester	Thrombin	50 µM	0	55
Antipain	Serine & Cysteine proteases	0.1 mM	0	55
PMSF	Serine & Cysteine proteases	1 mM	5	0
Aprotinin	Serine proteases	0.5 µM	23	35
Metal ion	Salt source	Final concentration	% Inhibition	% Inhibition
Zn ⁺⁺	ZnCl ₂	20 µM	21	N/T
Zn ⁺⁺	ZnCl ₂	100 µM	59	25
Fe ⁺⁺⁺	FeCl ₃ .H ₂ O	20 µM	25	N/T
Fe ⁺⁺⁺	FeCl ₃ .H ₂ O	100 µM	23	65
Cr ⁺⁺	Cr ₂ (SO ₄) ₃ .K ₂ SO ₄ .24H ₂ O	20 µM	6	N/T
Cr ⁺⁺	Cr ₂ (SO ₄) ₃ .K ₂ SO ₄ .24H ₂ O	100 µM	43	35

1. All dilutions from stock solutions were carried out using 10 mM Potassium Phosphate, 0.5 mM EDTA, 0.1 % Tween 20, pH 6.2 (Buffer A).
2. All dilutions from stock solutions were carried out using 10 mM Potassium Phosphate, 0.5 mM EDTA, 0.1 % Tween 20, pH 7.4.
3. In addition, the following compounds were tested and did not inhibit (Fig. 35); SBTI 50 µM, SBTI 50 µM and Hirudin 5 ATU.

Table 11. Summary of effects of inhibitors on the amidolytic assay of fI¹.

Full Name	Targets	Concentration	% Inhibition ² (pH 8.25)	% Inhibition ³ (pH 7.4)
1 ⁴ . PEFABLOC SC	Serine proteases	0.25 mM	100	100
2. PEFABLOC Xa	Factor Xa	0.25 mM	93	N/T
3. Suramin	Serine proteases	1 mM	87	95
4. Benzamidine	Serine proteases	20 mM	82	92
5. Z-D-Phe-Pro-methoxypropylboroglycin epinane diol Ester	Thrombin	50 µM	82	66
6. Antipain	Serine & Cysteine proteases	0.1 mM	80	73
7. PEFABLOC TH	Thrombin	0.25 mM	58	N/T ⁵
8. PMSF	Serine & Cysteine proteases	1 mM	42	34
9. Aprotinin	Serine proteases	0.5 µM	37	32
10. Soya bean Trypsin inhibitor	Serine proteases	50 µM	37 ⁶	0 ⁶
11. Leupeptin	Serine & Cysteine proteases	10 µM	24	25
12. Lima Bean Trypsin inhibitor IIL	Serine proteases	50 µM	20	0
13. Hirudin	Thrombin	5 ATU ⁷	19	0
14. ε-Amino-n-Caproic acid	Serine proteases	20 mM	10	10
15. Bestatin	Metalloproteases	0.1 mM	0	N/T
16. C1 inhibitor	Serine proteases	0.2 µM	0	N/T
17. Chymostatin	Serine & Cysteine proteases	0.1 mM	0	0
18. Pepstatin A	Aspartic acid proteases	1 µM	0	0
19. 1, 10 Phenanthroline	Metalloproteases	0.1 mM	0	0

1. The effects of inhibitors were examined in the amidolytic assay using FGR-AMC as the substrate.

2. The buffer used for the assay was: 25 mM Bicine, 146 mM NaCl, 0.5 mM EDTA, pH 8.25. The values presented correspond to the mean of 2 determinations. Reproducibility was ± 5-10 %. Boehringer Mannheim Complete™ Mini EDTA-free proteases inhibitor tablets: A stock solution made by dissolving 1 tablet in 1.5 ml of H₂O (according to manufacturer's instructions) and diluting 4X into the final reaction volume was found to inhibit the activity of fI by 100 %.

3. The buffer used for the assay was: 25 mM Bicine, 0.5 mM EDTA, 0.1% Tween 20, pH 7.4.

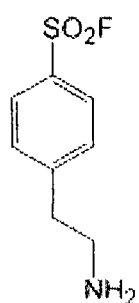
4. The structures of selected compounds are illustrated at the next page.

5. N/T=Not tested.

6. Different batches of SBTI were used for the assays at different pH values.

7. One anti-thrombin unit (ATU) neutralizes one NIH unit of thrombin (fibrinogen assay) at 37 °C, 1 NIH unit of thrombin clots a standard fibrinogen solution in 15 sec. at 37 °C; 5 ATU are expected to neutralise 2.5 µg of thrombin.

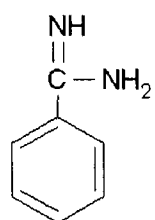
1



HCl

4-(2-Aminoethyl)benzenesulfonylfluoride HCl (Pefabloc SC)

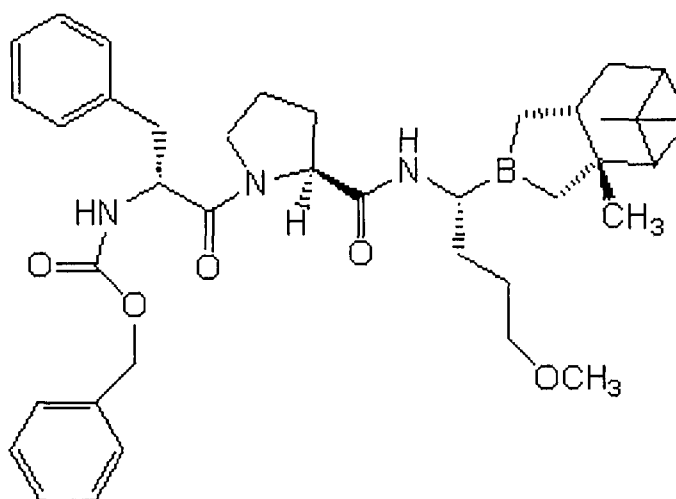
4



• HCl

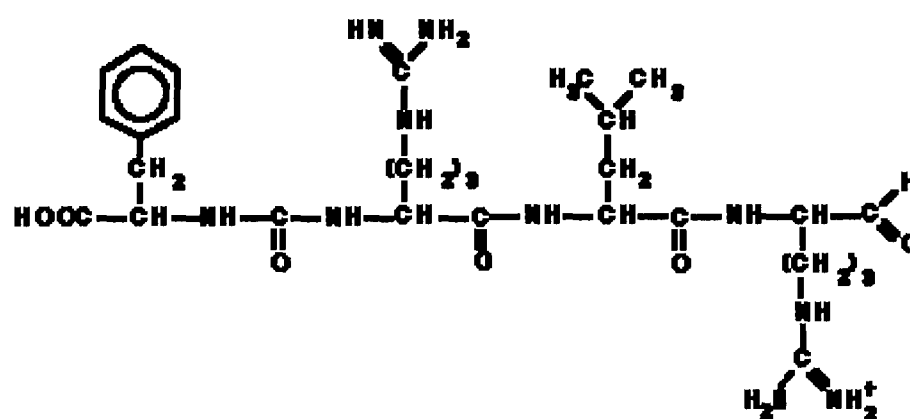
Benzamidine hydrochloride

5



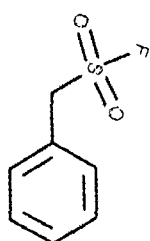
Z-D-Phe-Pro-methoxypropylboroglycinopinanediol Ester (BoroMPG)

6



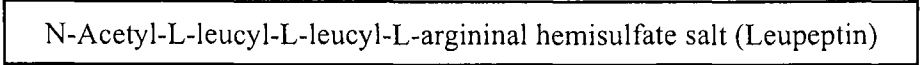
[(S)-1-Carboxy-2-Phenyl]-carbamoyl-Arg-Val-arginal (Antipain)

8

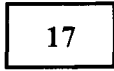


Phenylmethanesulphonyl fluoride (PMSF)

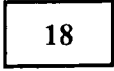
11



15



N-[(S)-1-carboxy-isopentyl]-carbamoyl- α -(2-iminohexahydro-4(S)-pyrimidyl)-L-glycyl-L-phenylalaninal (Chymostatin)



Isovaleryl-Val-Val-AHMHA-Ala-AHMHA where AHMHA= (3S, 4S)-4-amino-3-hydroxy-6-methyl-heptanoic acid. (Pepstatin)

Selected compounds from this screen were subjected to further analysis with dose-dependent responses in the amidolytic assay (Fig. 34) and in the proteolytic assay.

Initially, amidolytic assays were done at their optimum pH, pH 8.25 and proteolytic assays at pH 6.2. However, some anomalies were noted between the 2 sets of assays, which may have been pH dependent. Selected inhibitors were then tested at pH 7.4 in both proteolytic and amidolytic assays to resolve the anomalies. Amidolytic and proteolytic assays were done with selected inhibitors in low salt buffers at pH 7.4, to allow more direct comparison of the inhibitory effects (Tables 10 and 11). From the compounds initially examined at pH 8.25, EDTA-free protease inhibitor tablets, Pefabloc SC/Xa/TH, Suramin, Benzamidine, Boro MPG and Antipain were all found to inhibit strongly the amidolytic activity of fI (60-100% inhibition). In contrast, at the same pH, PMSF (phenylmethanesulphonyl fluoride), Aprotinin, Leupeptin, SBTI, LBTI, Hirudin and ϵ ACA, showed a more moderate inhibition of activity (10-40% inhibition). The remaining compounds: Bestatin, C1 inhibitor, Chymostatin, Pepstatin A and 1, 10 Phenanthroline, showed no evidence of inhibition. The dose-dependent nature of fI inhibition in the amidolytic assay at pH 8.25 (in physiological salt) by Leupeptin, Antipain, Boro MPG, Pefabloc SC, Suramin and Benzamidine was demonstrated (Fig. 34). Most notably, Antipain and Leupeptin showed strong inhibition, 80% at 25 μ M, although complete inhibition was not achieved. Fifty % inhibition was observed with Boro MPG, Pefabloc SC and Suramin at concentrations of 25, 40 and 125 μ M, respectively. Benzamidine reached a plateau of inhibition close to 80% at 10 mM. From all the compounds analysed only Pefabloc SC appeared to show complete enzyme inhibition.

The irreversible inhibitors Pefabloc SC, Suramin, Leupeptin and Aprotinin gave similar inhibitions regardless of pH. BoroMPG appeared to show strong pH dependence. It inhibited strongly in the amidolytic assay at pH 8.25, but did not inhibit at all at pH 6.2 in the proteolytic assay. At pH 7.4, it inhibited both assays by 55-66% (Tables 10, 11 and Fig. 35). Antipain showed a similar pattern, with no inhibition at pH 6.2, intermediate at pH 7.4 and highest at pH 8.25. These reflect the pH optimum of the catalytic activity (Fig. 32) and may indicate that the rate of the (irreversible) reaction of these inhibitors with the enzyme active site is very slow, such that, in the long incubation periods used, neither reacts significantly at pH 6.2.

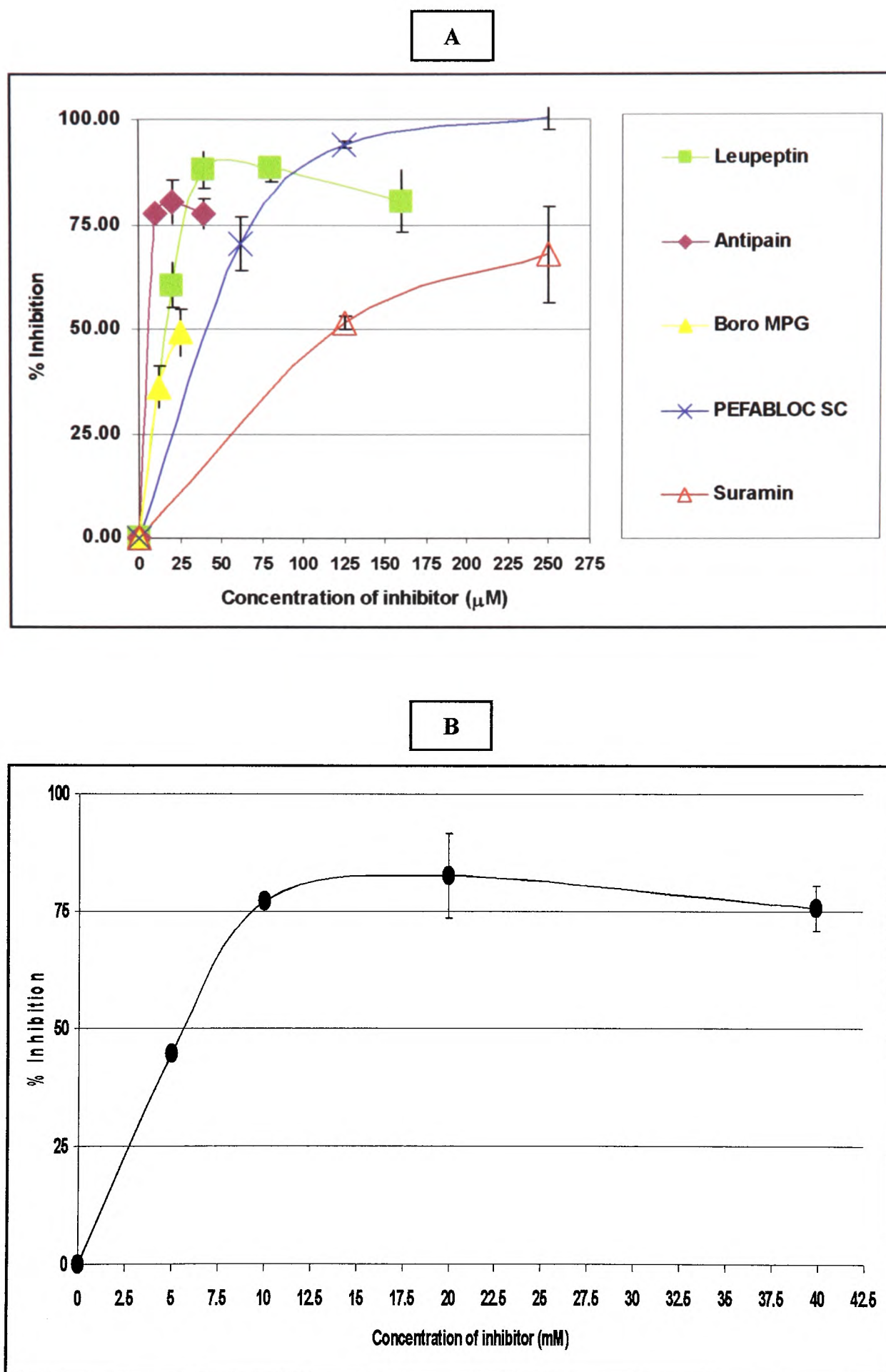


Fig. 34. Dose-dependent inhibition of fI amidolytic activity by selected compounds. A) Leupeptin, Antipain, Z-D-Phe-Pro-methoxypropylboroglycinepinadiol ester (Boro MPG), Pefabloc SC, Suramin and B) Benzamidine. Different dilutions from stock preparations of each compound in 50 μ l of 25 mM Bicine, 146 mM, 0.5 mM EDTA, pH 8.25 were co-incubated for 1 h at 37 ° C with 100 μ l of 0.4 μ M (35.2 μ g/ml) fI in the same buffer. Each mixture was transferred to 50 μ l of FGR-AMC substrate (25 μ M final concentration) in the same buffer in a Microfluor white plate well.

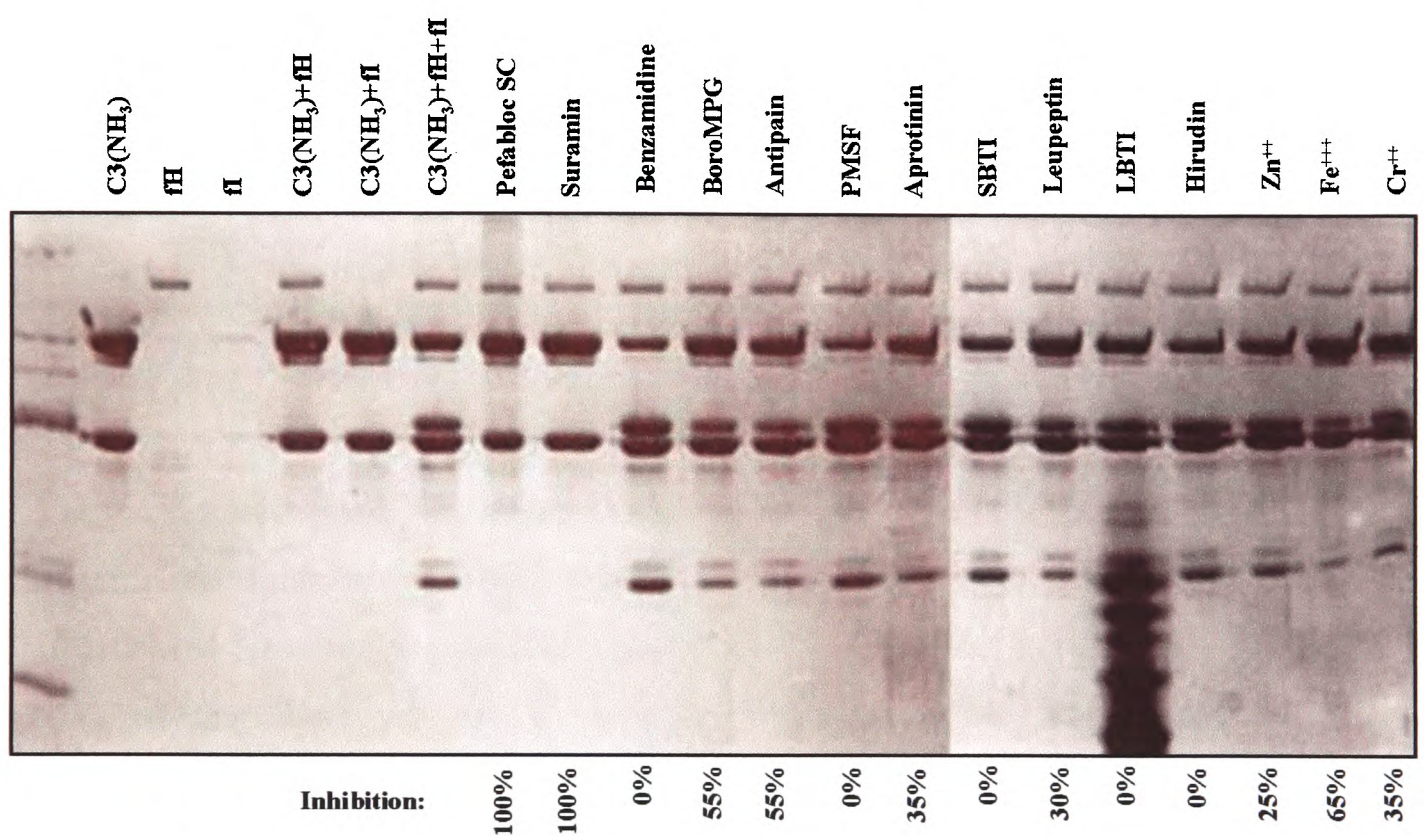


Fig. 35. Examination of the inhibition properties of selected compounds on the proteolytic assay. The SDS-8.5% PAGE gel shows the cleavage of C3(NH₃) by fI in the presence of fH and selected compounds (Table 10). The bands on the gel were visualised with Coomassie Blue staining as described in section 2.2.2. Selected compounds from the ones that were found to have an inhibitory effect on the amidolytic activity at pH 8.25, were tested for their effect against non-labelled C3(NH₃) on the proteolytic assays using 10 mM potassium phosphate, 0.5 mM EDTA, 0.1% Tween 20, pH 7.4 as buffer (section 3.2.2). A similar buffer (25 mM Bicine, 0.5 mM EDTA, 0.1% Tween 20, pH 7.4) was also used for examining the inhibitory effect on the amidolytic assay at pH 7.4 as described in section 3.2.5. Since, the inhibitory effects of the various compounds were initially examined on both the proteolytic and amidolytic assays using different ionic strength and pH conditions, for consistency and comparison reasons it was decided to study the particular effects on the two different system under similar buffer conditions.

PMSF inhibited the proteolytic assay very poorly at both pH 6.2 and 7.4, but it inhibited the amidolytic assay well at pH 7.4 and 8.25. In both types of assay, PMSF was preincubated with fI alone for 1 h at 37 ° C. This result suggests that PMSF is not acting as a irreversible inhibitor, but is instead acting in some unusual way as a competitor for the low molecular weight substrate used in the amidolytic assay. The natural substrates C3b and C4b may interact multiply with the enzyme, resulting in higher avidity which may displace non-covalently bound PMSF or its hydrolysis product. Benzamidine is a competitive (reversible inhibitor) and shows the same pattern as PMSF. As shown in Tables 10 & 11, SBTI and LBTI were found to inhibit partially the amidolytic assay at pH 8.25, but not at pH 7.4. They did not inhibit the proteolytic assay. The finding with SBTI is probably a minor anomaly caused by contaminant(s) in 1 batch of SBTI (Table 11).

The results from the proteolytic assays are in agreement with observations made by Crossley and Porter (Crossley and Porter, 1980) who reported no inhibition of the same reaction (the proteolytic assay) at low ionic strength and pH 6.5 by Benzamidine or PMSF at concentrations of 5 and 0.1 mM respectively. In the same report it was noted that there are no effects from chelating agents such as EDTA or 1, 10 phenanthroline at 50 mM and 1 mM, respectively.

3.3.5 Heat stability of factor I

The heat stability of fI was tested in both the proteolytic and the amidolytic assays. Fig. 36 shows the loss of enzymatic activity over an extended range of temperatures from 37 to 91 ° C. In both assays fI loses 50 % of its activity between 37 and 65 ° C, with more than 80 % loss at 75 ° C. In neither assay was the activity completely lost at temperatures close to 80 ° C indicating that the serine protease domain of fI shows a strong degree of thermal stability. In the more detailed analysis of the proteolytic assay, the loss of activity may occur in two stages, one between 37-48 ° C and another from 61 to 73 ° C. In the amidolytic assay, the loss of activity follows a linear trend above 48 ° C.

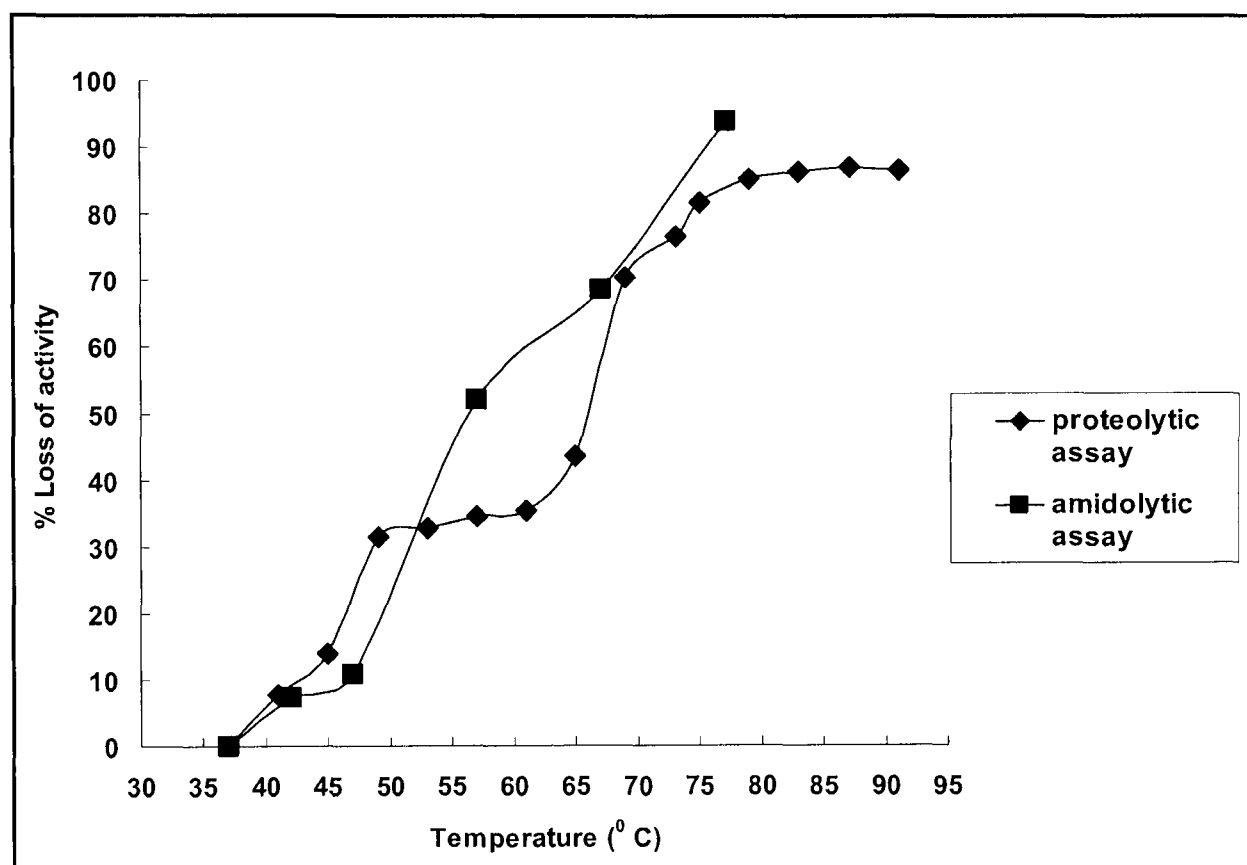


Fig. 36. Heat stability of fI. Two sets of fI samples, one in 10 mM potassium phosphate, 0.5 mM EDTA, pH 6.2 (buffer A) the other in 25 mM Bicine, 0.5 mM EDTA, pH 8.25 (buffer B), 145 mM NaCl were preheated at various temperatures from 37-91 ° C for 30 min. Each set was tested independently for its activity, the first in the proteolytic assay and the second in the amidolytic assay. For the proteolytic assay aliquots of 40 ng of fI in 75 μ l of buffer A were exposed to temperatures from 37 to 91 ° C and tested for activity at 37 ° C. The level of activity in each sample was expressed as % total activity lost compared to the 37 ° C result set as 0 % loss. For the amidolytic activity assay aliquots of 50 μ l containing 0.8 μ M fI in buffer B were heated at 37 to 77 ° C prior to testing on VPR-AMC. The final salt concentration was 145 mM NaCl. The activity data were plotted as a function of temperature.

In a multidomain protein it might be expected that the overall activity would be lost at the melting temperature of one particular domain, if the conformation of the particular domain was critical for the structure of the protein and/or for the interactions with other proteins. If that was the case for fI, a single denaturation event at one critical temperature would cause a very large loss of activity. This seems not to be the case for fI. In the complex formation with fH and C3(NH₃), the abolition of the natural conformation of the non-catalytic heavy chain appears to affect the interactions with the cofactor and the substrate, but since there are likely to be multiple sites of interaction between the heavy chain of fI with fH and/or C3(NH₃), the denaturation of the heavy chain may weaken the highly ordered interactions necessary for the efficient cleavage of C3(NH₃) (Soames and Sim, 1997). In the amidolytic assay of fI the activity is dependent on the function of the single SP domain; the activity is lost in a linear trend with the denaturation of the catalytic subunit, as the gradual increase of the temperature introduces a series of sequential alterations to the native conformation of the domain. Such alterations eventually lead to the complete abolition of function.

3.3.6 Calorimetric studies on factor I-Differential Scanning Calorimetry (DSC)

When the denaturation of human fI was examined between 20 and 80 ° C, the melting profile indicated that the enzyme denaturation occurs at two reproducible major melting peaks, one at approximately 44 ° C and the other at 62 ° C, each corresponding to a single symmetric curve (Fig. 37). The first peak was found to be reversible when a sample was examined from 20 to 50 ° C applying the second type of measurement (section 3.2.7) (Fig. 38), while the second peak was irreversible when the sample was heated up twice from 20 to 80 ° C using the first type of measurement (section 3.2.7). Since fI is a heterodimer the data suggested that each peak corresponded to a single denaturation event for each of the fI subunits. It is proposed that the first peak at 44 ° C corresponds to the denaturation of the non-catalytic heavy chain subunit, while the second peak at 62 ° C corresponds to the denaturation of the catalytic subunit, the SP domain. We were unable to make measurements on either of the two subunits separately, so could not confirm this hypothesis directly; the

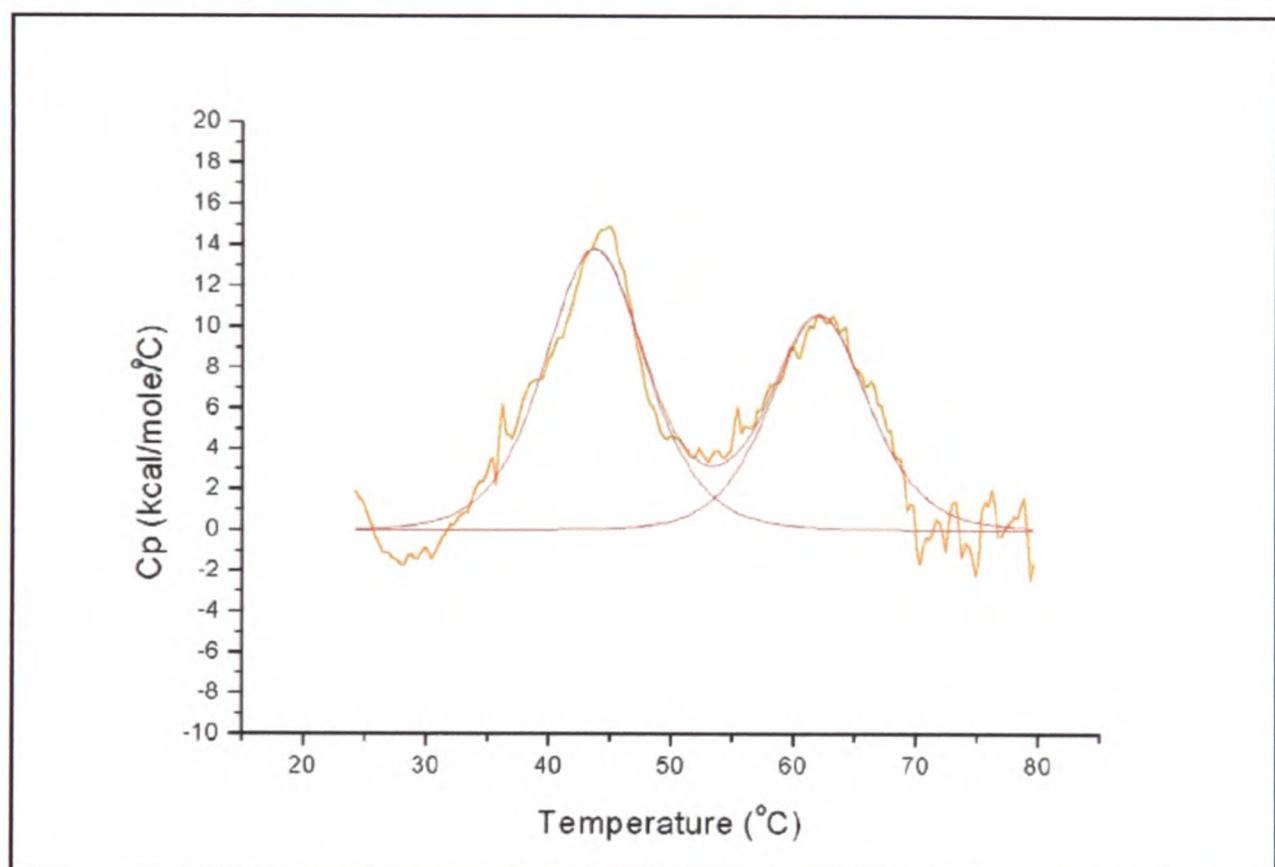


Fig. 37. DSC melting profile of human fI (I). Heating of fI from 20 to 80 ° C as described in section 3.2.7 showed that fI denatures in two major events, one reaching a peak at approximately 44 ° C and another at approximately 62 ° C. From the two peaks only the first one was found to be reversible with the drop of the temperature back to 20 ° C. It was proposed that each peak corresponds to the denaturation of a fI subunit, the first to the non-catalytic heavy chain and second to the catalytic SP domain. The results and derived hypothesis from the DSC studies appeared to be in agreement with the functional data obtained for the heat stability of fI. It is now believed that the two subunits of fI have distinct heat capacities (expressed in Kcal/mole/ ° C) and that both participate directly in the interactions with the substrate and the cofactor.

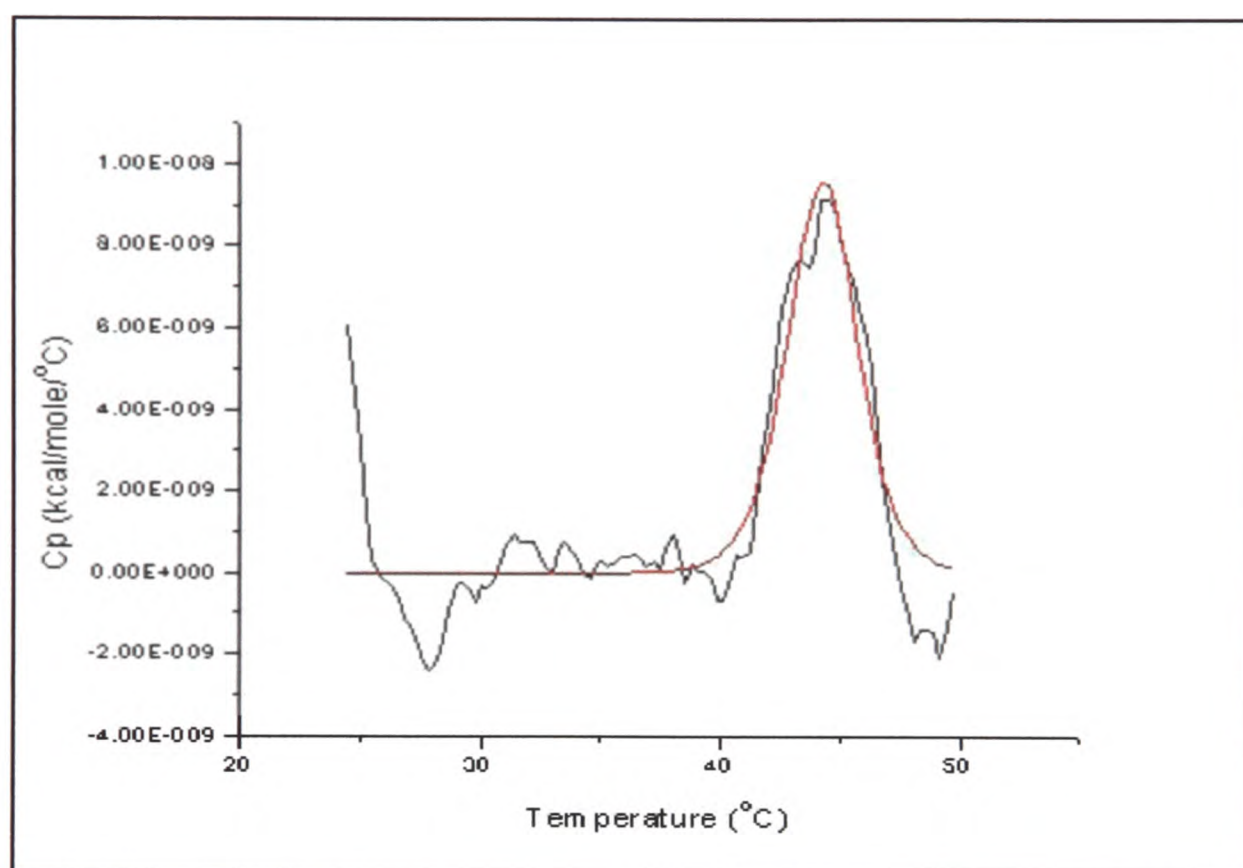


Fig. 38. DSC melting profile of human fI (II). Heating of fI from 20 to 50 ° C as described in section 3.2.7 showed that a major temperature-reversible denaturation event occurs reaching a peak at approximately 44 ° C. It was proposed that this peak corresponds to the denaturation of the non-catalytic heavy chain fI subunit. This part of the hypothesis derived from the DSC studies appeared to be in agreement with the functional data obtained for the heat stability of fI from the proteolytic assay. The non-catalytic subunit of fI appears to participate directly in the interactions with the cofactor and/or substrate in the complex formation through highly ordered interactions. Upon denaturation the interactions are believed to weaken up to approximately the region of 55 ° C where the heavy chain denaturation apparently reaches completion and the denaturation of the SP domain appears to begin. The fact that in both the proteolytic and amidolytic assays the activity is not lost below approximately the 80 ° C region, is due to the fact that the SP domain has still activity until that region.

calorimetric studies were found to be in agreement with the functional data obtained during the heat stability studies of fI (section 3.3.5).

In the proteolytic assay fI was found to lose activity in two main stages, one between 37-48 ° C and another between 61-73 ° C (Fig. 36), while in the amidolytic assay fI was found to lose activity in a linear trend above 48 ° C (Fig. 36). In both cases more than 80% of the fI activity was lost at 75 ° C. The match between the temperature areas of loss of activity in the proteolytic assay (Fig. 36) and the ones of the denaturation peaks (Fig. 37) suggest that the first peak corresponds to the non-catalytic heavy chain and the second to the SP domain. The denaturation of the non-catalytic subunit appears to reach completion at the region of approximately 55 ° C (Fig. 37), from where the denaturation of the SP domain begins. This can explain the loss of fI activity in the proteolytic assay as this depends on both the heavy and the light chains. The fact that in both the proteolytic and amidolytic assays the fI activity is not completely lost below approximately the 75 ° C region, may be due to the fact that the SP domain has still retained activity up to that temperature region as its denaturation has not reached completion. The temperature region around approximately 80 ° C is where the highly ordered SP structure, necessary for the catalysis, is completely and irreversibly damaged abolishing the ability of the SP domain to deliver any cleavage.

3.4 DISCUSSION

Although the physiological activity of fI has been broadly characterised, detailed descriptions of the potential range of its catalytic properties have not been available owing to the lack of identifiable synthetic substrates and high-resolution structural data. Previously, information available on the substrate specificity and likely structure of the active site has come from analysis of known cleavage sites in the natural substrates C3b or C4b, and the work on the development of selective inhibitors (Ekdahl et al., 1990; Fevig et al., 1998; Kam et al., 1992; Rupin et al., 1997). The lack of reactivity of fI against thioester substrates reported previously (Kam et al., 1992), suggested the possibility that the catalytic light chain of fI is functionally inactive prior to substrate-induced conformation change.

However, it is clear from the data presented here that fI has amidolytic activity in the absence of cofactors and natural substrates (Table 7). This shows that the enzyme in its native state has a conformation that accommodates substrate recognition and cleavage. The presence of excess cofactor (fH) does not modify the amidolytic activity (Fig. 31), so fH is unlikely to modify directly the active site of fI. The detection of amidolytic activity in the absence of fH and C3(NH₃), suggests that the requirement for fH in the cleavage of C3b *in vivo* is to support substrate orientation effects, but the possibility that fI-cofactor interaction alters the conformation of fI to provide a secondary substrate binding site has not been eliminated. This is in agreement with the data describing the interactions within the ternary complex formed between fI, fH and C3(NH₃) (DiScipio, 1992; Soames and Sim, 1997). In the experiment where all components (fI, fH and C3(NH₃)) and the synthetic substrate were present (Fig. 31), C3(NH₃) might be expected to act in competition with the synthetic substrate. However, the ratio of D-P-R (25 μ M) to C3(NH₃) varied from 50 to 250-fold molar excess, such that any competitive effect would be small.

The substrate specificity patterns of fI are dependent on the architecture of the SP domain of fI that aligns the desired arginyl bond to the active site area. The strong preference for R at P₁ (Fig. 29) is determined by the presence of an Aspartate residue at position 189, located at the bottom of the specificity pocket that interacts

electrostatically with positively charged residues like R or K. For the P₂ position there is a preference for Proline. This selectivity at S₂ is under the influence of Ser 214. Surprisingly, at the natural cleavage sites (Table 1) G, S and L are preferred in P₂ (P-S-R, L-L-R in C3b and T-G-R, R-G-R in C4b) instead of P. For position P₃, little information can be gained as, in the substrates that were cleaved best, the residues at the P₃ were diverse: (Table 7). In C3b and C4b cleavage sites, P, L, T and R all occur at the P₃ position. It is difficult to judge which residues are preferred in P₃, but those that can form two antiparallel β -strand hydrogen bonds with the G at 216 (Perona et al., 1995) are likely to be the optimal.

fI and Thrombin appear to have similar synthetic substrate specificities, but cleave at very different rates of catalysis (Table 8). The similar substrate specificities can be attributed to homologies within the substrate binding sites for both proteins (Fevig et al., 1998; Rupin et al., 1997). Earlier studies on the specific activity of fI on C3(NH₃) in the presence of fH had reported higher cleavage velocities than the ones reported here (Pangburn and Muller-Eberhard, 1983). The turnover rate reported for the cleavage of the natural substrate C3(NH₃) at 37 °C in physiological conditions is 900 pmoles C3b cleaved/min/ μ g fI, which is significantly higher from the values described for the cleavage of tripeptides by fI (Table 7; 0.5 & 0.35 pmoles AMC released/min/ μ g fI). Factor I has already been shown to exhibit a very tight binding to the C3(NH₃)-fH complex, but also a low rate of catalytic efficiency (section 1.5.4). The V_{max} values obtained for Thrombin are still considerably higher (Table 8; 49 & 32 nmole AMC released/min/ μ g fI). Although the two assays are significantly different, as are the two substrates (the synthetic one is a tripeptide and the natural one is a large protein (section 1.4.1)), making direct comparisons difficult, the efficiency of cleavage of the natural substrate is likely to be higher; conformational effects that occur during the complex formation with the substrate and the cofactor are likely to contribute to increase the efficiency.

Clotting proteases share ancestry with complement proteases (Patthy, 1990) (section 1.3.6). The limited substrate range and low catalytic activity of fI is most likely related to its narrow activity against only two natural substrates, in the presence of cofactors. A difference between fI and thrombin is the lack of allosteric regulation of fI activity by Na⁺ ions. According to Dang and Di Cera (Dang and Di Cera, 1996),

the presence of a Y at position 225 in thrombin supports the binding of a Na^+ ion in a designated binding loop, while the replacement of Y 225 with a P in fI results in no such regulation. In complete contrast, the proteolytic activity, which can be assessed only when a cofactor protein and a protein substrate are present, is very dependent on salt strength. The rate of reaction decreases very substantially between 10 mM and 150 mM NaCl (Sim and Sim, 1983). In addition, the pH optimum for the cleavage of $\text{C3}(\text{NH}_3)$ by fI when fH is the cofactor, is low (<5.5), but when CR1 is the cofactor, the optimum pH lies between 7-7.5 (Sim and Sim, 1983). These significant differences between the proteolytic and amidolytic assays presumably reflect the weak ionic interactions fI-cofactor, fI-substrate and substrate-cofactor (Soames and Sim, 1997). The interactions $\text{C3}(\text{NH}_3)$ -fI, $\text{C3}(\text{NH}_3)$ -fH and fI-fH are stronger at low ionic strengths and have pH optima lower than 6 (Soames and Sim, 1997).

The cleavage of synthetic substrates is optimal at pH 8.25 and is negligible at pH below 6.0. The bell shaped curve in Fig. 32 is typical among serine proteases, as at alkaline pH the catalytic triad operates at its most efficient mode. Chemically, this is mainly due to the fact that at alkaline pH the H^{57} of the serine protease domain catalytic triad can function as the base catalyst for the formation of an acyl-enzyme intermediate during the catalysis reaction. At alkaline pH the imidazole ring of H^{57} is deprotonated and can easily accept the proton from the $-\text{OH}$ group of the catalytic S^{195} that attacks the peptide bond between residues P_1 - P_1' (section 1.2.6.1). As the typical pK value of the imidazole ring for histidine in proteins is approximately 6.5 (Stryer, 1995) at pH values below pH 6.0, the H^{57} cannot act directly as a base and hence the activity of the serine protease drops largely. In accordance, at pH 5 the catalytic efficiency of the majority of serine proteases is very low. However, as has been shown in several studies, the pH optimum for the proteolytic cleavage of C3b by fI, is below 6.0 when fH is the cofactor, but is between 7-8 when CR1 is the cofactor (section 1.4). Although the pH optimum for the case of CR1 is explainable, the low pH optimum for the case of fH is in contrast with the established mechanism of catalysis and suggests the strong possibility that at the complex formation with C3b and fI, fH might influence the protonation state of the catalytic triad enabling it to continue functioning at low external pH. That could be achieved for example by creating a microenvironment in which fH may be affecting the relative distribution of charges locally. Although H^{57} is the critical base catalyst in the serine protease

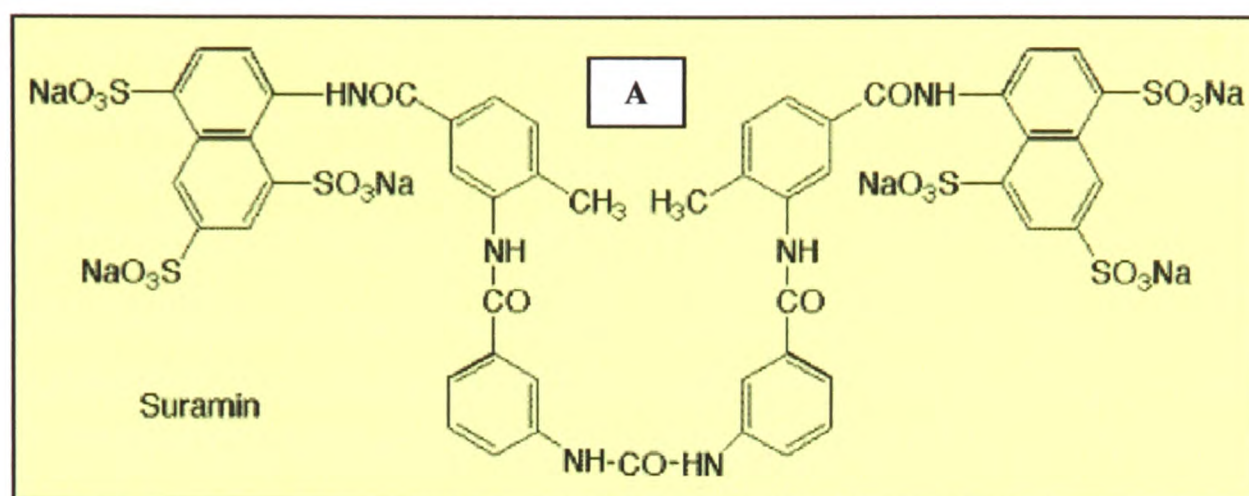
catalysis mechanism, the effect of fH could on one hand be direct towards H⁵⁷, or it could be indirect; that is, fH could affect the charge status of neighbouring residues in the area of the active site that may subsequently have an effect either directly on H⁵⁷ or could support the catalysis independently of H⁵⁷ at the acidic pH. As a molecule, fH is known to participate in interactions with charged groups such as sialic acids and glucosaminoglycans (section 1.5.3.1).

The effects of inhibitors on the amidolytic assay confirmed the serine protease nature of fI. When Crossley and Porter (1980) tested a series of inhibitors on fI, they reported poor reactivity with several serine protease inhibitors. DFP for example did not inhibit. fI was characterised as a serine protease from sequence determination (Catterall et al., 1987; Goldberger et al., 1987). The activity of additional inhibitors reported in this paper is consistent with the nature of fI. As expected, Bestatin, an amino- and exo-peptidase inhibitor and 1, 10 Phenanthroline, a metalloprotease inhibitor did not inhibit at all, since no metals were found that regulate positively the activity of the enzyme. Pepstatin, an aspartic acid protease inhibitor, and chymostatin a specific inhibitor of chymotrypsin also did not inhibit. No inhibition was found with C1-inhibitor, a well characterised serpin that inhibits C1s, C1r, MASP-1, MASP-2, plasma kallikrein, factor XIIa and tPA. The cleavage site in human C1-inh has the sequence S-V-A-R that is unlikely to be compatible with the fI active site architecture. ϵ -ACA, a lysine analog does cause slight inhibition, while Benzamidine, an arginyl analogue, gave much stronger inhibition in accordance with the arginyl preference. The moderate inhibition by SBTI and LBTI is probably due to trace contaminants in the preparations or perhaps due to substrate competition effects. Since these inhibitors form 1:1 complexes with their natural targets, it would be expected that if there was inhibition, at such high molar excess (250X for SBTI and LBTI) the percentage loss of activity should be 100%. This was not explored further. Hirudin gave a small effect at about 1:1 molar ratio.

From the other irreversible inhibitors examined, namely Pefabloc SC and Boro MPG, only the first one inhibited well at all ranges of pH values tested, while the inhibitory effect of the Boro MPG was found to be largely dependent on the pH of the reaction. This suggests that on a chemical basis the reactivity of both compounds with their target residues is dependent on the state of the target residue at the various pH

values. Although the strong and uniform inhibitory effect of Pefabloc SC, that reacts with the catalytic serine, can be explained on the basis that serine does not have any ionisable side chains, for the case of Boro MPG the basis for the lack of inhibition at pH 6.2 could be either due to the very slow rate of reaction with the enzyme active site or most probably due to its proposed reactivity with H^{57} (Claeson et al., 1993) (<http://www.merckbiosciences.co.uk/product/605200>); The changes of the ionisation state of the imidazole ring of H^{57} with pH can reasonably explain the changes in the inhibitory effect of Boro MPG which are consistent with the changes in the activity of the SP domain among the pH range examined (Fig. 32). The BoroMPG inhibitor was tested as a representative of the boropeptide thrombin inhibitors reported to cross react with fI (Fevig et al., 1998; Rupin et al., 1997). Unfortunately, the particular compound is of limited solubility (water soluble up to 0.4 mM). Of the Pefabloc variants examined, Pefabloc TH is sold as a more selective inhibitor for Thrombin, while Pefabloc Xa is relatively selective for factor Xa. Pefabloc SC was designed to cover a wider spectrum of proteases. Surprisingly, Pefabloc SC and Xa inhibited fI much better than Pefabloc TH, despite indications that fI had similar specificity to Thrombin. This variability could be due to the presence of a Y at position 99 in Xa and fI, whereas Thrombin has L. The larger Y 99 residue should make the aryl binding pocket of fI smaller than that of Thrombin (Fevig et al., 1998). It is notable that Pefabloc SC inhibits fI very effectively, while the other “wide-range” serine protease inhibitor DFP, does not inhibit fI (Crossley and Porter, 1980).

Suramin inhibits well in both assays at all pH ranges examined. It is a hexasulfonated naphthylurea with a symmetrical structure (Nakajima et al., 1991) (Fig. 39) that has been used for decades in the treatment of trypanosomiasis and onchocerciasis (Hawking, 1978; Voogd et al., 1993). It has proved useful as an anti-tumour agent (Cadene et al., 1997) and as a potent reversible inhibitor of the complement system (Fong and Good, 1972) and of C3b breakdown (Lachmann et al., 1973). Suramin has been reported to bind to proteins containing LDLR type a domains (Vassiliou, 1997; Vassiliou et al., 2001), two copies of which are present in the heavy, non-catalytic chain of fI (Fig. 9). It has also been found to inhibit simple proteases, like trypsin, with only SP domains (Cadene et al., 1997; Cutting, 1964). In addition, suramin has been reported to cause oligomerisation of complement components C8 and C9 thus blocking Membrane Attack Complex formation and



B

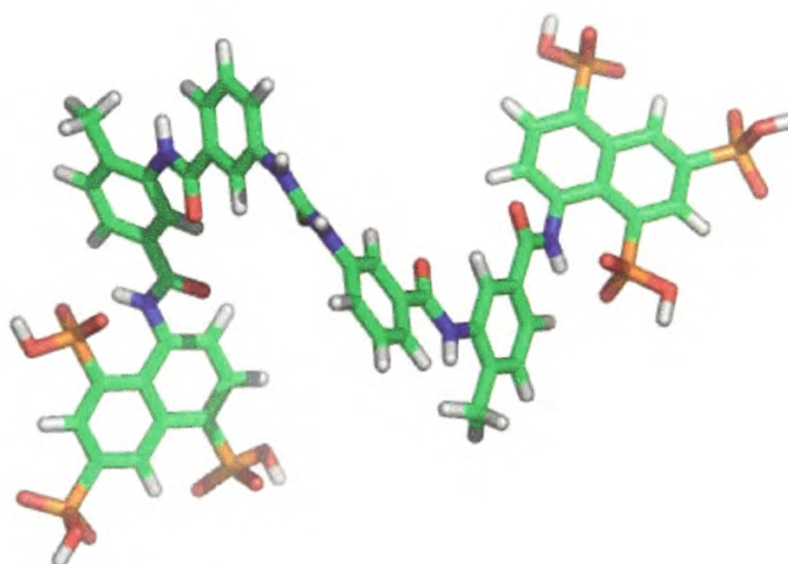


Fig. 39. The structure of Suramin. Suramin, the chemical name of which is hexasodium *sym*- bis [*m*-aminobenzoyl-*m*-amino-*p*-methylbenzoyl-1-aminonaphthalene-4,6,8-trisulfonate]carbamide, is a hexasulfonated naphthylurea with a symmetrical structure. A. The chemical formula, B. A stick representation of the 3D structure of suraminic acid. The structure was generated using the “CORINA” (COoRdINates) software [http://www2.ccc.uni-erlangen.de/software/corina/free_struct.html]; Sadowski, J., Schwab, C. H., and Gasteiger, J. (2003). 3D Structure Generation and Conformational Searching. In *Computational Medicinal Chemistry and Drug Discovery*, P. Bultinck, H. De Winter, W. Langenaeker, and J. P. Tollenaere, eds. (New York, Dekker Inc.), pp. 151-212.)] and the figure was produced using the “PyMOL Molecular Graphics System” by DeLano Scientific LLC [San Carlos, CA, USA] (<http://pymol.sourceforge.net/>). The colours correspond to different atoms: green for C, white for H, red for O, blue for N and orange for S.

preventing complement-mediated haemolysis (Saez et al., 1999). The first reports that suramin inhibits C3b breakdown to iC3b were from Tamura and Nelson (1967) and Lachmann et al. (1973). It was assumed then that binding of Suramin to C3b blocked the cleavage by fI, since treatment of C3b-coated cells with Suramin, prior to exposure to fI, inhibited breakdown of C3b to iC3b (Fong and Good, 1972). The affinity for Suramin may be related to the basicity of proteins (Cadene et al., 1997); the negatively charged sulfonate groups of the polyanion bind to cationic groups provided by the proteins through electrostatic interactions (Bos et al., 1990). Although in theory the binding is of electrostatic nature, hydrophobic interactions have also been implicated in interactions with the proteins (Saez et al., 1999). For the case of fI, it is clear that suramin inhibits human fI directly (Tables 10 & 11) while, the similar degree of inhibition in both assays also indicates that suramin does not have strong direct effects on fH or C3b. It was of interest to examine the effects of suramin on fI in more detail by additional experimental work using ^{125}I -fI. This work is discussed in detail in chapter 5.

Leupeptin and Antipain both inhibit fI effectively at or above the physiological pH of 7.4. Leupeptin has the sequence L-L-R that matches one of the natural cleavage sites in C3b and the synthetic substrate L-L-R-AMC. Antipain has an R-V-R sequence that resembles the R-G-R natural cleavage site in C4b. Leupeptin has previously been described to inhibit thrombin as has Aprotinin (Brass and Shattil, 1988). The moderate inhibition of fI by Aprotinin that bears a K-A active site, is consistent with the Arg preference of fI already discussed. A summary of the sequence similarities between the natural fI cleavage sites and the peptide substrates and inhibitor analogues examined on fI can be found in Table 12.

Benzamidine and PMSF hardly inhibited in the proteolytic assay but were effective in the amidolytic assay. Crossley and Porter (Crossley and Porter, 1980) also found Benzamidine ineffective in the proteolytic assay. Possibly its affinity with Asp 189 is much lower compared to the natural substrate and upon complex formation the analog is likely removed. The multiple interactions with substrate surfaces are likely to increase the stability and enhance the interactions that result in the removal of the low molecular weight analog. Strong pH effects were observed for the peptide analogs Antipain and Boro-MPG which did not inhibit at pH 6.2, but inhibited well at pH 7.4

Table 12. Summary of the sequence similarities between the natural fI cleavage sites and the peptide substrates and inhibitor analogues examined on fI.			
Natural substrate	Sequence P _n P ₄ P ₃ P ₂ P ₁ †P ₁ 'P ₂ 'P ₃ 'P ₄ 'P _n '	Synthetic substrates (at least two common residues)	Inhibitor analogues [At physiological pH 7.4]
C3b, C3(H ₂ O) ¹ , C3ma ²	L ¹²⁷⁸ P S R † S S K I ¹²⁸⁵	F S R † AMC (P ₁ & P ₂)	<u>In amidolytic assay:</u> Benzamidine (R analogue) Boro MPG (arginyl analogue) PMSF (R analogue) Aprotinin (xxK) <u>In proteolytic assay:</u> Boro MPG (arginyl analogue) Aprotinin (xxK)
	S ¹²⁹⁵ L L R † S E E T ¹³⁰²	L L R † AMC (P ₁ & P ₂)	<u>In amidolytic assay:</u> Benzamidine (R analogue) Boro MPG (arginyl analogue) PMSF (R analogue) Aprotinin (xxK) Leupeptin (LLR) <u>In proteolytic assay:</u> Boro MPG (arginyl analogue) Aprotinin (xxK) Leupeptin (LLR)
	S ¹³¹⁴ T G R † N G F K ¹³²¹	F G R † AMC (P ₁ & P ₂) I E G R † AMC (P ₁ & P ₂) G G R † AMC (P ₁ & P ₂)	<u>In amidolytic assay:</u> Benzamidine (R analogue) Boro MPG (arginyl analogue) PMSF (R analogue) Aprotinin (xxK) <u>In proteolytic assay:</u> Boro MPG (arginyl analogue) Aprotinin (xxK)
C4b, C4(H ₂ O) ¹ , C4ma ²	H ⁹³⁴ R G R † T L E I ⁹⁴¹	F G R † AMC (P ₁ & P ₂) I E G R † AMC (P ₁ & P ₂) G G R † AMC (P ₁ & P ₂)	<u>In amidolytic assay:</u> Benzamidine (R analogue) Boro MPG (arginyl analogue) Antipain (RVR) PMSF (R analogue) Aprotinin <u>In proteolytic assay:</u> Boro MPG (arginyl analogue) Antipain (RVR) Aprotinin (xxK)
1. C3(H ₂ O)/C4(H ₂ O): C3/C4 with a hydrolyzed thioester. 2. C3ma/C4ma: Methylamine treated C3/C4. Both forms 1 and 2 of C3 and C4, in the presence of cofactors, can adopt a conformation that supports their cleavage by fI.			

and even better at pH 8.25. Since these inhibitors are irreversible substrate analogues, presumably their rate of reaction with the fI serine protease domain active site is very slow, so that no reaction occurs at pH 6.2. In both tests Aprotinin was found to inhibit fI to a similar, moderate extent.

In conclusion, this work identifies means to assay fI alone without a cofactor. The novelty of the exploration of the active site of fI with substrates and inhibitors provides valuable information towards the elucidation of the configuration of the active site and the specificity regions. Mapping of certain specificity regions and characterisation of the interactions within and around the active site can provide powerful information that may guide the effort for synthesis of specific inhibitor compounds that could have important medical therapeutic applications. Future analysis of a 3D structure of fI will complement the current observations.

Chapter Four

CHAPTER 4: GENERATION OF THE HUMAN COMPLEMENT FACTOR I SERINE PROTEASE DOMAIN

4.1 INTRODUCTION

Factor I has restricted specificity limited to the cleavage of few arginyl bonds in its natural protein substrates C3b and C4b. Additional components such as factor H (fH), CR1, MCP or C4bp are required as cofactors. During natural substrate cleavage, fI forms a ternary complex with the substrate and the cofactor (DiScipio, 1992; Soames and Sim, 1997). However, fI has been shown to be active alone in the cleavage of synthetic amide substrates (Tsiftoglou and Sim, 2004), suggesting that the catalytic subunit of the enzyme in its resting state has a conformation that accommodates substrate recognition and cleavage. Certain aspects of the fI/cofactor/substrate complex formation remain unclear, such as, whether binding of the cofactor to both fI and the substrate is required for substrate orientation, or is necessary for inducing appropriate conformations in either the substrate or enzyme.

The FIMAC domain of the fI heavy chain (Diag. 1) contains a Kazal-type protease inhibitor module the role of which has not been elucidated. Kazal-type inhibitors are a diverse family of multi-domain proteins with high inhibitory potency which inhibit thrombin/trypsin-like proteases (Schlott et al., 2002). It has been suggested that the serine protease domain of fI might be inhibited in the isolated enzyme because of its proximity to the Kazal domain (Chamberlain et al., 1998). If this is so, cofactor-substrate-fI complex formation might cause separation of the SP and Kazal domains allowing fI to become fully active. However, fI has been shown to be active against synthetic substrates in the absence of cofactors (Tsiftoglou and Sim, 2004).

More information is required for understanding the protein-protein interactions between enzyme-cofactor-substrate. Little is known about the role of the fI heavy chain domains in complex formation, while the mechanisms of the cofactor activity have not been fully explored. It is unknown whether the catalytic subunit of fI can cleave the natural substrate in the absence of the heavy chain and in the presence or absence of cofactors.

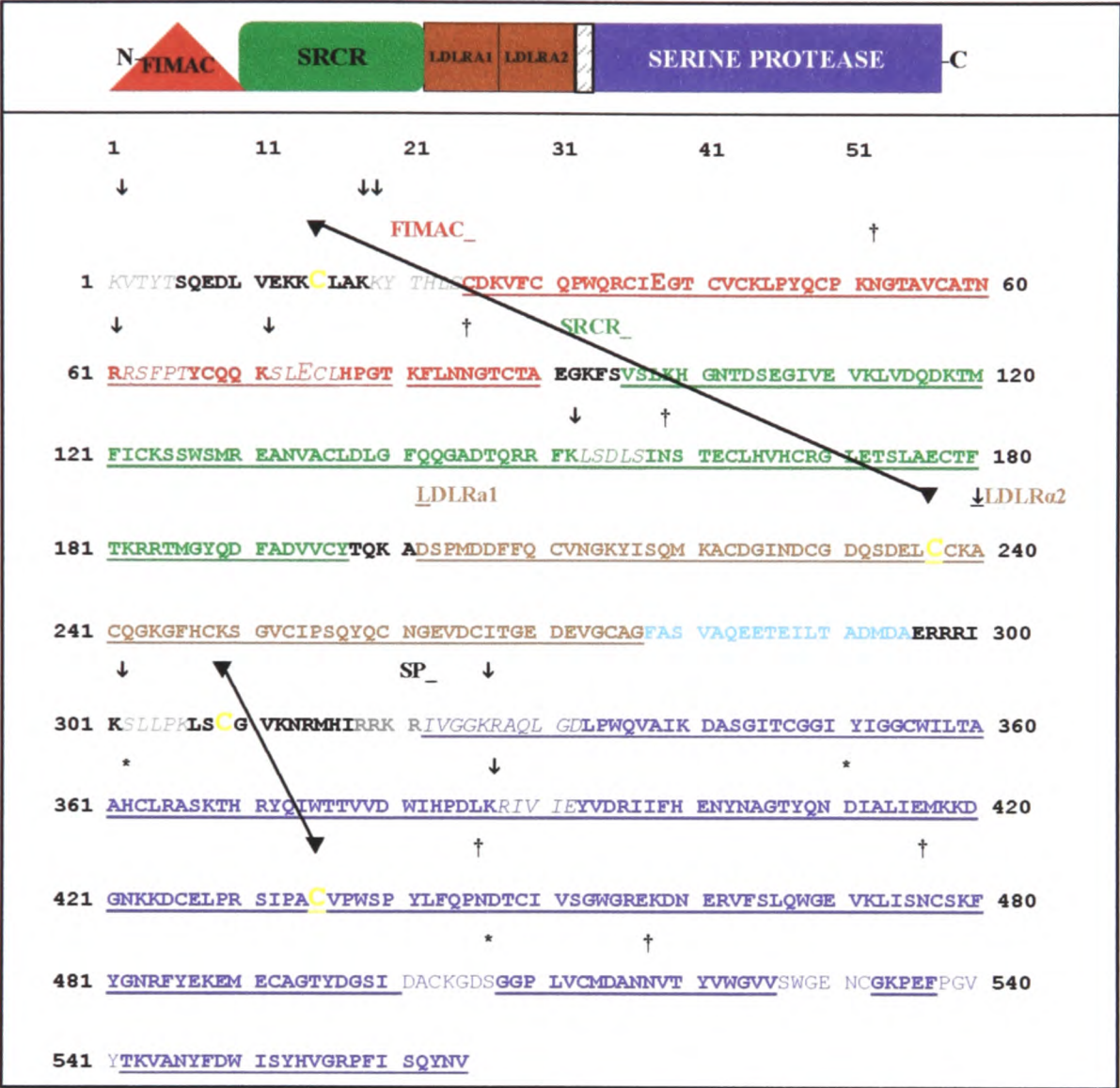


Diagram 1. The sequence and domain organisation of human fI.

fI exhibits a unique linear arrangement of domains; an N-terminal FIMAC (Factor I Membrane Attack Complex) domain (in red), a SRCR (Scavenger Receptor Cysteine Rich) domain (in green) and two LDLR-A (class A Low Density Lipoprotein Receptor) domains (in brown) in the non-catalytic heavy chain. The non-labelled portion represents a connecting segment. In the primary sequence, all domains are illustrated underlined and the start of each domain is indicated by an underscore beside the domain name (e.g. SRCR_). Non-underlined text in the heavy chain shows non-domain regions. The non-bold, non-underlined sequences in the Serine Protease (SP) domain (in blue) form the walls of the specificity pocket. The peptide bonds that have been identified by N-terminal sequence analysis as cleavage sites for plasmin (section 4.3.3) are indicated in italics and ↓ indicates each bond that is cleaved.

This diagram is a copy of Fig. 9 presented in chapter 1. For more a more detailed description please refer to the legend of Fig. 9 and section 1.3.2.

The central objective of this work has been the structural and functional characterisation of the fI SP domain. The first steps of this strategy focused on the isolation of the SP domain from the native enzyme. This chapter describes in detail, the generation of the SP domain from native human fI using proteolysis with plasmin and its purification for further characterisation of function studies.

4.2 MATERIALS AND METHODS

4.2.1 Proteolytic fragmentation of human factor I by V8 protease

Aliquots of 20 μ l containing 9 μ g of fI (0.45 mg/ml) in PBS, 0.5 mM EDTA, pH 7.4 were incubated with variable amounts of V8 protease at final concentrations from 0.1 % to 4 % w/w. The final reaction volume was 40 μ l, and reaction mixtures were incubated for 1 hr at 37 ° C. The reactions were stopped by the addition of 15 μ l of SDS-PAGE sample buffer and analysed by SDS-9.0% PAGE under non-reduced and reduced conditions.

4.2.2 Proteolytic fragmentation of human factor I by plasmin

Seven identical samples each containing 10 μ g of fI (1.185 mg/ml) in PBS, 0.5 mM EDTA, pH 7.4, were incubated with 0.6 μ g of Plasmin [6% (w/w)-Plasmin/fI] (1.2 μ l of 0.5 mg/ml) at 37 ° C for a variable time. The reaction was blocked by the addition of a 2-fold molar excess of SBTI over Plasmin and further incubation for 15 minutes under the same conditions. The samples were analysed by SDS-10% PAGE (Pre-cast 10% NuPAGE Bis-Tris gel system) under non-reduced and reduced conditions.

4.2.3 Characterisation of the factor I plasmin digest fragments by N-terminal sequencing

Digest samples were analysed on a 10% Novex Bis-Tris NuPAGE precast gel using MES buffer in a Novex X Cell II Mini-cell gel apparatus. The gel was electroblotted onto a Novex 0.2 μ m PVDF membrane (Invitrogen) on a Novex blot module. The membrane was stained with Coomassie Brilliant Blue. Target protein bands were excised from the membrane and washed with 10% methanol prior to N-terminal sequencing (Matsudaira, 1987). They were then were sequenced on an Applied Biosystems 494A 'Procise' protein sequencer (Applied Biosystems, Warrington, U.K.) for 10 cycles each using standard sequencing cycles. The N-terminal sequencing was performed by Mr. A. C. Willis, MRC Immunochemistry Unit.

4.2.4 Purification of the factor I light chain by antibody affinity chromatography

A digestion of fI with plasmin was prepared under optimized conditions. One mg (844 μ l) of human fI was incubated with 60 μ g (120 μ l) of plasmin at 37 ° C for 20 minutes in PBS, 0.5 mM EDTA, pH 7.4. The reaction was stopped by the addition of 30 μ g of SBTI (30 μ l). The digest was passed down a 2 ml MRC-OX21 antibody-Sepharose column (Sim et al., 1993) pre-equilibrated in 25 mM Tris, 140 mM NaCl, 0.5 mM EDTA, pH 7.4. The MRC-OX21 mAb recognizes an epitope on the heavy chain of fI. Antibody affinity chromatography was employed to ensure complete removal of uncleaved fI from the digest mixture. Fractions (1 ml) were collected (numbered 1-6) and the column was washed extensively with running buffer till OD_{280nm} of the eluate was < 0.04. Bound protein was then eluted with 3M MgCl₂, pH 6.9. The fractions containing 3M MgCl₂ were pooled and dialysed against 10 mM NaCl, pH 7.2 and concentrated by freeze drying. The unbound and bound fractions were analysed by SDS-10% PAGE under non-reduced and reduced conditions.

4.3 RESULTS

4.3.1 Proteolytic fragmentation of human factor I by V8 protease

The sequence and domain organisation of human fI are shown in Diag. 1. Several proteases (trypsin, chymotrypsin, thrombin and pepsin) were used in preliminary tests on fI cleavage, but these proteases did not produce suitable fragment sizes. V8 protease and plasmin produced more favourable results. Factor I was digested for a fixed time with increasing quantities of V8 protease. As shown in Fig. 40B, the fI SP domain (LC) is quite resistant to cleavage by V8, while the heavy chain is cleaved to fragments of about 40 and 20 kDa. Further degradation of the 40 kDa fragment is seen at high V8 concentration. With non-reduced samples (Fig. 40A), it can be deduced from the disulphide bridging pattern of fI (Diag. 1) that the major cleavage occurs in the region of the LDLR-A2 domain between C²³⁷ and C³⁰⁹ (Diag. 1). This creates an N-terminal fragment of ~40 kDa, leaving the SP domain attached to a ~15 kDa C-terminal fragment of the heavy chain. The digestion therefore left a large heavy chain fragment attached to the SP domain, which was unsuitable for further processing. Plasmin digestion was therefore explored.

4.3.2 Proteolytic fragmentation of human factor I by plasmin

Digests of fI with plasmin were done to observe the rate and patterns of cleavage. A typical time course is shown in Fig. 41A. Intact fI is cleaved (Fig. 41A) so that after 160 minutes less than 10% remains. An intermediate product of M.W. ~ 40 kDa (non-reduced) is formed (labelled “c”), reaches a maximum at ~ 30 minutes and then breaks down further. Concurrently with the appearance of “c”, a band of M.W ~38 kDa appears (“d”). It was shown by N-terminal sequence (Table 13) analysis that “d” contains as a major component a species with the intact N-terminus of the SP domain.

As illustrated in Fig. 41B, more than 90 % of the heavy chain (“a”) has been cleaved at 160 minutes and a large number of small fragments from ~31 kDa-4 kDa are formed. The light chain (“b”) appears more resistant to digestion although after

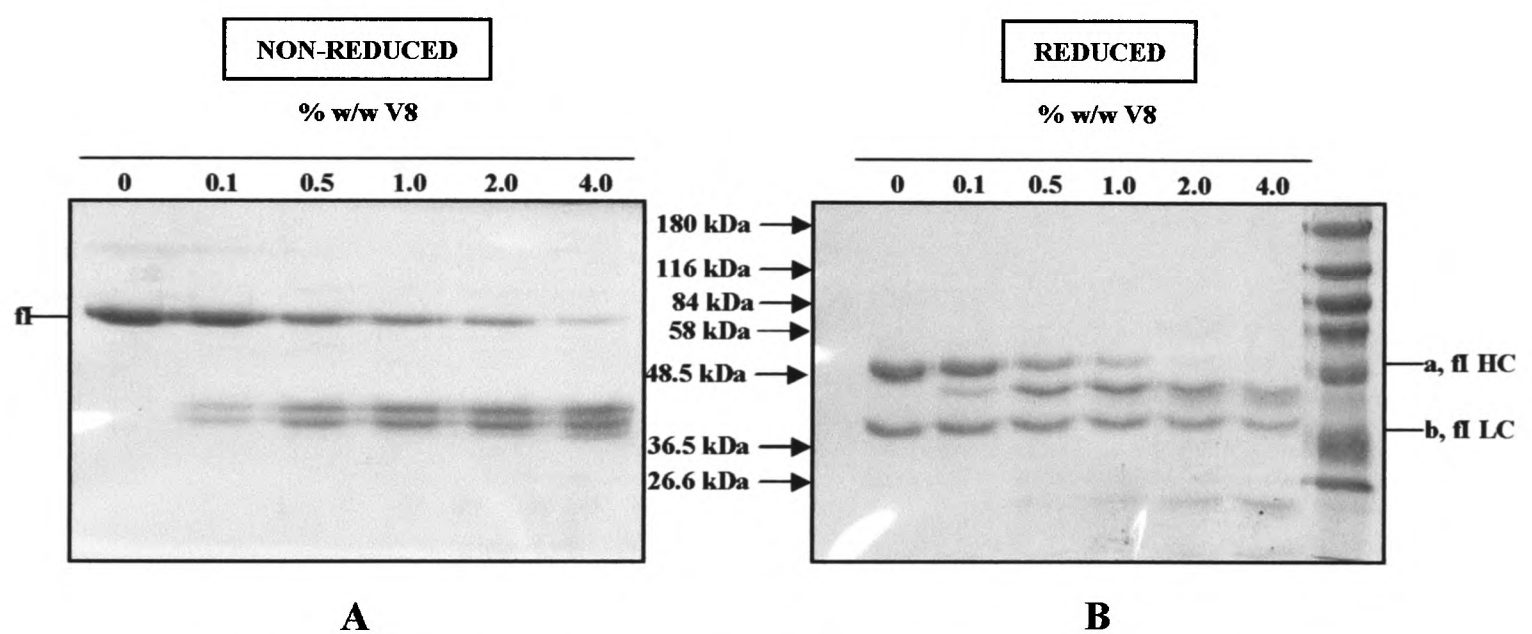


Fig. 40. Proteolytic fragmentation of human fI by V8. SDS-9.0% PAGE analysis of the digest mixtures under non-reduced (A) and reduced (B) conditions. Bands a and b represent the fI heavy and light chains, respectively.

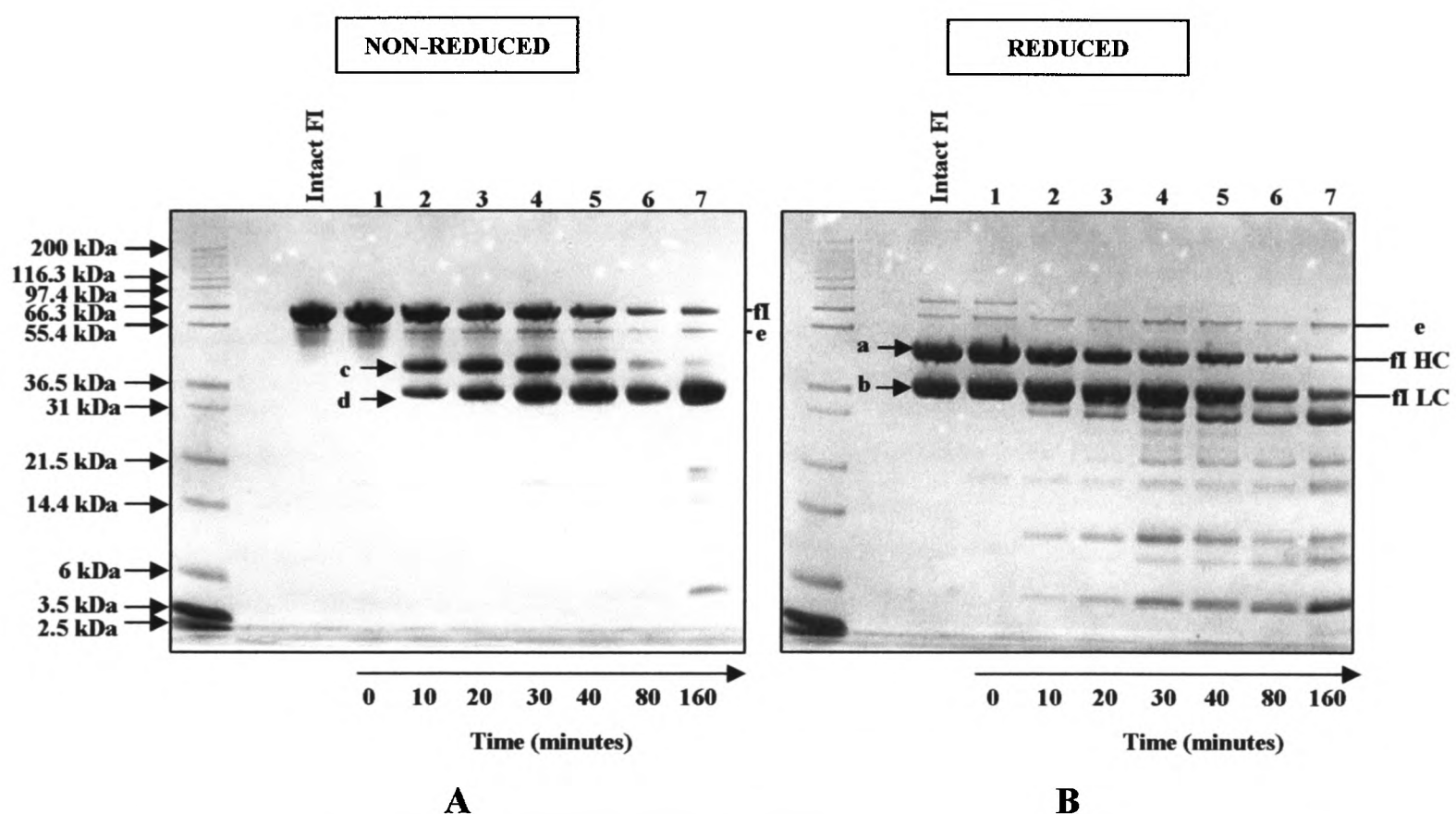


Fig. 41. Proteolytic fragmentation of human fI by plasmin. The reaction mixtures containing the digests were analysed by SDS-10% PAGE (Pre-cast 10% NuPAGE Bis-Tris gel system) under non-reduced (A) and reduced (B) conditions. Tracks 1-7 represent successive time points in the digestion of fI by plasmin. Track labelled "intact fI" shows fI without plasmin, unincubated. fI HC is labelled as "a" and the LC as "b". An intermediate product, "c", of M. W. ~40 kDa in A is formed in the initial phase of incubation, reaches a maximum at ~30 minutes and then breaks down further. N-terminal sequencing results showed that the product "d" in A contains the SP domain disulphide linked to a C-terminal fragment of the heavy chain through the disulphide bridge formed between C³⁰⁹ and C⁴³⁵. "e" is a minor contaminant of negligible amount compared to fI.

Table 13. Summary of results from the N-terminal sequence analysis of species “b” and “d” in Fig. 41.

Band species	N ² -terminal sequence (sequencing yield in pmoles)
Non-Reduced ¹ Band d, Lane 3, Fig. 41A.	I ³²² VGGKRAQLG (24.0) [Light Chain] R ³²⁷ AQLGDLPWQ (21.9) [Light Chain] R ³⁸⁸ IVIEYVDRI (17.7) [Light Chain] S ³⁰² LLPKLSCGV (38.4) [Heavy Chain]
Reduced ³ Band b, Lane 3, Fig. 41B	I ³²² VGGKRAQLG (16.4) [Light Chain] R ³²⁷ AQLGDLPWQ (15.3) [Light chain]

1. Plasmin-digested fI was subjected to N-terminal sequencing under reduced and non-reduced conditions. The sequencing revealed the presence of four different species, two of which carry N-terminal sequences (RAQLGDLPWQ and RIVIEYVDRI) that represent cleaved forms of the light chain. The sequence IVGGKRAQLG represents the N-terminus of the fI light chain. The sequence SLLPKLSCGV corresponds to a fragment that consists of a heavy chain piece disulphide bridged to the light chain. Of the total light chain material detected, only 35-40 % is intact (24 pmoles of the IVGGKRAQLG sequence compared with 24.0+21.9+17.7=63.6 pmoles) of the total light chain sequence.
2. The bold superscript numbers at each first amino acid residue of every identified N-terminal sequence, denote the position of the start residue of each proteolytic fragment within the sequence of human fI as illustrated in Diagram 1.
3. Extended incubation of fI with plasmin can result in the production of a partially cleaved light chain fragment that has its N-terminal IVGGK sequence removed. Removal occurs through the cleavage of 5 or 66 residues from the N-terminus. The sequencing of the reduced preparation provided a total yield of 31.7 pmoles and confirmed the light chain partial cleavage. For the reduced products the ratio of intact light chain to light chain lacking the first 5 residues was (16.4 : 15.3 =) 1.07, compared with (24.0 : 21.9 =) 1.09 calculated from the non-reduced material.

~40 minutes it clearly diminishes. Therefore timing is critical for the optimal generation of an intact SP domain product.

Further experiments were carried out to optimize the time-course and the concentration of plasmin, so as to obtain the maximum yield of band “d” (Fig. 41A), avoiding further degradation of the light chain (as seen in figure 41B, at 80 and 160 minutes).

The band labelled “d” (Fig. 41A) consists of a small fragment of the heavy chain disulphide linked to the light chain (see below). fl contains no unpaired cysteines (Chamberlain et al., 1998). All of the cysteines in fl can be assigned by homology to intra-domain disulphide bridges, except cysteines 15, 237 and 309 (numbering as in Diag. 1). C⁴³⁵ in the SP domain by homology should be involved in linkage to the heavy chain.

Sequence analysis of band “d” (Fig. 41A) (Table 13) showed that it consists of a single heavy chain fragment (N-terminus beginning with S³⁰²LLPK) that is linked to 3 SP domain-derived sequences (discussed below). This pattern is consistent with the interchain disulphide being between C³⁰⁹ and C⁴³⁵. Therefore the two other unassigned cysteines, C¹⁵ and C²³⁷, form a disulphide bridge linking a region close to the N-terminus of the heavy chain to the C-terminus of the LDLR-A1 (Diag. 1).

Band “d” (Fig. 41A) therefore contains the desired product, the SP domain disulphide linked to a small C-terminal heavy chain fragment. The heavy chain fragment may contain up to 20 residues (residues 302-321), depending on whether there is cleavage at the lysines in this region during the circulation of fl *in vivo*. This material therefore contains a much smaller heavy chain fragment than the product generated by V8 proteolysis.

4.3.3 Characterisation of the factor I plasmin digest fragments by N-terminal sequencing

fI was digested with plasmin for two different time periods (40 minutes to generate intermediate and end-point products and 16 hr to generate end-point fragments). The digests are shown in Fig. 42, as a stained blot after SDS-PAGE analysis.

The fragments formed during the 40 minutes digest were characterised by N-terminal sequencing (bands 1-8: no data were obtained for bands X and X'). N-terminal sequences of bands 1-8 are shown in Table 14 and the deduced cleavage sites are shown in Diag. 1. Eight cleavage sites were found in the heavy chain and two in the light chain (Diag. 1). All were located at the carboxy side of lysines, except for the R⁶¹R site in the heavy chain (numbering as in Diag. 1). The intact light chain is in band 1, but is contaminated with a form of the light chain that has been cleaved five residues from the N-terminus (R³²⁷AQLG). Considering the identified cleavage sites (Diag. 1), the largest end-point fragments, observed in the 16 hr digest, are the 33 kDa C-terminus of the light chain (starting at R³³⁸IVIE) and the 38 kDa light chain starting at I³²²VGGK or R³²⁷AQLG. The long end-point digest cannot however be used to generate intact SP domain because of the partial cleavage at position five of the light chain.

The results show that overall the SP domain exhibits higher resistance to plasmin than the heavy chain, but cleavages at two positions in the light chain (K³²⁶ and K³⁸⁷) do occur. These cleavages contribute to three forms of the light chain; R³²⁷AQLG and R³⁸⁸IVIE and the intact light chain I³²²VGGK. The additional sequence S³⁰²LLPK (Table 13) that was detected attached to the light chain in non-reduced samples, corresponds to a fragment from the C-terminal end of the heavy chain. The detection of this sequence shows that the disulphide interchain bridge forms between positions 309-435 (Diag. 1). In total 35-40 % of the whole light chain material (band d, Fig. 41A) had an intact N-terminus (Table 13).

4.3.4 Purification of the factor I light chain by antibody affinity chromatography

The plasmin digest of fI, done under conditions designed to optimise the yield of intact SP domain (as described in section 4.2.4), was processed further through an

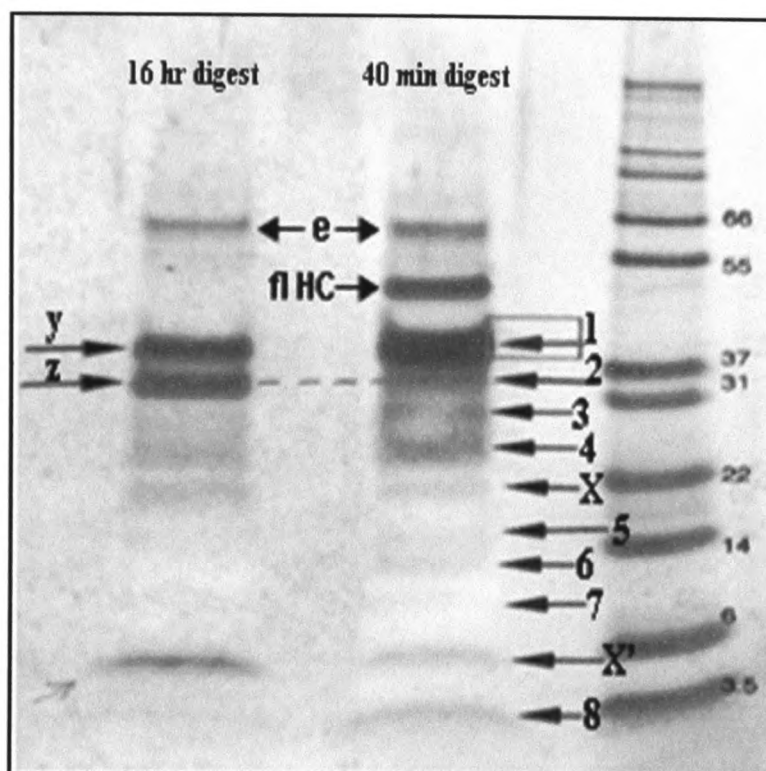


Fig. 42. PVDF blot generated for N-terminal sequence analysis of reduced fI fragments produced by proteolysis of fI with 6% (w/w) plasmin/fI. Two digest mixtures of fI were subjected to N-terminal sequence analysis. Both mixtures were prepared with the same composition (fI 50 µg, plasmin 3 µg in a final volume of 365 µl of PBS, 0.5 mM EDTA). One mixture was incubated for 40 minutes and one for 16 hours at 37 ° C. Each reaction was stopped by the addition of 1.5 µg SBTI in 150 µl of the same buffer (final volume 515 µl). Seventy µl from each preparation was concentrated with 7 µl of Strataclean resin each and both were run reduced on a 4-12% NuPAGE gel system with MES buffer. The gel was transferred to a Novex 0.2 µM PVDF transfer membrane via Western blotting and sequence analysis of selected bands (indicated by arrows and numbers 1-8) was done as described in the Materials and Methods section. The results of the analysis are summarised in Table 14. The presence of more than 8 visible bands running below the fI light chain (1) indicates a wide diversity of breakdown products. Some are intermediate products absent from the 16 hr digest and are therefore further cleaved on extended incubation. The intermediate products give rise to smaller fragments difficult to detect due to small M.W. There are clear differences in product yield between the two preparations. No sequence information was obtained for bands labelled X, X', but their sequence can be deduced (see Table 14).

Table 14. Characterisation of fI digest fragments by N-terminal sequencing¹.

Band in Fig. 42	Observed MW (kDa)	N ² -terminal sequence (sequencing yield in pmoles)
1	38	I ³²² VGGK (32.5), R ³²⁷ AQLG (7.9), S ⁷² LECL (7.9)
2	33	R ³³⁸ IVIE (5.4), S ⁷² LECL (2.1)
3	30	S ⁷² LECL (2.5)
4	25	S ⁷² LECL (7.4)
5	15	K ¹ VTYT (1.91), S ⁷² LECL (1.43)
6	12	K ¹ VTYT (6.12), S ⁷² LECL (4.07)
7	10	K ¹⁹ YTHL (10.0), Y ²⁰ THLS (9.29), L ¹⁵³ SDLS (2.69)
8	3	S ³⁰² LLPK (9.31), R ⁶² SFPT (3.63), I ³²² VGGK (3.24)

1. The protein fragments numbered 1-8 in Fig. 42 were sequenced. The position of these cleavage sites within the complete fI sequence is shown in Fig. 9 (Chapter 1). It can be deduced from the cleavage site pattern on Fig. 9 that band X (20 kDa) on Fig. 42 is likely to correspond to the glycopeptide sequence between cleavage sites K¹⁵²-K³⁰¹. Band X' might be the 62 or 63 aa segments starting at A²⁴⁰CQKG or R³²⁷AQLG.
2. The bold superscript numbers at each first amino acid residue of every identified N-terminal sequence, denote the position of the start residue of each proteolytic fragment within the sequence of human fI as illustrated in Diagram 1.

MRC-OX21-Sepharose affinity column. The MRC-OX21 mAb removes intact fl and some heavy chain fragments. As shown in Fig. 43, the mAb column bound and retained all of the intact fl, while the SP domain-containing fragment (d on Fig. 43A) was not bound on the column. A residual heavy-chain fragment of ~40 kDa (c_1 on Fig. 43A), also did not bind to the column. Upon reduction, c_1 was seen to break down to smaller fragments (Fig. 43B).

4.3.5 The MRC-OX21 epitope

From the data available (Table 14) and the high reproducibility of the NuPAGE Bis-Tris gel system, it was considered useful to identify the region where the MRC-OX21 mAb binds. Band 6 (Table 14 and Fig. 43B) contained 2 heavy chain fragments with N-terminal sequences of K^1VTYT and $S^{72}LECL$ of MW ~11.6 kDa. The SDS-PAGE analysis on Fig. 43B shows that from band 6, species 6_1 did not bind to the column and 6_2 did. Using the primary sequence data (Diag. 1) and the established plasmin cleavage sites, the information collected about the preferred cleavage sites, it was investigated which part of the heavy chain was likely to contain the epitope. A non-glycosylated fragment of ~11.6 kDa beginning from K^1VTYT would contain ~105 amino acids (numbering as in Diag. 1). However such a product would not form as there is a cleavage at $S^{72}LECL$ and a glycosylation at N^{52} . The MW of the fragment K^1VTYT to $S^{72}LECL$ (using 110 Da and 3,500 Da as average MW for each amino acid and N-linked carbohydrate attachment) is 11.6 kDa, which is the MW of band 6. The other band 6 fragment begins at $S^{72}LECL$ and with high probability ends within $K^{152}LSDL$ (Table 14), giving a fragment of 81 amino acids with 1 glycosylation site (approximately 12 kDa). The MRC-OX21 mAb epitope must be present in one of these two fragments. Band 2 (Table 14) also contains two species of which one binds to MRC-OX21 and one does not. Since the epitope is not in the light chain, the fragment from band 2 that must bind MRC-OX21 is the one with the N-terminal $S^{72}LECL$. Band 3 consists of only a single fragment with the same starting sequence, $S^{72}LECL$, and binds to MRC-OX21 (Fig. 43B). These findings indicate that the epitope is located within $S^{72}LECL-K^{152}$, the smallest fragment (band 6_2) which binds MRC-OX21. This represents the last few residues of the FIMAC and the first half of the SRCR domain.

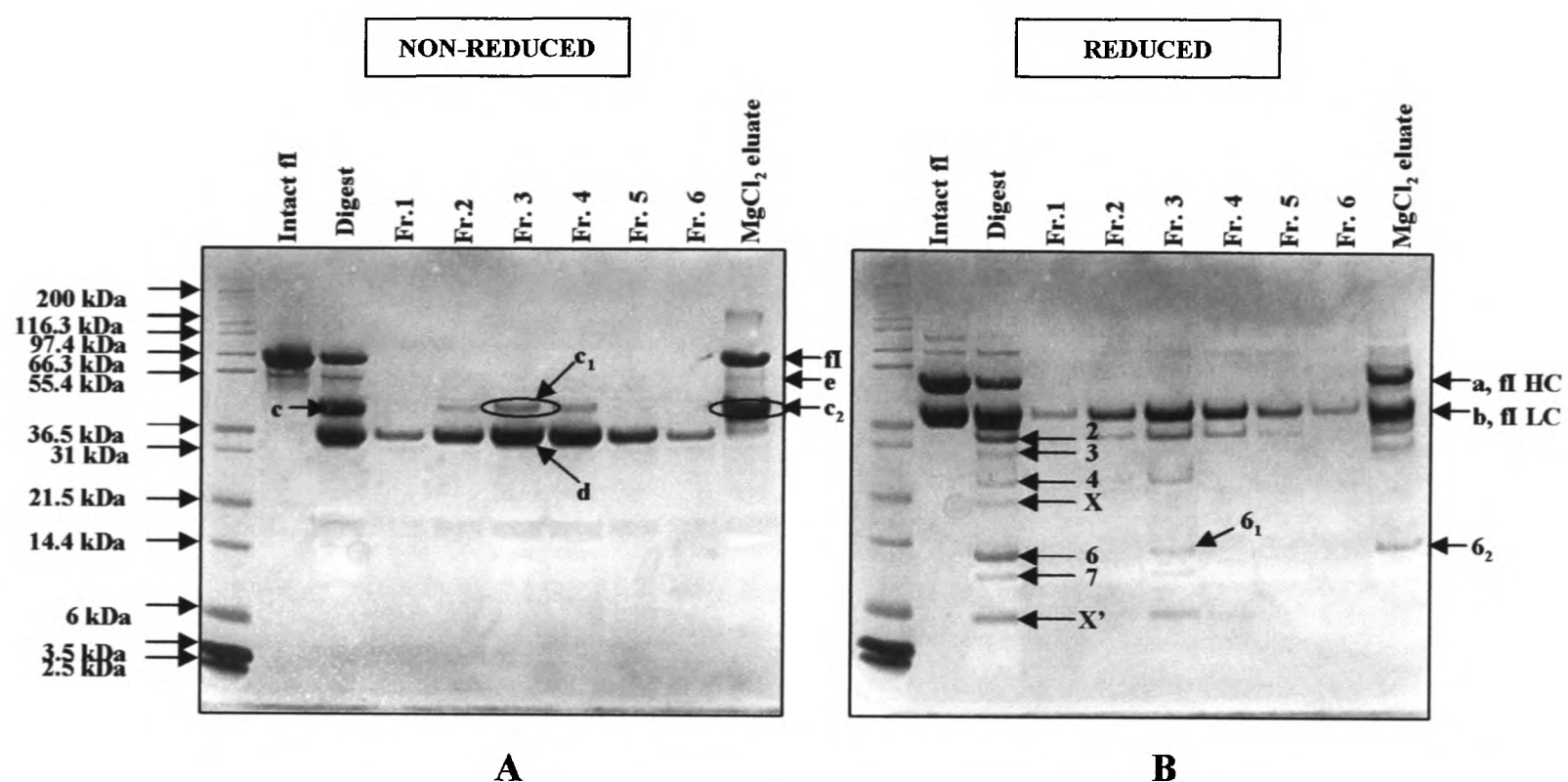


Fig. 43. Purification of the fI serine protease domain by antibody affinity chromatography. A digestion of fI with plasmin prepared under optimized conditions was processed further via affinity chromatography using an MRC-OX21 antibody-Sepharose column. The SDS-10% PAGE (Pre-cast 10% NuPAGE Bis-Tris gel system) analysis of the material obtained showed that the fractions 1-6 that contained all the unbound material, had no intact fI, while the MgCl₂ eluate appeared to contain all the uncleaved fI and heavy chain fragments that carry the mAb recognition epitope. N-terminal sequencing revealed that the species in B, indicated by the arrows (2, 3, 4, X, 6, 7 and X' – same numbering as in Fig. 42 and Table 14), are fragments originating from the fI heavy chain.

In conclusion, proteolytic fragmentation of fI by plasmin generated a range of fragments. Affinity chromatography was used to purify species “d” (Fig. 41 and 43), which contains the SP domain disulphide linked to a small heavy chain peptide. Product “d” however is heterogeneous since it contains partially cleaved SP domain forms. The presence of Ile at the N-terminus of SP domains is generally considered essential for the formation of a salt bridge with D¹⁹⁴ (chymotrypsin numbering) (D⁵⁰⁶ in Diag. 1) in trypsin and chymotrypsin (Freer et al., 1970; Khan and James, 1998; Kossiakoff et al., 1977; Stroud et al., 1977) as discussed in section 1.2.6.1. In the zymogens of trypsin-like proteases, the oxyanion hole (the amides of G¹⁹³ and S¹⁹⁵, (G⁵⁰⁵ and S⁵⁰⁷ in Diag. 1)) and about 50% of the substrate binding pockets are not in the catalytically competent conformation seen in the activated enzyme (Reiling et al., 2003). In contrast, the catalytic triad is. Upon zymogen activation, the establishment of a conserved and buried salt bridge between the newly formed N terminus (I-I/V-G-G) and D¹⁹⁴, contributes to the refolding of the molecule into the conformation desired for catalysis. This information is derived mainly from crystallographic structures, and can be assumed to be the case for fI, since the fI SP domain is homologous to and shares key functional conserved residues with the trypsin and chymotrypsin family (Perkins and Smith, 1993). However it is not known whether either of the detected cleavages at the SP domain causes dissociation of the peptide from the N-terminus of the SP domain, so the N-terminal Ile may still be present, and linked to D⁵⁰⁶ (Diag. 1), even in the cleaved forms. Thus the cleaved forms of the fI SP domain may or may not be active.

4.4 DISCUSSION

The work discussed in this chapter describes in detail the strategy of generating the fl SP domain from the native enzyme for further characterisation of its catalytic properties. The SP domain of fl was purified after limited proteolysis of fl by plasmin and the final yield of the reaction was high. From 1,000 µg of digested fl, approximately 430 µg of product “d” (Fig. 41A, Table 13) was obtained after affinity chromatography. This corresponds to > 90% recovery of material containing light chain disulphide-bridged to a small (no more than 20 residues) heavy chain fragment. However, only 35-40% of the light chain in this material was found to be intact: 60-65% was partially cleaved near the N-terminus (Table 13). Therefore the yield of material with intact SP domain was $(0.35-0.45) \times 90\% = 32-41\%$. The protein sequencing required to characterise the SP domain also enabled the location of the interchain disulphide bond formed between C³⁰⁹ and C⁴³⁵ and the MRC-OX21 binding site within the region S⁷²LECL-K¹⁵². The disulphide bond C³⁰⁹-C⁴³⁵ was originally predicted through solution neutron and X-ray scattering and homology modelling studies (Chamberlain et al., 1998) and its experimental characterisation was fundamental for confirming the V-shaped compact triangular molecular arrangement of fl. In the particular V-shaped architecture, the C¹⁵-C²³⁷ and C³⁰⁹-C⁴³⁵ pairs form inter-domain disulphide bridges which act as a scaffold for the arrangement of the domains (Fig. 17). The characterisation of the amidolytic and proteolytic properties of the purified SP domain are discussed in detail in the following chapter.

Chapter Five

CHAPTER 5: CHARACTERISATION OF THE CATALYTIC PROPERTIES OF THE ISOLATED HUMAN COMPLEMENT FACTOR I SERINE PROTEASE DOMAIN

5.1 INTRODUCTION

The previous chapter described in detail the generation of the SP domain from native human fI by proteolysis and its purification by antibody affinity chromatography. Towards the structural and functional characterisation of the fI SP domain, this chapter describes the experimental work for the characterisation of both the proteolytic and amidolytic catalytic properties of the domain.

5.2 MATERIALS AND METHODS

5.2.1 Characterisation of functional activity of human factor I and purified fI SP domain

A. The proteolytic assay: Cleavage of ^{125}I -C3(NH₃) by factor I or the SP domain

This assay was done as described in section 3.2.1 with minor modifications. Approximately 35,000 dpm of ^{125}I -C3(NH₃) (15 ng) was mixed with 1.25 µg of fH and 0.47 µg of SP domain or 1.08 µg of fI in a final volume of 100 µl of 10mM potassium phosphate, 0.5mM EDTA, 0.1 % Tween 20, 1.25 µM SBTI, pH 7.2. Factor I and the SP domain were added at a final concentration of 0.12 µM. Molarity was calculated on the assumption that all the material in the preparations is functional and on the observed MW of 88 kDa for fI and 38 kDa for the SP domain on the SDS-PAGE. All mixtures were incubated at 37 °C for variable time-periods of up to 480 minutes. Prior to analysis, reactions were stopped by the addition of 25 µl of SDS-PAGE sample buffer with DTT (for reduced samples only). Analysis was carried out by SDS-8.5% PAGE and autoradiography as described in section 3.2.1 and Fig. 27.

In some experiments, unlabelled C3(NH₃) was used instead and adjustments were made to compensate for the observation that the SP domain preparation contained some partially cleaved material. Here 10 µg of C3(NH₃) (31 µl) were mixed with 0.36 µg of fI (3.15 µl) or 0.47 µg of SP domain (10 µl) - to compare on an equimolar basis the intact molecules – in the presence or absence of 1.246 µg of fH (10 µl) in 80 µl of final reaction volume made 1.56 µM with SBTI. It was assumed for these analyses that the SP fraction contained ~1/3 SP domain with an intact N-terminus. For pattern comparison of the C3(NH₃) cleavage by the isolated SP domain or fI, the reactions were analysed by SDS-PAGE reduced and non-reduced.

Various compounds were tested as inhibitors in the proteolytic assay. As described above, fI or the SP domain were pre-incubated with the test compound at 37 °C for 60 minutes prior to the addition of fH. Incubation was continued for 60 minutes at 37 °C, then ¹²⁵I-C3(NH₃) (17,500 dpm) was added, and incubation continued for further 480 minutes. Final reaction volume was 100 µl. Samples were analysed by SDS-PAGE and autoradiography as described above.

B. The amidolytic assay: Cleavage of the synthetic substrate FGR-AMC by factor I or the SP domain

The amidolytic activities of fI and the SP domain were compared with FGR-AMC. This substrate is cleaved by fI in the absence of cofactors (section 3.3.1 and (Tsiftoglou and Sim, 2004)). Measurements were carried out in pairs of equimolar concentrations for comparison. For each reaction the substrate, 100 µM in 100 µl of 20 mM HEPES, pH 8.5 was added to 100 µl of fI or SP of variable concentration in the same buffer in white Microfluor plate wells. The amidolytic activity was measured using a microtitre plate reader (Fluoroskan, Thermo Life Sciences Ltd., Basingstoke, UK) by excitation at 355 nm and continuous monitoring of emission at 460 nm for 2 hours at 37 °C.

5.2.2 Examination of the physical effect of suramin on human factor I

Following the strong inhibitory effects of suramin (Fig. 39) on both the proteolytic and amidolytic activities of human fI (section 3.3.4), it was decided to examine the effects of the compound on the enzyme in more detail; following earlier reports that suramin causes the aggregation of other proteins like human aFGF, bFGF, PDGF (Middaugh et al., 1992) and IL-4 (Leland et al., 1995), it was investigated whether suramin causes fI to aggregate or oligomerise. In the first series of experiments, to examine whether suramin causes fI to aggregate, aliquots of 500 μ l in 1.5 ml Eppendorf-type tubes each containing 350,000 dpm of 125 I-fI in PBS, 0.5 mM EDTA, pH 7.4, 0.1% Emulphogene BC720, were incubated for 1 hr at 37 ° C alone (control) or with suramin at final concentrations of 0.5 and 1 mM. Upon completion of the incubation, the aliquots were subjected to centrifugation (8,000 g, 5 min, RT) and the recovered supernatant was transferred with care to a new tube; the total radioactivity of both the supernatant and the original tube were separately measured in triplicate for all samples. The derived average values from the two types of measurement for each sample were compared; for a suramin treated sample, any reduction in the total radioactivity of the supernatant with a parallel increase in the total radioactivity of the empty tube, would show that suramin causes fI to aggregate and eventually precipitate. All the tubes containing the recovered supernatant were further incubated for 16hr at 4 ° C. Similarly, measurements as before were then taken for all samples. The second incubation was carried out to examine the possibility whether suramin causes fI to aggregate slowly.

In the second series of experiments, to examine whether suramin cross-links fI causing it to oligomerise, but not to precipitate, suramin-treated samples of radioiodinated fH, fI, recombinant MASP-2-CCP1-CCP2-SP (Ambrus et al., 2003) and non-labelled human serum were subjected to size exclusion chromatography. Apart from fI, the rest of the samples were used as markers of known molecular weight and pattern of elution. Aliquots of 500 μ l in 1.5 ml Eppendorf-type tubes each containing 350,000 dpm of 125 I-fI or 125 I-fH or 125 I-MASP-2-CCP1-CCP2-SP or 200 μ l of human serum in PBS, 0.5 mM EDTA, pH 7.4, 0.1% Emulphogene BC720, 1 mM suramin (reaction buffer), were incubated for 1 hr at 37 ° C; upon completion of the incubation, all samples were subjected to centrifugation (8,000 g, 5 min, RT) and measurements of the total radioactivity followed as above. The recovered supernatant of each sample was loaded and analysed separately in a Superose 12 HR (10/30, 10

mm diam x 30 cm) column pre-equilibrated with the reaction buffer containing 1 mM suramin (PBS, 0.5 mM EDTA, pH 7.4, 0.1% Emulphogene BC720, 1 mM suramin). The same buffer was also used as running buffer. One ml fractions were collected for all runs. Upon completion of each run, the radioactivity of each fraction was measured separately in triplicate and the derived mean values were used to plot the elution profile for each run. The profiles from the analysis of the samples containing ^{125}I -fI, ^{125}I -fH and ^{125}I -MASP-2-CCP1-CCP2-SP were plotted together in the same chart. The sample that contained human serum was run last and selected fractions were analysed under non-reduced and reduced conditions by SDS-PAGE (NuPAGE 4-12% gradient gel system with MES running buffer); each sample contained 7 μl from every selected fraction, while the bands were detected using Coomassie Blue staining.

5.3 RESULTS

5.3.1 Functional activity of the purified human factor I SP domain

A. The proteolytic assay: Cleavage of ^{125}I -C3(NH₃) by factor I SP domain

The enzymic properties of the purified SP domain were tested in the proteolytic assay, using C3(NH₃) or ^{125}I -C3(NH₃) as a substrate in the presence or absence of factor H. Native fI or the SP domain with or without fH were incubated with ^{125}I -C3(NH₃) for up to 480 minutes (8 hours) at 37 °C, in the presence of SBTI as described in the Materials and Methods section. Results are shown in Fig. 44. The molar ratios of the reaction components used were fH:SP=0.65, fH:C3(NH₃)=100.00 and SP:C3(NH₃)=155.00. In the absence of fI, ^{125}I -C3(NH₃) is stable in the presence or absence of fH during this incubation period. When ^{125}I -C3(NH₃) is incubated with fI, but no fH, there is very slight cleavage, visible at 480 minutes of incubation (Figure 44C). In the presence of fH, fI cleaves ^{125}I -C3(NH₃) at a high rate (at least 300X faster than in the absence of fH) resulting in nearly complete cleavage of the α chain within 15 minutes (Fig. 44D). The cleavage gives the well-established pattern of products (Harrison and Lachmann, 1980; Sim et al., 1981). Even after 480 minutes, there is some residual uncleaved α chain. This is probably due to disulphide rearrangement in the C3(NH₃) which leads to a form of C3(NH₃) that is not cleaved by fI (Seya and Nagasawa, 1988).

Surprisingly, the results obtained for the SP domain differ qualitatively from those obtained with intact fI. In the presence of fH, (Fig. 44F) the cleavage of ^{125}I -C3(NH₃) by SP proceeds at a very low rate with barely detectable cleavage of the α chain after 480 minutes of incubation. This is at least 300-fold lower activity than intact fI in the presence of fH. Remarkably, however, in the absence of fH, the SP domain cleaves ^{125}I -C3(NH₃) quite rapidly yielding initially a similar ~43 kDa product, but with further cleavage to an end-product about 5-8 kDa smaller (Fig. 44E). The rate of cleavage is still about 16-fold slower than the cleavage by intact fI in the presence of fH (Fig. 44D). The controls (Figs. 44A, B and C) showed the expected response. Since there could be traces of plasmin in the SP domain material, controls of the

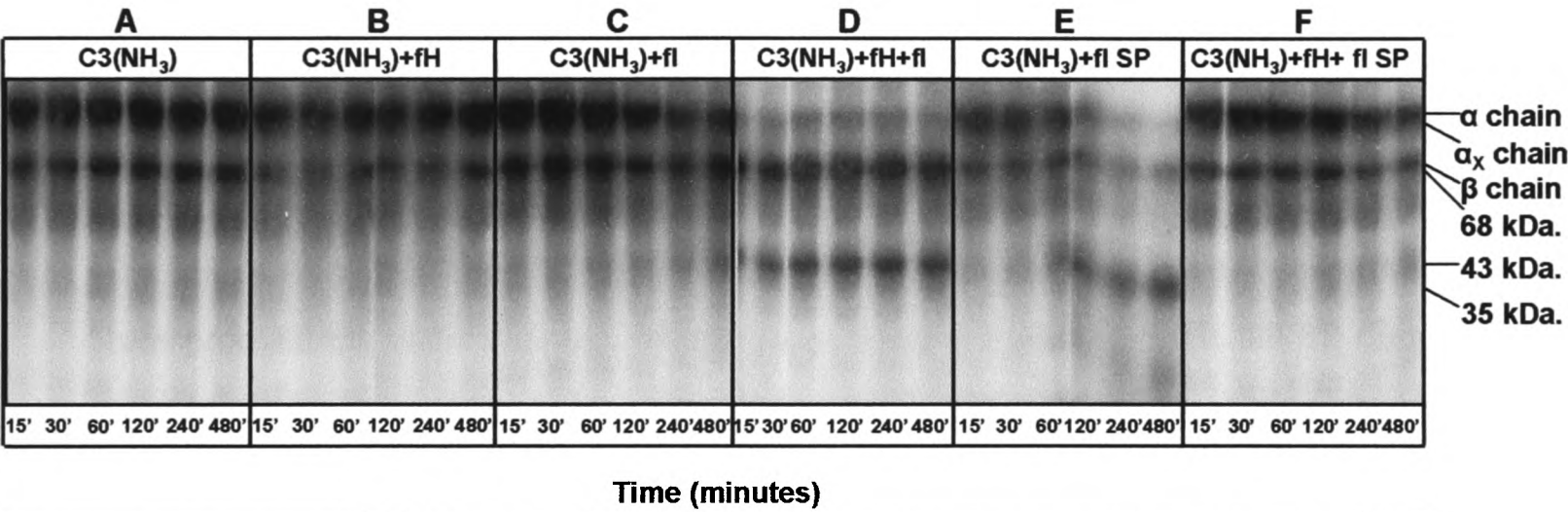


Fig. 44. Activities of factor I or the SP domain in the proteolytic assay using ¹²⁵I-C3(NH₃). The autoradiograph shows the cleavage of ¹²⁵I-C3(NH₃) by fI or the fI SP domain in the absence or presence of fH. The procedure and C3(NH₃) fragmentation pattern are as described in the Materials and Methods section.

cleavage of C3(NH₃) by plasmin were carried out. In the presence of SBTI (which is present in all of these assays), no cleavage of C3(NH₃) by plasmin was observed (data not shown). The major feature of Fig. 44 is that, while the action of intact fI is barely measurable without fH, the activity of the SP domain is in contrast much greater in the absence of fH than in its presence.

The fragmentation pattern of C3(NH₃) by the SP domain was investigated more thoroughly and the results are shown in Fig. 45. The molar ratios of the reaction components used in this experiment were fH:SP=0.65, fH:C3(NH₃)=0.15 and SP:C3(NH₃)=0.23. In Fig. 45A, B, cleavage of ¹²⁵I-C3(NH₃) by the SP domain in the absence of fH is shown. Fig. 45B shows a pattern of cleavage similar to Fig. 44E. The α chain of ¹²⁵I-C3(NH₃) is cleaved progressively, with appearance of a product at approximately 43 kDa, which is trimmed to 35 kDa. This is distinct from the pattern of C3(NH₃) cleavage by intact fI in the presence of fH (Fig. 45D or Fig. 45F). In addition, it appeared that there was cleavage of α chain to an α' – like product. Examination of non-reduced samples (Fig. 45A) confirmed that the SP domain does not produce the same cleavage pattern as is observed with fI + fH: the SP domain cleaves ¹²⁵I-C3(NH₃) to a C3c – like product (145 kDa) (Fig. 45A), while fI + fH do not (Fig. 45E). The SP domain also appears to produce a general degradation of ¹²⁵I-C3(NH₃), as the total material in high molecular weight bands diminishes (Fig. 45A, B) with accumulation of low molecular weight fragments. This suggests the SP domain cleaves many peptide bonds in ¹²⁵I-C3(NH₃).

The early stages of cleavage of C3(NH₃) by the SP domain were examined using unlabelled C3(NH₃) (Fig. 45C-F) with a substrate concentration 670 times greater than in Fig. 45A, B. In panels C and D, cleavage without fH is shown. fI produces no cleavage of C3(NH₃) over an 18 hour period (Fig. 45C, D). SP domain however, cleaves the α chain of C3(NH₃) (Fig. 45D) producing a fragment of similar molecular weight to C3 α' chain (108 kDa). Since we did not obtain sequence data to prove this is α' , it is designated α_x . Analysis of non-reduced samples (Fig. 45C) shows that the SP domain produces slight fragmentation of C3(NH₃) to a fragment of ~ 145 kDa.

In the presence of fH (Fig. 45E, F), fI as expected rapidly (5 minutes) cleaves C3(NH₃) to iC3(NH₃) with no further cleavages up to 18 hours. iC3(NH₃) comigrates with

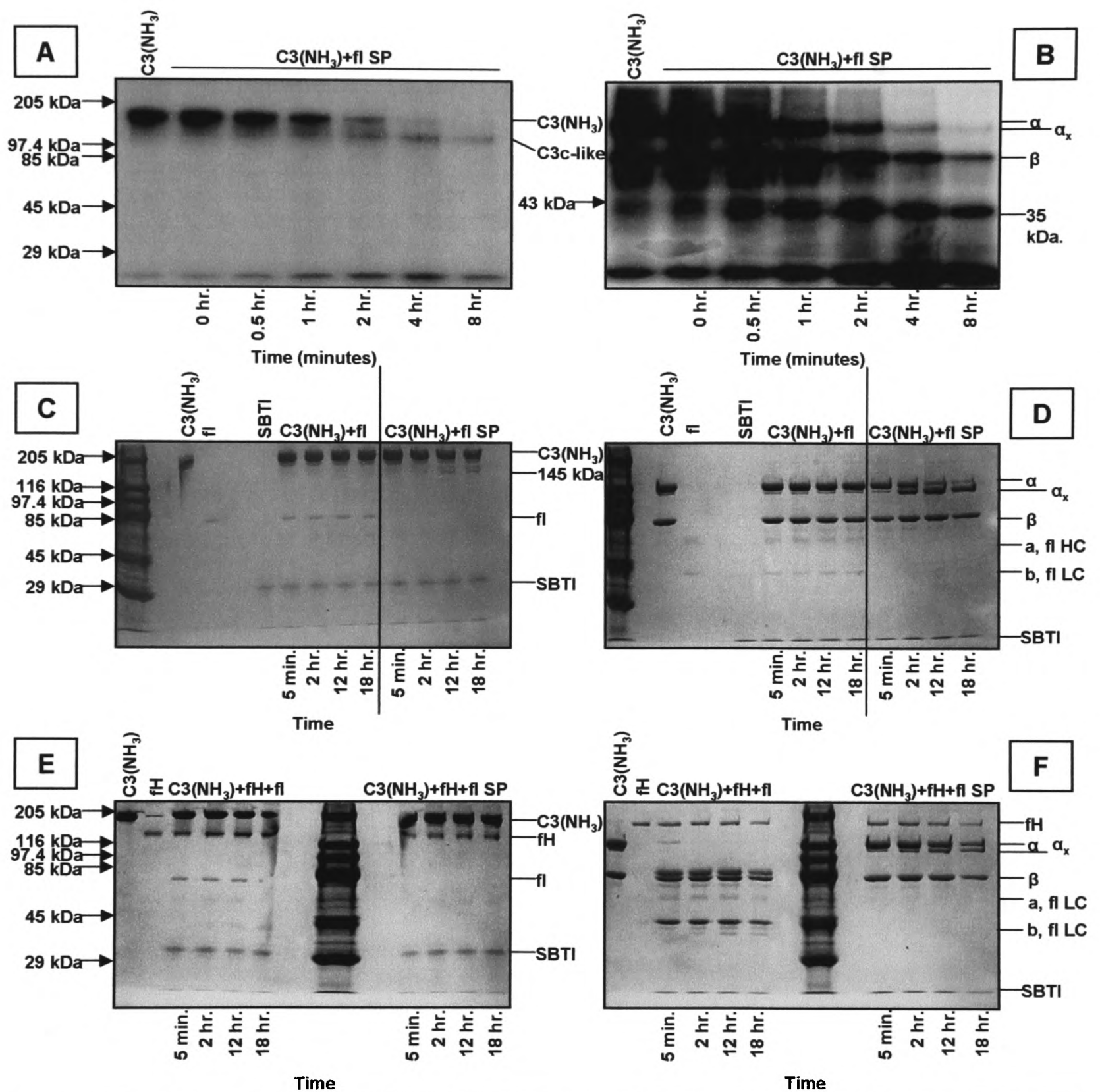


Fig. 45. Fragmentation pattern of ^{125}I -C3(NH₃) or C3(NH₃) by fl or fl SP domain.

A, B. Digestion of ^{125}I -C3(NH₃) by fl SP domain. Conditions are as described in the Materials and Methods section except that the SP domain concentration was 2-fold lower than in Fig. 44. The reaction mixtures that were generated were analysed by SDS-PAGE and autoradiography both under reduced (A) and non-reduced (B) conditions. Under non-reduced conditions uncleaved C3(NH₃) runs as a single species of 185 kDa.

C, D, E, F, Digestion of unlabelled C3(NH₃). C3(NH₃) was digested with fl or the SP domain in the absence (C, D) or presence (E, F) of fH as described in the Materials and Methods section. Coomassie Blue stained SDS-PAGE gels are shown: C, D non-reduced; D, F reduced.

C3(NH₃) without reduction (Fig. 45E) and on reduction runs as β chain plus 68 kDa and 43 kDa α chain fragments. The SP domain however does not produce iC3(NH₃) (Fig. 45F): instead it produces the same cleavage to a_x fragments as are produced without fH (Fig. 45D, F). Cleavage to a C3c-like fragment is obscured by fH on Fig. 45E. As seen in Fig. 44, the presence of fH slows the rate of cleavage by the SP domain. In Fig. 44, where [fH] was 100 – fold greater than [C3(NH₃)], the cleavage by the SP domain is almost completely stopped. In Fig. 45F, where fH is present at much lower concentration than C3(NH₃) (0.15 : 1.0 molar ratio) cleavage does occur, but is 2-3 fold slower than in the absence of fH (Figs. 45D and 45F).

These results show that the SP domain cleaves C3(NH₃) relatively slowly at multiple sites (overall loss of high molecular weight material in Fig. 45A, B), and that fH protects C3(NH₃) from this cleavage. fI, in contrast does not cleave C3(NH₃): this may be because fI interacts via its heavy chain with C3(NH₃) in a specific orientation which predominates over random interaction. fH allows fI to cleave C3(NH₃) rapidly at only two sites to produce the stable iC3b. fH binding to C3(NH₃) may be required to expose these sites to fI in the ternary fI – fH - C3(NH₃) complex.

It seems that the conversion of C3(NH₃) to iC3(NH₃) is a highly specific process in which the heavy chain of fI must play an important role by primarily orientating the catalytic domain to the R¹²⁸¹ - S¹²⁸² and R²⁹⁸ - S¹²⁹⁹ cleavage sites in C3(NH₃). In the absence of the fI heavy chain, prolonged incubation results in the cleavage of bonds that are not cleaved by intact fI.

B. The amidolytic assay: Cleavage of FGR-AMC by factor I SP domain

The SP domain and native fI were compared for their amidolytic activity with the substrate FGR-AMC (Fig. 46). Their activities are similar. If the cleaved forms of the SP domain present in the SP preparation are inactive, it would be expected that the SP domain material would be only 40 % as active as fI on a molar basis. In fact the SP domain activity is only 15-20 % lower than fI. It can be concluded that the Kazal domain in the heavy chain does not inhibit catalytic activity, as absence of the heavy chain does not increase activity.

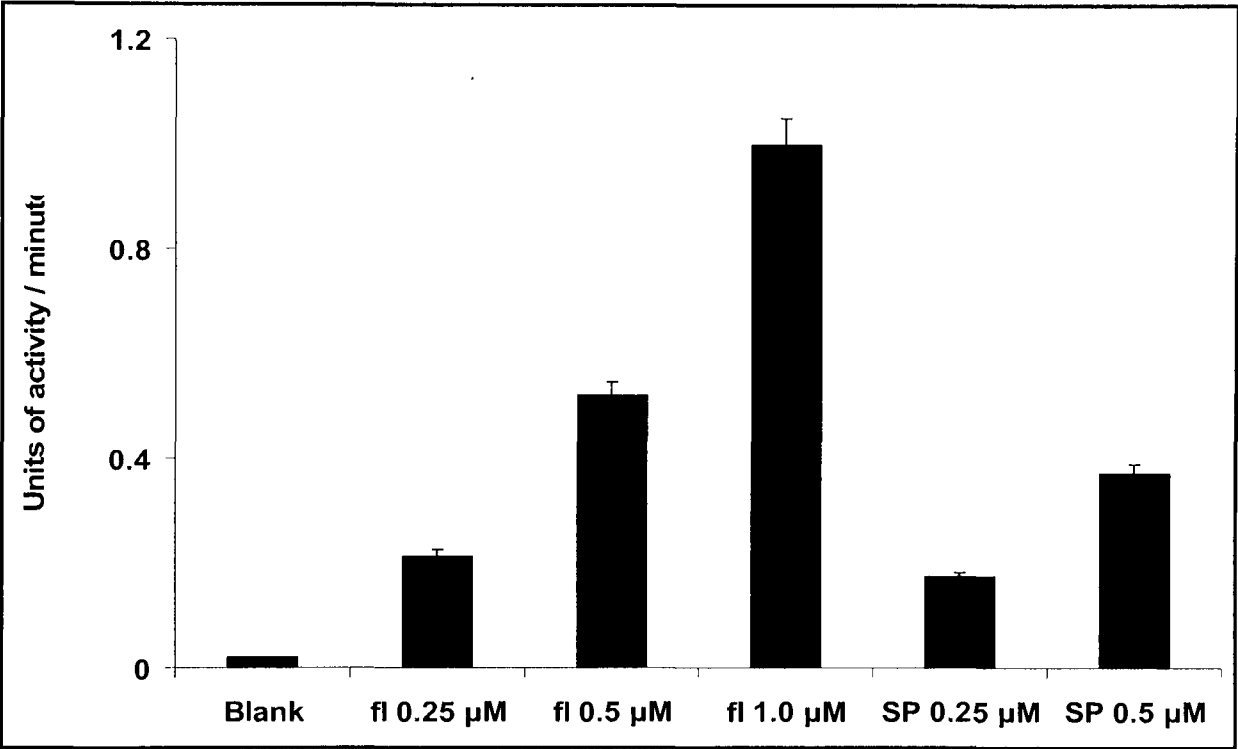


Fig. 46. Comparison of activity of fl and the SP domain on the synthetic substrate FGR-AMC. The amidolytic assay was performed as in the Materials and Methods section. For every reaction the substrate, 0.1 mM in 100 µl 0.1 mM of 20 mM HEPES, pH 8.5 was added to 100 µl of fl or SP solution in the same buffer in white Microfluor white plate wells and incubated for 2 hrs at 37 ° C.

5.3.2 Effect of inhibitors on factor I SP domain proteolytic activity

The effect of compounds previously tested on the fI proteolytic activity (section 3.3.4), were also tested on the purified fI SP domain. The compounds were used at a single concentration and the level of inhibition was estimated from end point-reactions. The results are shown in Table 15. From the compounds examined only Pefabloc SC was found to inhibit strongly the proteolytic activity of the SP domain. This is in agreement with the result from testing on fI. Suramin, however, which strongly inhibits fI, did not inhibit the SP domain. Antipain, Aprotinin and Leupeptin were all found to cause moderate inhibition of both fI and the SP domain. As expected Hirudin, ϵ ACA and 1, 10 Phenanthroline were all found not to inhibit either. With the exception of Suramin, these results are in agreement with recent extensive testing of inhibitor compounds on the proteolytic activity of fI (section 3.3.4).

5.3.3 Effect of suramin on human factor I

The first series of experiments with suramin showed that the compound did not cause any aggregation of fI. As shown in Table 16, when suramin was tested at the concentrations of 0.5 or 1 mM, it did not cause any fI to precipitate, either at short (1 hr) or at long incubation (16 hr) as judged by the measurements of radioactivity of all the supernatant solutions. Although suramin did not cause any precipitation of fI at 1 mM, the concentration at which it was found to inhibit strongly the proteolytic and amidolytic activities of the enzyme (Tables 10 & 11), the possibility that it may cause fI to oligomerise was explored in the second series of experiments. The size exclusion chromatography experiments indicated that suramin does not have any effect on the monomeric state of fI in the physiological buffer conditions. This conclusion was reached by the comparison of the elution profiles of 125 I-fH (155 kDa), 125 I-MASP-2-CCP1-CCP2-SP (42 kDa) and non-labelled human serum (Fig. 47A). In the presence of 1 mM suramin, fI (88 kDa) eluted as a monomer reaching a peak at fraction 10. Factor I eluted later than fH and earlier than HSA and the rMASP-2 construct. Suramin did not have any effect on the markers used either, as all the elution profiles generated were as originally expected. Factor H, due to its elongated structure (section 1.5.1), has a MW of 220 kDa in size exclusion experiments; fH eluted as a single peak

Table 15. Summary of effects of selected compounds on the proteolytic activities of fI and the SP domain ¹ .			
Compound	Final concentration	Inhibition ²	
		fI	fI SP domain
Pefabloc SC	0.25 mM	**	**
Suramin	1 mM	**	-
Benzamidine	20 mM	-	-
Antipain	0.1 mM	*	*
Aprotinin	0.5 µM	*	*
Leupeptin	10 µM	*	*
Hirudin	5 ATU ³	-	-
εACA	20 mM	-	-
1, 10 Phenanthroline	0.1 mM	-	-

1. Potential inhibitors were tested in the proteolytic assay as described in section 5.2.1.1. fI and the SP domain were tested in parallel and the inhibition was compared with untreated samples.

2. Classification of inhibition:
Strong inhibition [******] (60-100%)
Moderate inhibition [*****] (10-40%)
No inhibition [**-**] (0-10%)

3. Unit definition: One anti-thrombin unit (ATU) neutralizes one NIH unit of thrombin (fibrinogen assay) at 37 °C, 1 NIH unit of thrombin clots a standard fibrinogen solution in 15 sec. at 37 °C.
5 ATU are expected to neutralise 2.5 µg of thrombin.

Table 16. Examination of the physical effect of suramin on human fI¹

Sample	Total radioactivity ² (dpm)	Radioactivity (dpm) after incubation for 1 hr at 37 ° C (% total radioactivity)	Radioactivity (dpm) after incubation for 16 hr at 4 ° C (% radioactivity of supernatant A) ³
fI only	343,967	Supernatant A: 343,215 (<u>99.8%</u>) Tube: 643 (0.2%)	Supernatant B: 337,982 (98.4%) Tube: 643 (0.1%)
fI + 0.5 mM suramin	307,482	Supernatant A: 305,062 (<u>99.2%</u>) Tube: 974 (0.3%)	Supernatant B: 297,465 (97.5%) Tube: 357 (0.1%)
fI + 1 mM suramin	352,737	Supernatant A: 348,289 (<u>98.7%</u>) Tube: 1394 (0.4%)	Supernatant B: 343,589 (98.7%) Tube: 399 (0.1%)

1. All measurements were carried out in triplicate and the derived mean values were used for all calculations.
2. Total radioactivity for each sample prior to the first incubation.
3. The radioactivity of Supernatant A was the total radioactivity for each sample prior to the second incubation.

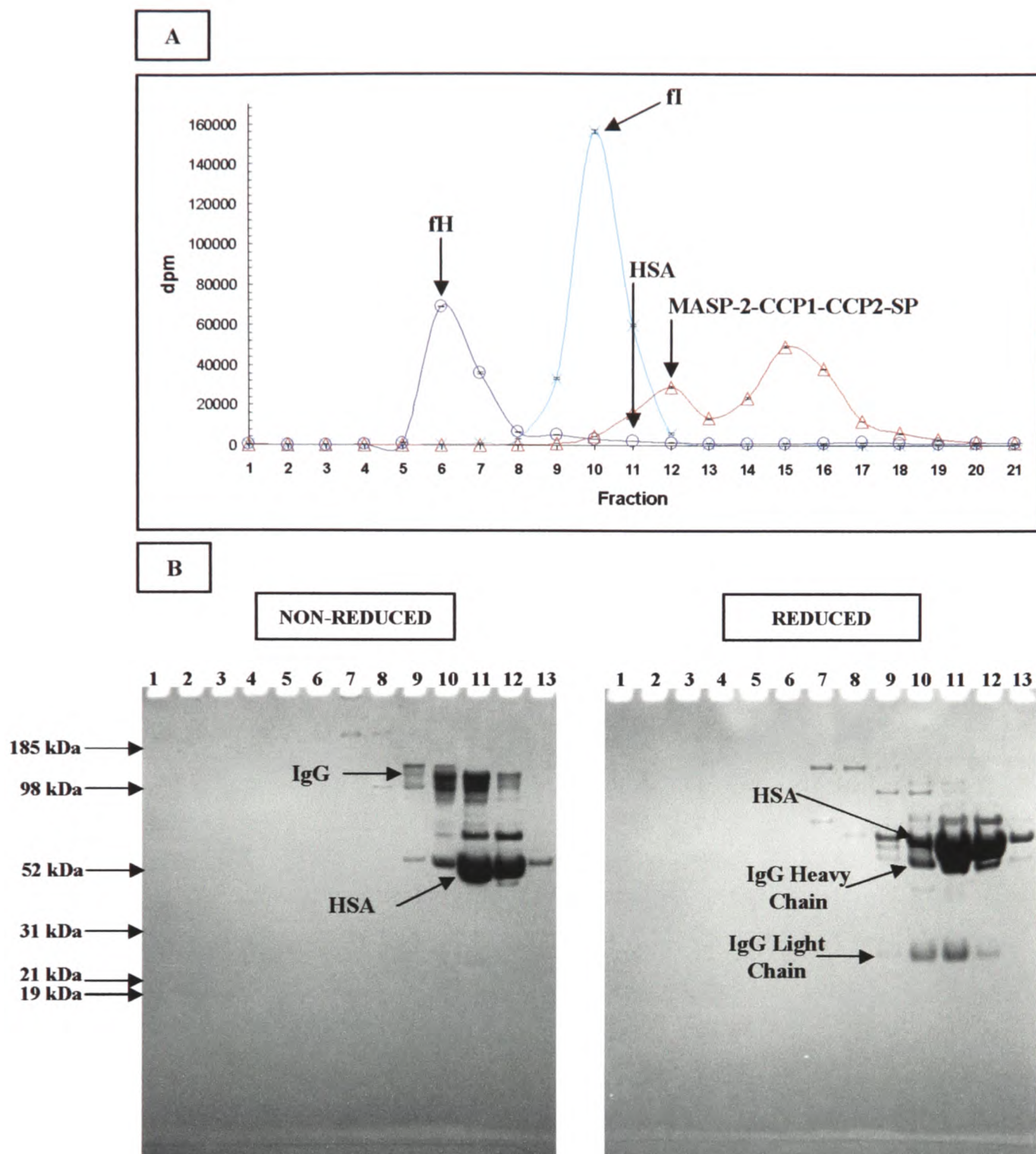


Fig. 47A. Size exclusion chromatography for the examination of the effect of suramin on the physical state of human fI. The effect of suramin on fI was assessed by analysing suramin treated samples of fI, fH, recombinant MASP-2-CCP1-CCP2-SP and human serum by size exclusion chromatography. Each component was examined separately under identical conditions. Aliquots of 500 μ l each containing 350,000 dpm of 125 I-fI or 125 I-fH or 125 I-MASP-2-CCP1-CCP2-SP or 200 μ l of human serum in PBS, 0.5 mM EDTA, pH 7.4, 0.1% Emulphogene BC720, 1 mM suramin, were incubated for 1 hr at 37 ° C, then centrifuged and the recovered supernatants were loaded and analysed separately in a Superose 12 HR (10/30) column pre-equilibrated with the same buffer. One ml fractions were collected. Upon completion of each run, the radioactivity of each fraction was measured in triplicate and the derived mean values were used to plot the elution profile for each run. The elution profiles obtained from the analysis of the samples containing 125 I-fI, 125 I-fH and 125 I-MASP-2-CCP1-CCP2-SP were plotted in the same chart for comparisons. Similarly, the position of the elution peak of HSA, as determined by SDS-PAGE analysis below, is also indicated.

47B. SDS-PAGE analysis of selected human serum fractions obtained by size exclusion chromatography (see Fig. 47A for HSA). A 500 μ l sample containing 200 μ l of human serum in PBS, 0.5 mM EDTA, pH 7.4, 0.1% Emulphogene BC720, 1 mM suramin was analysed by size exclusion chromatography using the same experimental conditions as above. Seven μ l from each selected fraction were analysed under non-reduced and reduced conditions. HSA was found to reach an elution peak at fraction 11.

in fraction 6, while albumin (69 kDa) that is the most abundant protein of serum, reached an elution peak in fraction 11 as judged by the SDS-PAGE analysis (Fig. 47A, B). The elution profile of ^{125}I -MASP-2-CCP1-CCP2-SP was characterised by two main peaks, the major occurring at fraction 12 and the second at fraction 15 corresponding to contaminants and/or some degradation products.

5.4 DISCUSSION

The work described in this chapter focuses on the enzymic properties of the SP domain of fI. This was studied to elucidate the roles of the fI heavy chain in restricting catalytic activity or substrate specificity. The SP domain material was found to have amidolytic activity against the synthetic substrate FGR-AMC. This activity was similar in magnitude to that of intact fI (Fig. 46) so the SP domain and the intact enzyme have similar affinity and catalytic activity for the synthetic substrate. In contrast, the proteolytic activities of the intact fI and the SP domain were strikingly different. Intact fI showed (almost) no cleavage of C3(NH₃) if fH was absent (Fig. 44C and Fig. 45C, D). When fH was present, cleavage was rapid and specific, generating iC3(NH₃) (cleavage of C3(NH₃) at two sites (Davis and Harrison, 1982; Sahu and Lambris, 2001; Sim et al., 1981)) (Fig. 44D and 45E, F). The SP domain however can cleave C3(NH₃) in the absence of fH (Fig. 44E and Fig. 45C, D). Cleavage was slow and less specific in that more sites were cleaved than with intact fI plus fH. Further, cleavage was inhibited, not stimulated by fH (Fig. 44F and Fig. 45E, F). The inhibitors Pefabloc SC, antipain, aprotinin and leupeptin all act similarly on the proteolytic activity of intact fI or the SP domain (Table 15). Suramin however, does not inhibit the SP domain although it is a potent inhibitor of fI. The observed lack of inhibition of the SP domain may indicate that Suramin binding involves both chains of fI. Suramin has been reported to bind to proteins containing LDLRa domains (Vassiliou, 1997; Vassiliou et al., 2001), but also inhibits simple proteases, like trypsin, with only SP domains (Cadene et al., 1997; Cutting, 1964). Suramin may therefore bridge between the chains of fI, possibly with higher affinity binding to the heavy chain. Suramin has been reported to cause oligomerisation of complement components C8 and C9 thus blocking Membrane Attack Complex formation and preventing complement-mediated haemolysis (Saez et al., 1999). Suramin that has been reported to cause aggregation of proteins like human aFGF, bFGF, PDGF (Middaugh et al., 1992) and human IL-4 (Leland et al., 1995), was found not to cause any aggregation of fI. Using analytical size exclusion chromatography we concluded that fI remains monomeric in the presence of 1 mM Suramin (Fig. 47, Table 16).

The contrast in the proteolytic activities of intact fI and the SP domain confirms that the heavy chain and cofactor have a role in contact with the substrates. The SP domain cleaves C3(NH₃) at several sites, so it is less “specific” than intact fI + cofactor. This strongly suggests that the fI heavy chain binds to substrate and orients the SP domain of intact fI towards the two cleavage sites in C3b which are cleaved to form iC3b.

Intact fI alone does not cleave C3b at all, while direct binding between them has been demonstrated (Soames and Sim, 1997). The interaction involves both chains of C3(NH₃), presumably indicating two or more sites of interaction. At least one binding interaction in the fI heavy chain may orient fI to the two specific cleavage sites. However, in the absence of fH, no cleavage occurs indicating that fH may be required to alter substrate conformation such that these two sites are exposed.

To understand in more detail how fH affects the C3b-fH-fI system, whether it binds to the SP domain/light chain of fI preventing it from cleaving C3b non-specifically, or whether it binds only to C3b hiding the cleavage sites that the isolated SP domain cleaves, the molar ratios of fH:SP, fH:C3(NH₃) and SP:C3(NH₃) from the experiments of Figs. 44 and 45 were compared (section 5.3.1). In both experiments, the ratio fH:SP is the same (0.65); in Fig. 45 however, the ratios of fH:C3(NH₃) and SP:C3(NH₃) are 667x and 674x lower than in Fig. 44. The relative ratio [SP:C3(NH₃):[fH:C3(NH₃)] in both experiments is similar; for Fig. 44 it is 1.55 and for Fig. 45 it is 1.53. These ratio values in parallel with the results presented earlier, suggest that the ratio fH:C3(NH₃) is important for determining the rate of C3(NH₃) breakdown; when the ratio fH:C3(NH₃) is higher, the inhibition of the C3(NH₃) breakdown is stronger; this supports the hypothesis that fH binds to C3b and “conceals” the sites cleaved by the isolated SP domain either by steric hindrance or through conformational change(s).

Thus, fI binds to C3b, probably via its heavy chain: this binding may be of sufficient affinity and duration to prevent the more random proteolysis seen with the SP domain. The binding of fH to both C3b and fI may enhance the binding of fI to C3b, while it may alter C3b to expose the 2 specific cleavage sites and also influence the orientation of fI. The activity of the SP domain is inhibited by fH (Fig. 44F and

Fig. 45E, F). This suggests that the conformational change in C3b induced by fH “hides” the bonds which are susceptible to cleavage by the SP domain either by steric interference or by altering C3(NH₃) conformation.

iC3b has not been reported to be generated from C3b by other proteases: several proteases however cleave C3b in the less specific pattern similar to that seen with the SP domain. Thus, the fI heavy chain contacts and the cofactor serve to restrict the specificity of fI to produce iC3b through a specialised catalysis event crucial for the innate immune response.

Chapter Six

CHAPTER 6: MOLECULAR CLONING AND EXPRESSION OF THE SERINE PROTEASE DOMAIN OF HUMAN COMPLEMENT FACTOR I AS RECOMBINANT PROTEIN-DEVELOPMENT OF AN ANTI-FI SP DOMAIN POLYCLONAL ANTISERUM

6.1 INTRODUCTION

The studies presented in chapters 3, 4 and 5 indicated that the fI SP domain has a native conformation that accommodates substrate recognition and cleavage, supporting both proteolytic and amidolytic activities. For further characterisation studies of the SP domain it was decided to clone and express a recombinant form of the particular domain. The expression of such a recombinant form was considered as an alternative procedure, compared to the proteolysis approach (Chapter 4), of obtaining yields of intact fI SP domain at high yield. The experimental approach employed was based on the earlier successful cloning and expression of recombinant truncated forms of human MASP-1 and MASP-2 in *Escherichia coli* (Ambrus et al., 2003). Recombinant forms of both of these proteins were expressed as inclusion bodies, but were further refolded at very low yields.

This chapter describes a series of experiments designed to clone and express the SP domain of human fI as recombinant protein in *Escherichia coli*. The largest part of this work was carried out in the Institute of Enzymology of the Hungarian Academy of Sciences in Budapest, Hungary under the supervision of our collaborator Dr. P Gál. The cloning and expression of a fI SP domain form was successful although the refolding of the recombinant protein from inclusion bodies was not possible. However, the produced inclusion bodies were used for the raising of an anti-fI SP domain polyclonal anti-serum. The quality of immunoreactivity of the raised anti-serum was tested by ELISA and was found positive both against surface bound native intact human fI and solubilised recombinant human fI SP domain.

6.2 MATERIALS AND METHODS

6.2.1 Materials

The full length cDNA of human complement factor I inserted in recombinant plasmid pSP64 was available in our laboratory (Catterall et al., 1987) and used as template for PCR. Plasmid pSP64 was propagated in *Escherichia coli* XL-1 Blue cells grown under the selection of ampicillin. pET-17b was purchased from **Novagen** [**Merck Biosciences Ltd.**, Beeston, Nottingham, UK] and used as the expression vector (Fig. 48). For all cloning and transformant selection applications all plasmids were propagated in *Escherichia coli* XL-1 Blue cells under the selection of ampicillin. *Escherichia coli* strains XL-1 blue by **Stratagene** [La Jolla, CA, USA] and BL-21(DE3)pLysS by **Novagen** were used as hosts for cloning and expression experiments, respectively; ready-to-use competent cells from these strains were employed for all transformations. The Pwo Master PCR kit by **Roche Diagnostics Ltd.** [Lewes, East Sussex, UK] was used for the basic DNA amplification reactions; the primers were from MWG-Biotech AG. For site-directed mutagenesis the QuickChange™ site-directed mutagenesis kit by **Stratagene** was employed. The primers for mutagenesis were from **Invitrogen** [Carlsbad, CA, USA]. Agarose was from **Sigma** [St. Louis, MO, USA]. Enzymes and reagents for restriction and ligation reactions were purchased from **New England Biolabs** [Ipswich, MA, USA]. Nutrient components for culture medium were purchased from **Oxoid Limited** [Basingstoke, Hampshire, UK]. Iso-propylthio- β -galactopyranoside (IPTG) was from **Calbiochem** [EMD Biosciences, Inc., San Diego, CA, USA].

6.2.2 Methods

6.2.2.1 Extraction of plasmid DNA from *Escherichia coli* cells

For all plasmid DNA extractions from *E. coli* cells the QIAprep Spin Miniprep kit by **QIAGEN Ltd.** [Crawley, West Sussex, UK] was used. The protocol supplied with the kit can support the purification of up to 20 μ g of highly pure plasmid DNA from 5



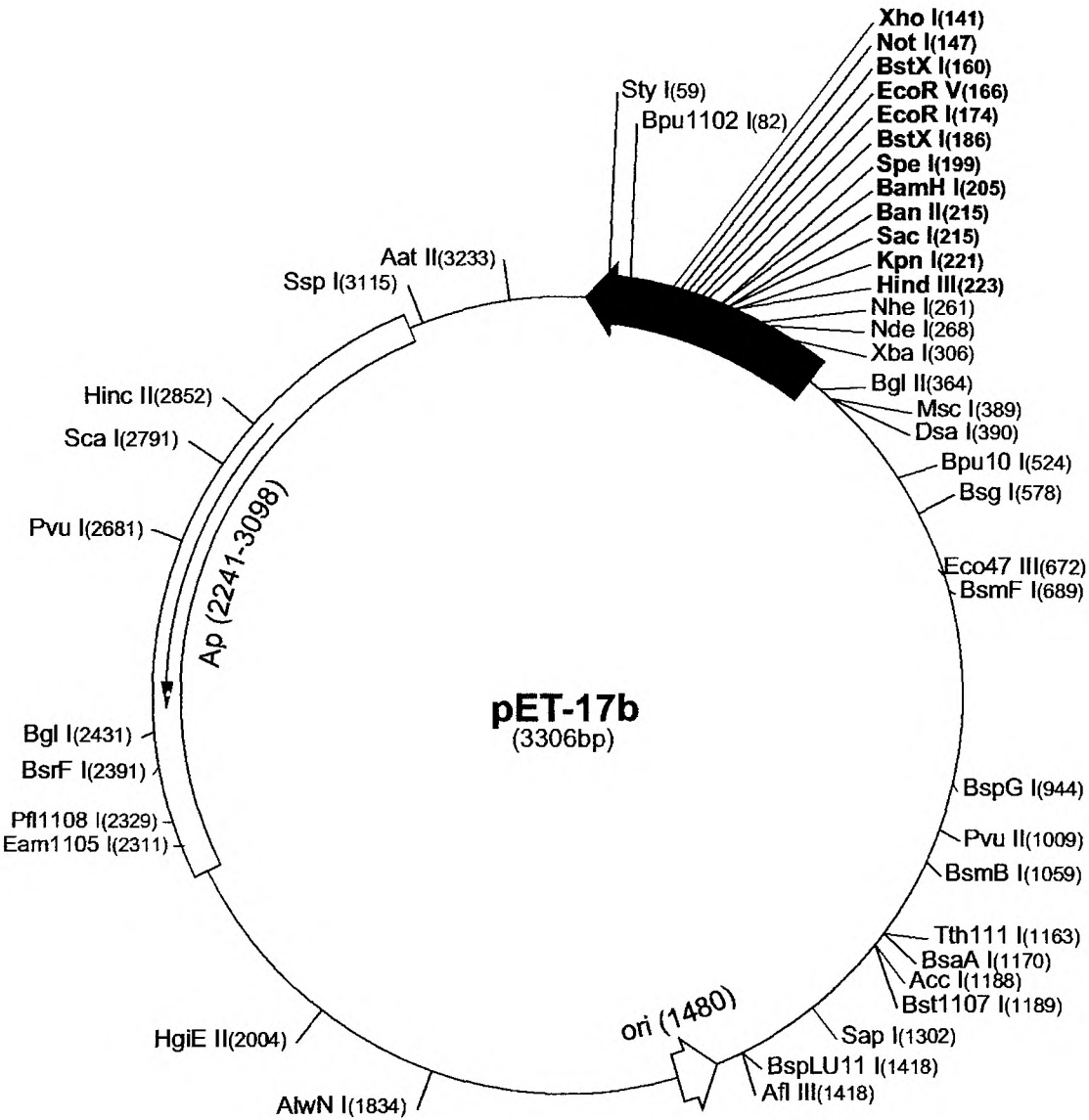
pET-17b Vector

TB047 12/98

The pET-17b vector (Cat. No. 69663-3) carries an N-terminal 11aa T7• Tag[®] sequence followed by a region of useful cloning sites. Included in the multiple cloning region are dual *Bst*X I sites, which allow efficient cloning using an asymmetric linker (1). Unique sites (except for the two *Bst*X I sites) are shown on the circle map. Note that the sequence is numbered by the pBR322 convention, so the T7 expression region is reversed on the circular map. The cloning/expression region of the coding strand transcribed by T7 RNA polymerase is shown below.

1. Sced, B. (1987) *Nature* 329, 840.

pET-17b sequence landmarks	
T7 promoter	333-349
T7 transcription start	332
T7• Tag coding sequence	237-269
Multiple cloning sites	
(<i>Hind</i> III - <i>Xho</i> I)	141-228
T7 terminator	28-74
pBR322 origin	1480
<i>bla</i> coding sequence	2241-3098



T7 promoter primer #69348-3

Bgl II

T7 promoter

Xba I

rrs

AGATCTCGATCCGCGAAATTAATACGACTCACTATAGGGAGACCACAACGGTTTCCCCTAGAAATAATTTGTTTAACTTTAAGAAGGA3A

Nde I

Nhe I

T7•Tag

Hind III

Kpn I

Sac I

BamH I

Spe I

BstX I

EcoR I

EcoR V

BstX I

Not I

Xho I

TATACATATGGCTAGCATGACTGGTGGACAGCAAATGGGTCGGGATTCAAGCTTGGTACCGCTCGGATCCACTAGTAACGGCGCCAGT3TGCTGGAATTCTGCAGATATCCATCAGACT3GCGGCCGCTCGAG

MetAlaSerMetThrGlyGlyGlnGlnMetGlyArgAspSerSerLeuValProSerSerAspProLeuValThrAlaAlaSerValLeuGluPheCysArgTyrProSerIleTrpArgProLeuGlu

Bpu1102 I

T7 terminator

CAGATCCGGCTGCTAACAAAGCCGAAAG3AAGCTGAGTTGGCTGCTGCCAC3GCTGAGCAATAACTAGCATAACCCCTTGGG3CCTCTAACCGGCTCTTGAGGGGTTTTTG

GlnIleArgLeuLeuThrLysProGluArgLysLeuSerIroLeuLeuProProLeuSerAsnAsnEnd

T7 terminator primer #69337-3

pET-17b cloning/expression region

227



pET-17b Restriction Sites

TB047 12/98

Enzyme	# Sites	Locations					
AatII	1	3233					
AccI	1	1188					
AceIII	4	927	1068	1370	2610		
Acil	44						
AflIII	1	1418					
AluI	16						
AlwI	13						
Alw21I	6	215	412	1236	1736	2897	
		2982					
Alw44I	3	1232	1732	2978			
AlwNI	1	1834					
ApoI	1	174					
AvaI	2	141	368				
Avall	5	382	424	703	2449	2671	
BamHI	1	205					
BanI	2	217	2259				
BanII	1	215					
BbsI	2	536	3289				
BbvI	20						
BccI	4	162	2348	2472	2759		
Bce83I	5	23	1509	1807	2048	2916	
Bcefl	2	179 1920					
BcgI	6	224	258	995	1029	2816	
		2850					
Bfal	8	72	200	262	307	432	
		1913	2166	2501			
BglI	1	2431					
BglII	1	364					
Bpml	2	945	2381				
Bpu10I	1	524					
Bpu1102I	1	82					
BsaI	2	331	2372				
BsaAI	1	1170					
BsaBI	3	164	363	615			
BsaHI	2	2848	3230				
BsaJI	3	59	390	1578			
BsaWI	5	4	607	1624	1771	2602	
Bsbl	2	1134	2854				
BscGI	9	46	394	779	1112	1745	
		2091	2312	2336	2858		
BsgI	1	578					
Bsil	3	1591	2975	3282			
BsiEI	6	150	194	1334	1758	2681	
		2830					
BsII	10	52	391	754	845	887	
		1067	1440	1458	1624	1903	
BsmAI	4	331	1059	2372	3148		
BsmBI	1	1059					
BsmFI	1	689					
BsoFI	31						
Bsp24I	8	231	263	1911	1943	2089	
		2121	3215	3247			
Bsp1286I	6	215	412	1236	1736	2897	
		2982					
BspEI	2	4	607				
BspGI	1	944					
BspLU11I	1	1418					
BsrI	16						
BsrBI	3	146	1351	3152			
BsrDI	2	2372	2546				
BsrFI	1	2391					
Bst1107I	1	1189					
BstXI	2	160	186				
BstYI	10	134	205	364	610	2059	
		2070	2156	2168	2936	2953	
Cac8I	12						
CjeI	14						
CjePI	14						
CviJI	52						
CviRI	14						
Ddel	10	82	103	524	686	1226	
		1693	2102	2268	2808	3234	

Enzyme	# Sites	Locations					
DpnI	19						
DraI	3	2177	2196	2888			
DrdI	2	1111	1526				
DsaI	1	390					
EaeI	4	147	191	387	2699		
EagI	2	147	191				
Eam1105I	1	2311					
EarI	2	1302	3106				
Ecil	3	1492	1638	2466			
Eco47III	1	672					
Eco57I	2	1966	2978				
EcoO109I	4	55	382	424	3287		
EcoRI	1	174					
EcoRII	4	384	1444	1565	1578		
EcoRV	1	166					
FauI	6	348	433	714	900	1121	
		1131					
FokI	8	637	699	777	963	1104	
		2277	2458	2745			
FspI	2	399	2533				
GdiII	3	147	191	2699			
HaeI	4	389	1433	1444	1896		
Haell	4	591	674	1296	1666		
HaellI	13						
Hgal	5	954	1111	1529	2107	2837	
HgiEII	1	2004					
HhaI	17						
Hin4I	3	210	2310	2384			
HincII	1	2852					
HindIII	1	223					
Hinfl	8	229	339	468	972	1318	
		1393	1789	2306			
HphI	8	463	1038	1047	2154	2381	
		2797	3003	3038			
KpnI	1	221					
Maell	8	489	513	743	1169	2121	
		2537	2910	3230			
MaellI	14						
MbolI	8	536	1289	2080	2151	2906	
		2984	3093	3289			
MmeI	2	1633	1817				
MnlI	19						
MscI	1	389					
MseI	16						
MslI	6	405	600	991	2563	2722	
		3081					
MspI	16						
MspA1I	6	86	1009	1128	1760	2005	
		2946					
Mwol	15						
NciI	7	428	756	1062	1097	1798	
		2494	2845				
NdeI	1	268					
NheI	1	261					
NlaIII	16						
NlaIV	13						
NotI	1	147					
NspI	3	763	1055	1422			
Pfi1108I	1	2329					
PleI	4	347	1312	1797	2300		
Psp5II	2	382	424				
Psp1406I	3	743	2537	2910			
PstI	2	173	2556				
PvuI	1	2681					
PvuII	1	1009					
RcaI	3	2138	3146	3251			
RsaI	3	219	1224	2791			
SacI	1	215					
SapI	1	1302					
Sau96I	10	55	382	424	703	890	
		2353	2432	2449	2671	3287	
Sau3AI	19						

Enzyme	# Sites	Locations					
Scal	1	2791					
ScrFI	11						
SfaNI	13						
Sfcl	5	169	332	1683	1874	2552	
SpeI	1	199					
SspI	1	3115					
StyI	1	59					
TaqI	4	142	361	1518	2962		3014
TaqII	5	1320	2659	2844	2997		
TfiI	4	229	468	972	1393		
ThaI	12						
TseI	20						
Tsp45I	5	857	1070	1165	2567	2778	
Tsp509I	7	174	298	348	2178	2484	
		2739	3304				
Tth111I	1	1163					
Tth111II	4	879	2008	2015	2047		
UbaII	11						
VspI	2	347	2483				
XbaI	1	306					
XhoI	1	141					
XmnI	2	976	2910				
Enzymes that do not cut pET-17b:							
AflII	AgeI	ApaI	ApaBI		AscI		
AvrII	BaeI	BclI	BmgI		BsaXI		
BseRI	BsmI	BspMI	BsrGI		BssHII		
BstEII	Bsu36I	ClaI	DraIII		DrdII		
EcoNI	FseI	HpaI	MluI		MunI		
NarI	NcoI	NgoAIV		NnuI	NsiI		
NspV	PacI	PfiMI		PmeI	PmlI		
PshAI	RleAI	RsrII		SacII	SalI		
SexAI	SfiI	SgfI		SgrAI	SmaI		
SnaBI	SphI	SrfI		Sse8387I	StuI		
SunI	Swal	XcmI					

ml cultures of *Escherichia coli* grown in LB medium. A typical extraction is described below.

Bacteria from a 5-ml overnight culture were harvested in a single 1.5 ml Eppendorf-type tube by centrifugation (9,300 g, 2 min, RT). The cell pellet was resuspended in 250 µl buffer P1 by vortexing. The cells were lysed with 250 µl buffer P2 and the tube was gently inverted 6 times until the solution became viscous and slightly clear. The tube was gently mixed to preserve genomic DNA from shearing. Both chromosomal DNA and proteins were precipitated from the bacterial lysate by adding 350 µl buffer N3. The solution was immediately inverted to avoid precipitation of potassium dodecyl sulphate. The mixture was centrifuged as above for 10 min. During centrifugation, a spin column supplied in the kit was transferred to a 2ml collection tube. The supernatant containing the supercoiled vector of interest was transferred carefully by pipetting onto the spin column. The DNA was bound to the column by centrifugation at 13,500 rpm for 1 min. The eluate was discarded and then the column was washed by adding 500 µl buffer PB. Centrifugation was followed as above. This step removes salt from the DNA on the column. Residual buffer PB was removed by spinning the empty column again (Buffers N3 and PB contain chaotropic salts). The spin column was carefully transferred into a 1.5 ml Eppendorf-type tube and after adding 50 µl of MilliQ H₂O and incubating at room temperature for 1 min, the bound DNA was eventually eluted by centrifugation at 13,500 rpm for 1 min.

6.2.2.2 Agarose gel electrophoresis (AGE) protocol

A standard gel electrophoresis protocol was used for all AGE experiments. A 1% (m/v) agarose solution was prepared. 1g agarose in 100 ml of TBE buffer (1 L of 10 X stock solution contains: 108.0 g Tris base, 55.0 g Boric acid, 40 ml 0.5 M EDTA, adjusted to pH 8.0 with NaOH). The agarose powder was dissolved in 1 X TBE by heating in the microwave on full power for 5 min and left at room temperature to cool. The gel was cast in a tray after fixing tape across the ends, and placing a suitable comb at the desired location.

When the agarose solution had cooled to about 45 ° C it was poured into the gel tray and left to set. When solidification was completed, the tapes were removed and the tray was then placed in a horizontal electrophoresis tank filled with TBE buffer. A volume of TBE buffer enough to just cover the gel surface was added so that the gel carried most of the current that passed when the potential difference was applied between the two poles. For all experiments a 6X stock of gel loading buffer [12% (v/v) glycerol, 60 mM EDTA, pH 8.0, 0.6% (w/w) SDS, 0.25% (w/v) Bromophenol blue sodium salt and 0.25% (w/v) Xylene cyanol FF] was used. The gel was run at a constant voltage of 200 V/cm. When electrophoresis was completed the DNA bands, stained with EtBr, were visualized under UV-irradiation (320_{nm}).

6.2.2.3 DNA purification from TBE agarose gels

The method followed is based on the protocol supplied with the QIAgen gel extraction kit by **QIAGEN Ltd.** The agarose slice containing the PCR product was excised from the gel using a clean sharp scalpel. Care was taken not to overexpose the gel to UV radiation as this may damage DNA.

The excised gel slice was placed in a pre-weighed 1.5 ml Eppendorf-type tube. Weighing again provided the mass of the gel slice. Three vol. buffer QG was added to 1 vol. gel (100mg~100µl); thus, for a 180 mg gel slice, 540 µl buffer QG was added. After vortex-mixing the tube contents were incubated at 50 °C for 10 min and vortex-mixing was repeated every 2.5 min. When the gel slice was completely dissolved (the colour of the solution remained pale yellow), the solution was transferred by pipetting into a 1.5 ml QIAquick column placed in a 2 ml collector tube and centrifuged at 13,500 rpm for 1 min (IEC Micromax) at room temperature. The DNA was bound to the column, while most contaminants including the agarose dissolved in the buffer passed through the column during centrifugation. The column was then washed with an additional 500 µl buffer QG to remove any residual traces of agarose. The waste was again discarded and 500 µl buffer QX was then added to the column to remove carbohydrates from DNA. Salts were next removed via the addition of 750 µl buffer PE and centrifugation followed as above. Removal of all contaminants was necessary, as the purified DNA was prepared for cloning experiments. At the final stage of

purification, the column was transferred to a 1.5 ml Eppendorf-type tube. Bound DNA was eluted from the column by adding 50 µl MilliQ H₂O, leaving to stand for 1 min at room temperature, and centrifuging as above. The 50 µl eluate contained highly purified DNA that was stored at -20 °C until required.

6.2.2.4 Construction of recombinant plasmids for the expression of factor I SP domain fragments

It was decided to express two recombinant forms of the human fI SP domain. One construct would contain the region A²⁸²-V⁵⁶⁵ (CONSTRUCT 1) and the other the region R³¹⁸-V⁵⁶⁵ (CONSTRUCT 2) (Fig. 9). Both of these constructs would contain the R³²¹-I³²² peptide bond that under physiological conditions is post-translationally cleaved inducing the activation of the Serine Protease domain (section 1.3.4). The cDNA encoding the particular regions was amplified by standard PCR technology directly from the pSP64 recombinant template. For the two amplification reactions the forward primers used were: 5'-gCg gCT AgC ATg ACT **gCT CAA gAA gAA ACA gAA ATT TTG**-3' for construct 1 and 5'-AgA ATg CTA gCC **gAA ggA AAC gAA TTg Tgg gA**-3' for construct 2; the same reverse primer 5'-CgA ATT CTT **ATA CAT TgT ACT gAg AAA TAA AAg**-3' was used for both amplifications. All three primers contained restriction sites (underlined) that facilitated the cloning of the two inserts into pET-17b. Upon completion of the amplification, the forward primers would generate on each product a site of cleavage for NheI restriction endonuclease, while the reverse primer a site of cleavage for EcoRI; with the particular cloning strategy employed (Table 17) the two PCR generated insert products could be inserted into the expression vector (Figs. 48 & 49) after both the inserts and the vector were digested with NheI and EcoRI to create identical sticky ends for ligation. The regions of the primers that anneal to the template are highlighted in bold; the melting temperatures (T_m) for these primer regions were calculated using the standard formula $T_m (^{\circ} C) \approx 2(N_A + N_T) + 4(N_G + N_C)$ [assuming that the annealing occurs under the standard conditions of 50 nM primer, 50 mM Na⁺, and pH 7.0] (N represents the number of residues of the respective nucleotide within the oligonucleotide) and were found to be 64, 62 and 64 ° C, respectively. The two expressed constructs would contain some extra residues at their N-termini; construct 1

Table 17: Summary of the cloning strategy for the generation of two recombinant forms of human factor I SP domain
Aim: Express two recombinant forms of the fI SP domain in <i>Escherichia coli</i>; construct 1 containing the region A²⁸²-V⁵⁶⁵ and construct 2 containing the region R³¹⁸-V⁵⁶⁵ (Fig. 9, Chapter 1). Refold the protein products from inclusion bodies and purify them to homogeneity for further structural and functional studies as well as for developing of an anti-fI SP domain polyclonal anti-serum.
<u>Stage 1:</u> Amplify the target regions from the full-length cDNA of human complement factor I via standard PCR. The primers carry cleavage sites for NheI and EcoRI restriction endonucleases that will facilitate the cloning of the inserts into the pET-17b expression vector.
<u>Stage 2:</u> Purify, digest with NheI and EcoRI and repurify the two inserts; clone them into linearised pET-17b with identical sticky ends.
<u>Stage 3:</u> Screen for successful recombinant clones via partial digestion with NheI and EcoRI. Confirm quality status via DNA sequencing.
<u>Stage 4:</u> Isolate target clones and propagate them in XL-1 Blue <i>E. coli</i> strain. Site-directed mutagenesis of C⁴³⁵ to S⁴³⁵ in construct 2. Screen again for mutant clones. Isolate a clone and confirm the mutagenesis via DNA sequencing.
<u>Stage 5:</u> Propagate both constructs in XL-1 Blue <i>E. coli</i> strain, purify and transform the <i>E. coli</i> expression strain BL21(DE3)pLysS.
<u>Stage 6:</u> Prepare cell cultures from colonies of transformants, grow cells and induce expression of the two constructs; monitor the expression profile over time.
<u>Stage 7:</u> Establish clones with stable expression patterns; Express the recombinant SP constructs and attempt to refold and purify one or both of them.
<u>Stage 8:</u> Raise in rabbits an anti-fI SP domain polyclonal anti-serum using one of the expressed constructs either in refolded or in inclusion bodies form.

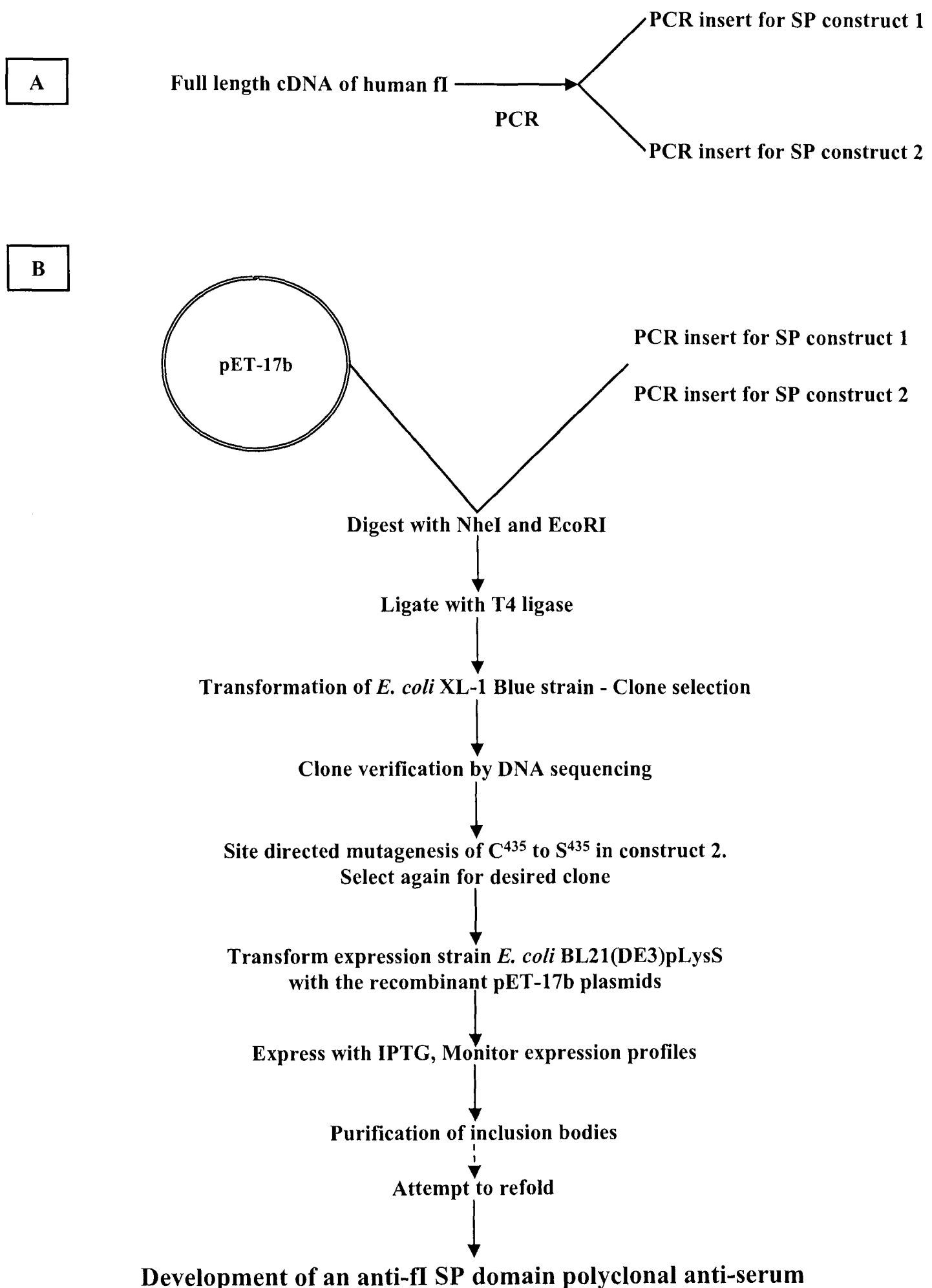


Fig. 49. Strategy of PCR cloning and expression of two recombinant forms of human complement factor I SP domain-Development of an anti-SP polyclonal antibody. A. Generation of inserts for two recombinant constructs of fI SP domain using PCR technology; full length cDNA of human fI was used as template. B. Construction of recombinant plasmids and expression in *E. coli* cells; production of recombinant proteins and development of an anti-fI SP domain polyclonal anti-serum.

would contain the four extra amino acids ASMT of the T7-Tag sequence that increases the efficiency of expression, while construct 2 the two extra amino acids AS of the T7-Tag sequence. For each PCR reaction a 100 µl reaction mixture was prepared as illustrated in Table 18. The cycling parameters used were the same for both reactions and are listed in detail in Table 19. At the end of each PCR reaction the quality of the products was accessed by 1% AGE; 10 µl was analysed from each PCR reaction mixture and the desired PCR products were gel purified as described above. Following the purification, both inserts were digested with NheI and EcoRI simultaneously (Table 20), repurified using the QIAquick DNA Cleanup SystemKit by **QIAGEN Ltd.** and ligated separately with the digested expression vector. The host vector had been also treated with Calf intestinal alkaline phosphatase (CIP) to minimise the possibilities of self-ligation. A typical ligation reaction is summarized in Table 21. Ligation reactions with only vector and only inserts were also prepared separately and served as negative controls.

6.2.2.5 Transformation

Upon completion of the ligation reactions, selection of the positive clones was carried out. Briefly, 2 µl from each 10 µl ligation reaction were used to transform a 50 µl aliquot of *E. coli* XL-1 Blue competent cells. The aliquot was placed on ice for 1 min and it was then mixed with 2 µl of the ligation mixture. After mixing by pipetting, the mixture was placed on ice for 60 min. Then the tube was placed in a rack and then heat-shocked in a 42 °C water bath for 90 s to allow the plasmid DNA to enter the cells. Immediately after the shock was completed, the mixture was incubated with 450 µl of pre-warmed SOB medium, which contains 20 g Bacto-tryptone, 5g Bacto-yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂ and 10mM MgSO₄ (added after autoclaving) per litre, in a shaking incubator at 37 °C for 60 min. Following the incubation, up to 100 µl of the transformation mixtures (including negative controls) was aseptically spread on LB agar plates [Ap100-100µg/ml] using a glass rod; the plates were then incubated for 16hrs at 37 °C and were then taken to 4 °C to avoid development of satellite colonies due to the gradual degradation of antibiotic around the developing colonies. The control plates were used to evaluate physically the success of the ligation reactions prior to the screening of

Table 18: Typical composition of a PCR reaction mixture for the amplification of the SP domain from the full-length cDNA of human factor I

Component	Volume (μl)
Buffer¹	10.0
dNTPs²	8.0
Forward primer³	5.0
Reverse primer	5.0
Template DNA⁴	4.0
Pwo polymerase	0.5
H₂O	67.5
Final volume	100.0

1. The reaction buffer was 10X, contained 20 mM MgSO₄ and was supplied with the Pwo Master PCR kit.
2. The working concentration of the dNTPs mixture used was 2.5 mM.
3. The primers were originally reconstituted with water to prepare a 100 pmole/μl (100μM) stock solution. To prepare a solution of working concentration each primer stock solution was diluted 10X.
4. Template DNA was at a stock concentration of 400 μg/ml. To prepare a solution of working concentration the template stock solution was diluted 20X.

Table 19: PCR cycling parameters for the amplification of the SP domain from the full-length cDNA of human factor I				
	Phase	Period (sec.)	Temperature (° C)	No. of cycles
Step 1	Hot start-Denaturation	300	94	
Step 2	Denaturation	30	94	
	Annealing	30	54	X 4
	Extension	45	72	
Step 3	Denaturation	30	94	
	Annealing	30	60	X 8
	Extension	45	72	
Total number of cycles ¹ :				X 12
1. Each reaction was set to proceed for a maximum of 12 cycles to minimise any proof-reading errors resulting in nucleotide base misincorporation.				

Table 20: Typical composition of a restriction endonuclease reaction mixture		
For plasmid DNA purified with the Miniprep kit (10 ml LB culture)		
NheI	2.0 µl	(10 Units)
EcoRI	0.5 µl	(10 Units)
NEB buffer 3 (10X) ¹	5.0 µl	Incubation for 3 hr. at 37 °C.
100xBSA	0.5 µl	
DNA	20.0 µl	
MQ H ₂ O	17.0 µl	
Final volume	45.0 µl	
Prior to cloning the linearised expression vector was also treated	+ 2.5 µl CIP Alkaline Phosphatase ² + 2.5 µl H ₂ O	Incubation for 2 hr. at 37 °C.
Final volume	50.0 µl	
For insert DNA generated in a single PCR reaction mixture and purified with AGE and gel extraction kit		
NheI	4.0 µl	(20 Units)
EcoRI	1.0 µl	(20 Units)
NEB buffer 3 (10X) ¹	5.0 µl	Incubation for 3 hr. at 37 °C.
100xBSA	0.5 µl	
DNA	10.0 µl	
MQ H ₂ O	29.5 µl	
Final volume	50.0 µl	
1. New England Biolabs buffer 3 (1X): 50 mM Tris-HCl 100 mM NaCl, 10 mM MgCl ₂ , 1 mM dithiothreitol, pH 7.9 at 25°C. 2. Alkaline Phosphatase catalyzes the removal of 5' phosphate groups from DNA. Since CIP-treated fragments lack the 5' phosphoryl termini required by a ligase, they cannot self-ligate. This property can be used to decrease the vector background in cloning strategies.		

Table 21: Typical composition of a ligation reaction ¹			
Components	Reaction 1 (μl)	Reaction 2 (μl)	Reaction 3 (μl)
5X Ligation Buffer ²	2	2	2
Digested vector DNA	3	2	1
Digested insert DNA	1	2	3
MQ H ₂ O	3	3	3
T4 ligase	1	1	1
Final volume	10 μl		

1. Prior to the setting up of a ligation, the relative concentrations of the two samples (vector + insert) were empirically determined by 1% AGE. Optimally, for a successful ligation an approximate molar ratio 3:1 of insert to vector is usually necessary. For each ligation, three reactions, excluding controls, were prepared containing different molar ratios of insert to vector; this strategy practically increased the possibilities of covering the spectrum of approximate molar ratios leading to a successful ligation.

2. 50 mM Tris-HCl, 10 mM MgCl₂, 10 mM dithiothreitol, 1 mM ATP, 25 μg/ml BSA, pH 7.5 @ 25 ° C

any transformants from colonies obtained. For each round of screening, 12 colonies from each insert+vector ligation reaction mixture were used for inoculation 5 ml LB cultures for plasmid extraction. The extracted plasmids were examined by partial digestion using the NheI and EcoRI restriction endonucleases. Two clones harbouring the inserts of interest were eventually selected; both the sequence and orientation of the inserts were confirmed by DNA sequencing.

6.2.2.6 Site directed mutagenesis

Site-directed mutagenesis was carried out to replace cysteine with serine at position 435 ($C^{435} \rightarrow S^{435}$) in the recombinant construct 2. The particular cysteine participates in the interchain disulphide bond between C^{309} - C^{435} that naturally connects the heavy and the light chains of fI together (Fig. 9). As C^{309} would be absent from the shorter recombinant construct of human fI SP domain, C^{435} was replaced to avoid causing any complications due to oligomerisation phenomena. The point-mutation was carried out using the QuickChange™ according to the manufacturer instructions. A sequenced plasmid containing the insert for construct 2 in pEt-17b was used as template; the primer pair used was: 5'-gTT CCA TCC CTg CCA gTg TCC CCT ggT CTC-3' (sense primer) and 5' gAg ACC Agg ggA CAC Tgg CAg ggA Tgg AAC-3' (anti-sense primer) (the codon that encodes for S is underlined). DNA sequencing confirmed the presence of the particular mutation in the selected mutants.

6.2.3 Expression of recombinant plasmids in *Escherichia coli* cells

Following the verification of the correct sequences of the two recombinant clones constructed, plasmid DNA was generated for each clone using the QIAprep spin miniprep kit protocol described earlier. The recombinant plasmids were transfected into BL21(DE3)pLysS host strain and the transformants were selected on LB medium plates containing ampicillin (Ap100, 100 µg/ml) and chloramphenicol (Chl170, 170 µg/ml). For each transformation, 2 µl from each prep were used to transform 20 µl of ready-to-use aliquots of *E. coli* cells. A few colonies derived from *E. coli* cells transformed separately with either constructs were randomly selected and used to inoculate 10 ml cultures. For each bacterial culture set for an expression experiment,

only 20% of the total flask volume was used for adequate air supply. The expression was conducted according to the instructions written in the pET system manual [10th edition, 2003, <http://www.merckbiosciences.co.uk/docs/docs/PROT/TB055.pdf>] published by **Novagen**. All cultures were grown at 37 ° C with vigorous shaking (250 rpm) until the OD_{600nm} of the culture reached 0.6-1.0 and then split into half cultures with one of them made up to 238.3 µg/ml (1 mM) with Iso-propylthio-β-galactopyranoside (IPTG) (always used fresh directly from – 20 ° C) for the induction of expression of the recombinant plasmids. Growth of all cultures was continued under the same conditions for 4 hours. IPTG was used to induce the T7 promoter of the recombinant plasmids by disturbing the repressor-operator interaction; In *E. coli* DE3 lysogens that carry a pET plasmid, a lac repressor limits the bacteriophage T7 transcription; this is achieved either indirectly by repression of the transcription of the T7 RNA polymerase gene or directly by blockage of transcription of the target gene by any T7 RNA that is present *in vivo*.

Upon completion of the incubations, 1 ml from each culture was removed, transferred to 1.5 ml Eppendorf-type tube and subjected to centrifugation (8,000 g, 5 min, RT) for harvesting of the cells. The supernatant was discarded and the cell pellets were washed by pipetting and centrifuging 3X with PBS buffer using 1/10 of the original culture volume (100 µl). The cells were eventually resuspended in 100 µl of Bugbuster buffer reagent by **Novagen** containing 1 µl/ml benzonase nuclease and the mixtures were transferred to -20 ° C for 30 min. The samples containing the total fraction of cell components were then thawed at RT and 100 µl of reducing sample buffer was added to each for SDS-PAGE analysis. The cells of the BL21(DE3)pLysS *E.coli* strain, once thawed, lyse due to the presence of T7 lysozyme encoded by the pLys plasmid. The T7 lysozyme can cut the peptidoglycan of the bacterial cell wall, thus making the cell lysis easier. From each sample, 40 µl (1/5 of final volume) were used for the analysis.

Following the examination of the expression profiles of a few selected colonies, expression was carried out in larger scale. For each construct, a colony was used to inoculate a 10 ml LB culture (Ap100, [100 µg/ml], Chl170, [170 µg/ml] as described above. This culture was used as a starter culture for a 500 ml culture. Following induction with IPTG, the culture was further incubated for 16 hr (overnight) at 37 ° C

with vigorous shaking (250 rpm). Upon completion of incubation, cells were harvested by centrifugation (7,500 g, 15 min, RT) and washed 3X with ice-cold PBS buffer using 1/10 (50 ml) of the original culture volume.

6.2.4 Purification of inclusion bodies

Following the washings, the cells from each culture were resuspended in the same buffer, then frozen and eventually thawed. Next, in every 50 ml suspension sample obtained, 2 ml of 1M MgCl_2 and 50 μl of DNase I (50 mg/ml) were added. The cell suspension sample was then subjected to mild vortexing and sonication at a Sanyo MSE Soniprep 150. Sonication was carried out in ice cold conditions six times (20 sec. each) using amplitude 14. After sonication, each sample was then left to mix at a bench flat rotor for 30 min. thus allowing DNase I to digest the cell extracted DNA. Upon completion of the mixing, each 50 ml sample was subjected to centrifugation, to harvest the inclusion bodies (IBs) of the expressed construct. The inclusion body pellets were thoroughly washed up to 5 times via pipetting using ice-cold PBS, 0.5 mM EDTA, 0.5% (v/v) Triton X-100 in order to remove any cell debris and inclusion body incorporated soluble protein contaminants. Following the thorough washings, a small volume of the inclusion body suspension was analysed by SDS-12.5% PAGE under reduced conditions to assess the amount and purity of the expressed and washed recombinant forms of the fl SP domain. The inclusion body suspension was eventually stored at -20°C with a minimal volume of buffer.

6.2.5 Development of an anti-hfl SP domain polyclonal anti-serum- Screening ELISA for the detection of antiserum

Inclusion bodies containing the expressed construct 2 were harvested from cells grown in a 50 ml cell culture; once purified they were resuspended in PBS, pH 7.2 and then were processed by passage through a 19G syringe needle. This step reduced the size of the inclusion bodies from small flakes into injectable fine particles suitable for immunisation. The inclusion body (IB) suspension containing the fine particles was then split into 4 equal aliquots of 500 μl that would be used for immunisation. Rabbits were immunised with a single dose of IB suspension in Freund's complete

adjuvant followed by booster injections in Freund's incomplete adjuvant. Booster injections of a single dose were done at days 14, 28 and 42. A test bleed was taken on day 35 and the final bleeding was on day 49. The immunisation was carried out by **Charles River Laboratories** [Romans, France].

The anti-fI SP domain polyclonal anti-serum raised was then tested in an ELISA system for specificity against the native human fI and the recombinant form of fI SP domain (Construct 2 product). Wells in a Maxisorb Nunc-Immuno™ 96 MicroWell™ Plate by **Nalge Nunc International** [Rochester, NY, USA] were coated for 16 hr (overnight) at 4 ° C with 50 µl of highly purified native human fI (10 µg/ml) in 0.1 M Na₂CO₃ pH 9.5 or recombinant fI SP domain (Construct 2) (10 µg/ml) solubilised in 2M GuHCl, 0.1 M Tris-HCl, 0.1 M DTT, pH 9.5. The wells were washed thoroughly three times with PBS, 0.5 mM EDTA, 0.1% (v/v) Tween-20 (PBS-TEDTA). Non-specific binding sites were blocked for 2 hours at RT using 400 µl of PBS-TEDTA, 1% (w/v) BSA per well. Wells were also washed thoroughly three times with PBS-TEDTA prior to addition of the antiserum obtained from the test bleed of day 35 (100 µl/well) at five-fold serial dilution (1:50-1:1250). All the samples were incubated in the wells for 1 hr at RT. The antiserum was diluted in PBS-TEDTA, 1% (w/v) BSA. All the wells were then washed thoroughly three times with PBS-TEDTA prior to incubation with 100 µl/well monoclonal mouse anti-rabbit IgG γ chain specific alkaline-phosphatase conjugate (Sigma, Cat. No. A2556) diluted 1:5,000 in PBS-TEDTA, for 1 hr at RT. The particular monoclonal antibody binds only to the rabbit IgG class of antibodies; it reacts with an epitope on the heavy chain of rabbit IgG, while it does not cross-react with the other light chains of rabbit IgM or IgA. In aspects of cross-species reactivity, no interactions have been detected with human, guinea pig, rat, bovine, goat, sheep, horse, dog, feline, pig or chicken IgGs. At the end, the wells were developed with 100 µl/well p-nitrophenyl phosphate substrate (pNPP, 1mg/ml in 0.2 M Tris-HCl, pH 8.0) at RT until yellow colouration started to appear. The absorbance was read at 405 nm using a microtitre plate reader.

6.3 RESULTS AND DISCUSSION

6.3.1 Construction of recombinant plasmids for the expression of factor I SP domain fragments

The construction of the two designed recombinant plasmids was straightforward and eventually successful. The annealing temperatures of the PCR cycling parameters were slightly modified to improve the reaction specificities. In particular, raising the annealing temperatures of steps 2 and 3 to the values presented in Table 17, led eventually to the generation of discrete products as illustrated in Fig. 50. The PCR derived inserts were highly purified and prepared for cloning experiments. A typical experiment of successful purification of insert DNA is shown in Fig. 51. Next, each DNA insert was ligated with the linearised pET-17b expression vector using T7 DNA ligase and the recombinant plasmids obtained were transfected into *E. coli* cells as described in the materials and methods section of the current chapter. At the end, the multiple rounds of selection yielded clones of recombinant plasmids suitable for protein expression studies.

6.3.2 Expression of recombinant plasmids in *Escherichia coli* cells

The primary protein expression experiments with constructs 1 and 2 indicated that the pET expression system had the ability to produce both of the genetically engineered recombinant forms of the hfl SP domain. The identity of the synthesized products was confirmed in both cases by N-terminal sequencing performed by Mr. A. C. Willis, (MRC Immunochemistry Unit). For the case of construct 1, at the final rounds of selection, six randomly selected plate colonies of transformants, designated 1, 4, 5, 10, 11 and 12, were found to have similar patterns of protein expression (Fig. 52). The analysis of the expression profile of construct 2 over time indicated, that the encoded fl SP protein was synthesised in high yields within 2-3 hours following induction of expression with IPTG (Fig. 53); the amount of protein produced however, reached maximum after approximately 3-4 hours and remained unchanged for longer periods of cell growth. Overall, all cultures, were grown overnight to achieve high yields of recombinant hfl SP production.

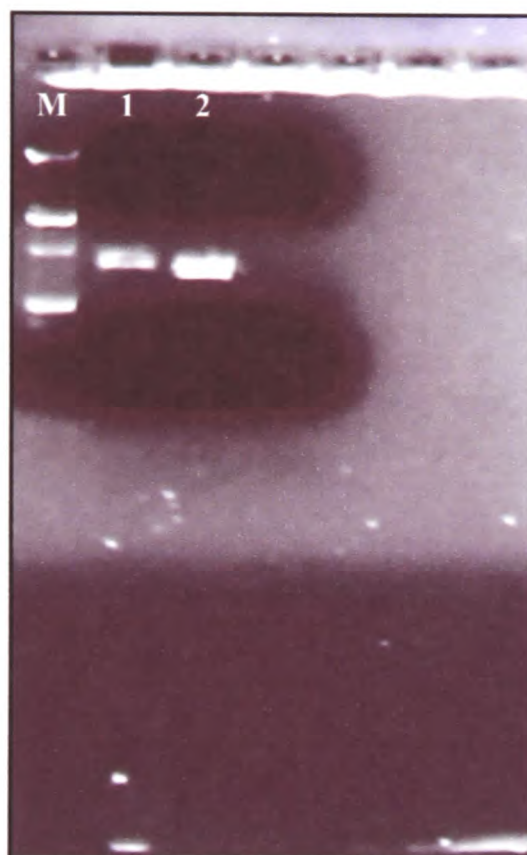


Fig. 50. Detection of the isolated PCR products by 1% AGE. Following optimisation of the PCR amplification conditions (Table 19), the inserts required for constructs 1 (Lane 1) and 2 (Lane 2) expressing two recombinant forms of human fl SP domain, were isolated from the full length cDNA of human fl. For the analysis 10 μ l from each PCR reaction mixture were loaded. The two fragments differ in size since the larger product in lane 1 contains an additional region that encodes for the C-terminal region of the fl heavy chain. The PCR inserts were stained with EtBr and visualised under UV-irradiation (320_{nm}).

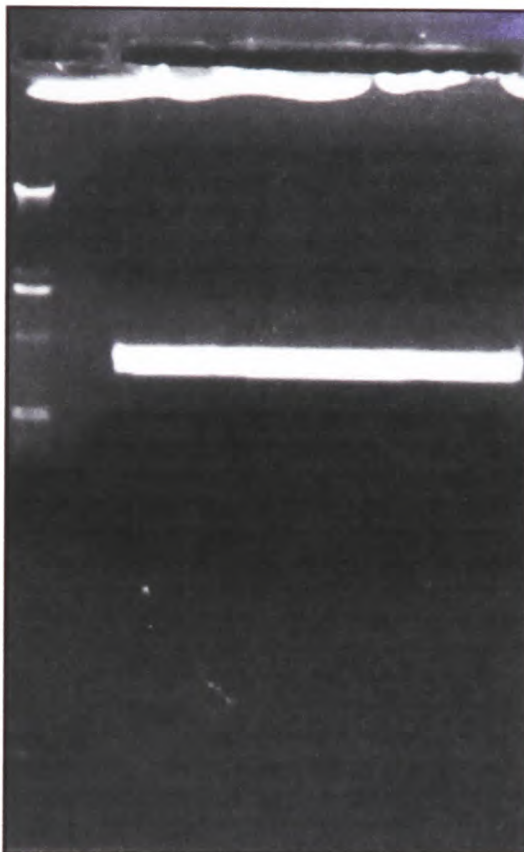


Fig. 51. Purification of PCR generated insert DNA used for the engineering of construct 2. Following the optimisation of the PCR amplification conditions for the generation of inserts for constructs 1 and 2, both inserts were subsequently purified via gel extraction from 1% AGE gels. The gel was stained with EtBr and the band of interest was visualised under UV-irradiation (320_{nm}). The exposure to UV-irradiation was short to avoid any damage of DNA. The band containing the insert was excised aseptically and further processed for cloning experiments.

For both constructs, expression of the target protein occurred only in cell cultures treated with IPTG as expected. Despite the fact that the related *E. coli* BL21(DE3) strain can sometimes exhibit leaky basal expression of a T7 promoter-driven RNA even without induction with IPTG [McKinney et. al, 2002], the *E. coli* BL21(DE3)pLysS strain employed appeared suitable for controlled expression experiments. The results illustrated in Figures 52 and 53 indicated that the genetically engineered cells harbouring construct 2 expressed the shorter recombinant form of the SP domain in higher amounts than construct 1; this conclusion was based on the comparison of samples obtained from cultures of similar cell density that were treated with IPTG and grown under the same conditions. On average, four hours after the induction of expression, the cells expressing construct 2 produced at least 20X more protein compared to the ones expressing construct 1. The reason for this discrepancy was not obvious.

Unfortunately, in both cases of protein expression of the two constructs, all of the generated protein was produced exclusively in the form of inclusion bodies (IBs) as for the case of human MASP-1 and MASP-2 (Ambrus et al., 2003). Intense efforts to obtain any of the two recombinant forms of hfl SP domain in soluble form by i) lowering the temperature of growth for expression to 20-25 ° C, ii) using rich growth medium, iii) reducing the target concentration of IPTG used, or iv) expressing the two constructs in *E. coli* Origami™(DE3)pLysS expression strain [Novagen] that enhances disulphide bond formation during the protein folding, were unsuccessful. Apparently, the *E. coli* strain used, despite its capacity (at the levels of transcription and amino-acid assembly) to synthesize the two recombinant forms of the hfl SP domain in high yields, it lacked the molecular machinery that would support the folding of these particular recombinant SP protein forms into a soluble form. The possibility that the synthesized protein aggregated *in vivo* due to the exposure of hydrophobic surface regions caused by the lack of any attached *N*-linked terminal carbohydrates, has not been ruled out. Additional strenuous and long efforts to refold any of the two recombinants proteins into their soluble forms using various buffer conditions as described by Ambrus and co-workers (Ambrus et al., 2003) or suggested by the pET system manual [Novagen, 10th edition, 2003], were not successful. Any future efforts to express the particular region of hfl SP domain as soluble recombinant

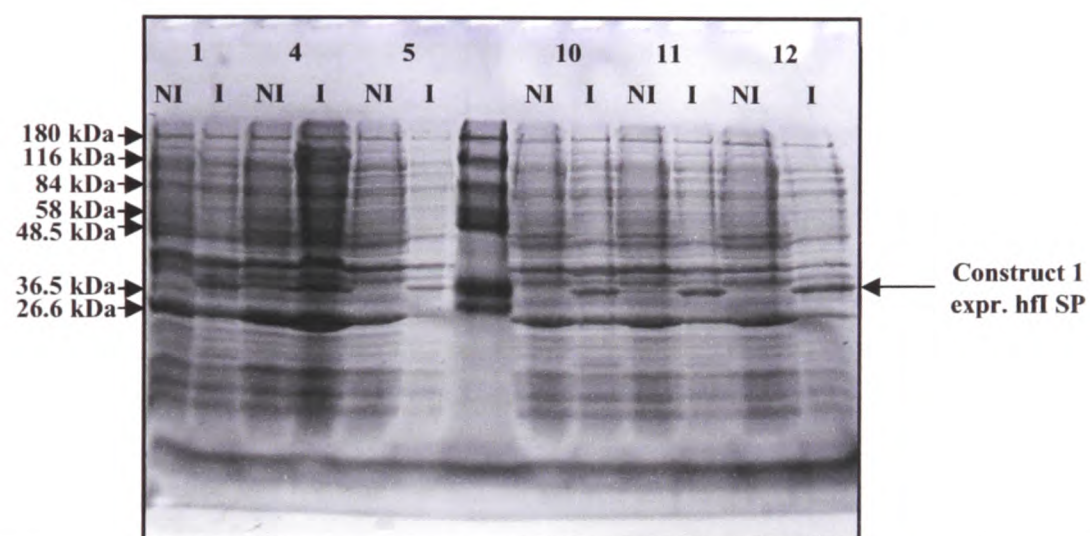


Fig. 52. SDS-10% PAGE analysis of the expression pattern of proteins produced by a few selected colonies of transformed BL21(DE3)pLysS *E. coli* cells containing construct 1. Following transfection with construct 1 (encoding an extended form of recombinant human fl SP domain), few plate colonies (designated by 1, 4, 5, 10, 11 and 12) were selected during screening for examination of the expression of the target protein. All cultures were grown until the OD_{600nm} reached 0.6-1.0 and then split into half cultures with one of them made up to 1 mM IPTG for the induction of expression of the recombinant plasmids and the other half remained unchanged. Growth of all cultures was continued for 4 hours; NI: Not induced, I: Induced; the analysis of the total fraction of all bacterial lysate samples indicated that all six colonies contained transformants with the capacity to express the desired protein only in the presence of IPTG in the growth medium. This also indicated that there was no 'leaky' expression. The gel band containing the product of interest, is indicated by the arrow on the left hand side. The identity of the recombinant hfl SP domain product was confirmed by N-terminal sequencing.

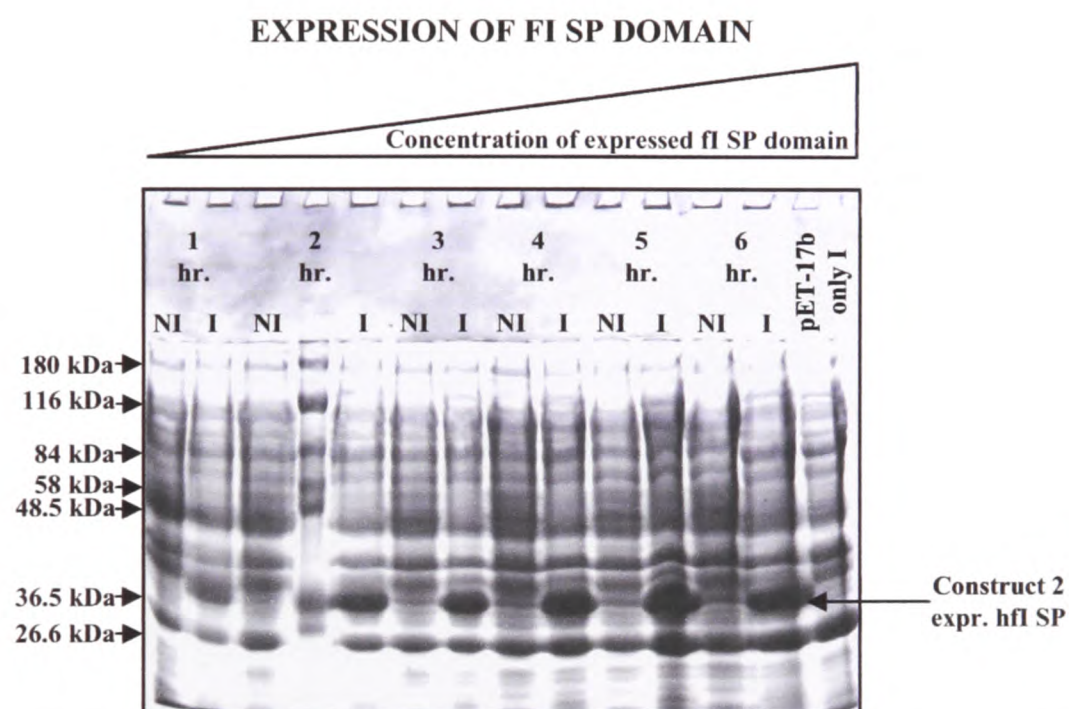


Fig. 53. SDS-10% PAGE analysis of the expression pattern of proteins produced by a colony of transformed BL21(DE3)pLysS *E. coli* cells containing construct 2. A selected colony of transformant cells containing construct 2 (encoding a shorter recombinant form of human fl SP domain) was examined for its expression profile over a 6 hr. period of time. A culture was grown until the OD_{600nm} reached 0.6-1.0 and then split into half cultures with one of them made up to 1 mM IPTG for the induction of expression of the recombinant plasmids. Growth of all cultures was continued for 6 hours with 1 ml aliquots removed for analysis at the end of each hour following the induction of expression with IPTG. NI: Not induced, I: Induced; the analysis of the total fraction of all bacterial lysate samples indicated that the selected colony overexpressed the desired protein. The expression appeared to reach a plateau after approximately three hours following induction with IPTG. Protein expression was detected only in the presence of IPTG in the growth medium indicating that there was no 'leaky' expression, as for the case of construct 1. The gel band containing the product of interest, is indicated by the arrow on the left hand side. The identity of the recombinant hfl SP domain product was also confirmed by N-terminal sequencing.

protein, should be based in other reliable eukaryotic expression systems such as insect cells or mammalian cell lines that have the capacity of producing soluble mammalian glycoproteins, at least in the 500-1000 µg scale from 1 L of medium.

6.3.3 Development of an anti-hfI SP domain polyclonal anti-serum- Screening ELISA for the detection of antiserum

With both recombinant forms of hfI SP domain produced as inclusion bodies, it was decided to use purified inclusion bodies containing the shorter recombinant form (expressed by construct 2) to raise a polyclonal anti-serum in rabbits (Fig. 54). Such an anti-serum, compared to another one raised previously against native intact hfI, in theory would be much more selective for hfI; that is because the heavy chain of human fI contains several regions that share high homology with other complement and plasma components (sections 1.2.6 & 1.2.6.6-1.2.6.8); the polyclonal IgG population species that would be generated against native intact fI, would have more possibilities of cross-reacting with other proteins that contain domain regions that structurally resemble some of those of hfI. Such an anti-hfI SP antiserum that cross-reacts both with hfI and the hfI SP domain, would be a useful tool for future applications such as protein purification and functional assays.

Examination of the specificity of the anti-hfI SP domain antiserum raised against hfI and the recombinant form of hfI SP domain, showed that the particular anti-serum recognises both (Table 22); the response against the recombinant product was quite high, but dropped sharply below the 1:250 dilution. However, the response against native hfI was much weaker, approximately 10X lower at the 1:50 dilution factor; this response was largely weakened at larger dilution factors. The anti-serum contained in the final bleed of day 49 was anticipated to have a similar pattern of specificity, but most likely a stronger one against nfl. The weaker response against hfI was probably due to the fact that the inclusion bodies contained misfolded protein aggregates; the majority of the exposed surfaces of the recombinant protein in such aggregates were either 'masked' or misfolded. In the 'masking' effect there can be a more limited repertoire of antigen recognition areas available for the raising of antibodies, while for the case of the protein folding, raised antibodies are likely to have limited capacities

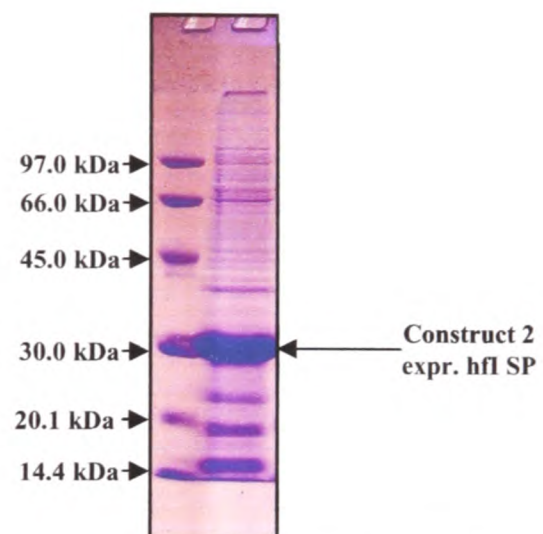


Fig. 54. SDS-12.5% PAGE analysis of the purified preparation of hfl SP inclusion bodies used for the development of an anti-fl SP domain polyclonal rabbit anti-serum.

Table 22: Screening ELISA for examination of the anti-hfI SP antiserum specificity¹

1° anti-serum	Coating	D0 ² , <u>1:50</u> (Control)	Mean	D35, <u>1:50</u>	Mean	D35, <u>1:250</u>	Mean	D35, <u>1:1250</u>	Mean
Anti-hfI SP	hfI	0.097	0.097	0.200	0.195	0.097	0.098	0.082	0.080
Anti-hfI SP	hfI	0.097		0.189		0.098		0.077	
Anti-hfI SP	Recombinant FI-SP (Construct 2)	0.135	0.137	2.034	1.982	1.625	1.653	0.713	0.724
Anti-hfI SP	Recombinant FI-SP (Construct 2)	0.138		1.930		1.681		0.734	

1. The values reported in this table correspond to absorbance readings at OD_{405nm}. All experiments were done in duplicate and the mean values were used for comparison studies.

2. D0: Day 0 (day of rabbit immunisation), D35: Day 35; the results presented in this table were obtained after testing the specificity of anti-hfI SP antiserum contained in the test bleed of day 35.

for recognising surface patterns of folding in the native conformation. Anti-hfI specific polyclonal IgG antibodies can be purified from the anti-serum of the final bleed (approximately 40 ml) via affinity chromatography; the primary purification of the mixed rabbit IgG population can be carried out using a Protein G column as described in section 2.3.5, while the specific anti-hfI IgG antibodies can be purified on a hfI-Sepharose resin. Such antibodies could be assayed for an inhibitory effect on the catalytic activity of hfI using either the proteolytic or the amidolytic assays described in Chapter 3.

Chapter Seven

CHAPTER 7: *N*-LINKED GLYCAN ANALYSIS OF HUMAN COMPLEMENT FACTOR I

The experimental work and data analysis described in this chapter were undertaken in the Institute of Glycobiology, Department of Biochemistry, University of Oxford with the kind assistance of Mr. James N. Arnold. The author wishes to acknowledge Mrs. Catherine M. Radcliffe, Dr. Pauline M. Rudd and Professor Raymond A. Dwek for their help and use of experimental facilities.

7.1 INTRODUCTION

On each chain of fI there are three potential sites of attachment of *N*-linked oligosaccharides located in loop regions; these sites in the Heavy chain are at N⁵², N⁸⁵, N¹⁵⁹ and in the Light chain at N⁴⁴⁶, N⁴⁷⁶, N⁵¹⁸ (section 1.3.4) (Figs. 9, 17 & 55) in accordance with the known characteristic sequences of potential *N*-glycosylation sites: -N-X-S- or -N-X-T- (Bause, 1983) (Fig. 55). In the primary human fI characterisation stages, the MW difference of approximately 23 kDa (25-27% w/w) that was detected by SDS-PAGE analysis between the observed and the estimated MW, had supported the notion that all six sites are occupied (Goldberger et al., 1984). Based on the most detailed model of human fI to date (Chamberlain et al., 1998), the fitting of *N*-linked glycans to the central location of the interlobal region of the fI model (section 1.6), has suggested that the glycans may have both structural and/or functional roles; they may be able to affect the interactions with the natural substrates C3b and C4b and cofactors by controlling the protein surfaces that are left exposed for protein-protein interactions, and/or may have the ability to participate directly in the substrate-cofactor-enzyme interactions that are mainly ionic in nature (DiScipio, 1992; Soames and Sim, 1997). Work on recombinant human fI (rhfI) expressed in insect cells had supported the hypothesis that the *N*-glycans may have some functional participation in the interactions as the recombinant fI exhibited only 55% of the C3(NH₃) cleavage activity compared to the native enzyme purified from human serum (Ullman et al., 1998). It is clear that to understand the molecular nature of the particular interactions, these *N*-linked glycans should be characterised. In addition,

towards obtaining an atomic structure for human fI (Chapter 8), the characterisation of the glycans of each chain is of great importance. The most detailed model of human fI to date (Chamberlain et al., 1998) does not include the actual fI glycan structures, but instead models the glycans as a complex –type structure (A₄G₄S₄), since the composition of the total glycans of fI was not yet known.

So far, the only available information concerning the composition of the fI N-glycans was from the analysis of the total glycan content of fI (Ritchie et al., 2002). The total fI glycan pool was found to contain predominantly complex biantennary glycans, 46% of which are disialylated and 26% of which are monosialylated. The analysis showed that the composition resembles the glycan pool compositions of proteins and enzymes that are predominantly synthesized in the liver which, as a common site of synthesis tissue for the majority of complement components (Crispin et al., 2004), influences the carbohydrate structures observed on the mature secreted products (Rudd and Dwek, 1997). Since glycosylation may be different between the Heavy and the Light chains of fI and as local 3D structure around a glycosylation site influences the glycan processing, it was therefore decided to analyse the N-glycans of each chain separately.

NetNGlyc 1.0 Server - prediction results

Technical University of Denmark

SignalP-NN euk predictions are as follows:
name Cmax pos ? Ymax pos ? Smax pos ? Smean ? D ?
Sequence 0.151 18 N 0.033 40 N 0.091 23 N 0.031 N 0.032 N

SignalP output is explained at <http://www.cbs.dtu.dk/services/SignalP/output.html>
#####

Human complement factor I Length: 561

KVTYTSQEDLVEKKCLAKKYTHLSCDKVFCQPWQRCIEGTCVCKLPYQCPKNGTAVCATNRRSFPTYCQQKSLECLHPGT 80
KFLNNGTCTAEGKFSVSLKHGNTDSEGIVEVKLVQDKTMFICKSSWSMREANVACLDLGFQGGADTQRRFKLSDLINS 160
TECLHVCRCGLETSLAECTFTKRRTMGYQDFADVVCYTQKADSPMDFFQCVNGKYISQMKACDGINDCGDQSDCLCKA 240
CQKGKGFHCKSGVCIPSQYQCNGEVDCTITGEDEVGCAGFASVAQEETEILTADMDAERRRIKSLLPKLSGKVNRMHIIVG 320
GKRAQLGDLPWQVAIKDASGITCGGIYIGGCWILTAACHLRASKTHRYQIWTTVVDWIHPDLKRIVIEYVDRIIFHENYN 400
AGTYQNDIALIEMKKDGNKKDCELPSPACVPWSPYLFQPNDCIVSGWGREGDNERNVFLQWGEVKLISNCSKFGYGNR 480
FYEKEMECAGTYDGSIDACKGDSGGPLVCM DANNTYVWGVVSWGENCGKPEFPGVYTKVANYFDWISYHVGRPFISQYN 560
V 640

.....N..... 80
...N.....N..... 160
..... 240
..... 320
..... 400
.....N.....N..... 480
.....N..... 560
..... 640

(Threshold=0.5)

SeqName	Position	Potential	Jury	N-Glyc agreement result
Sequence	52 NGTA	0.6007	(8/9)	+
Sequence	85 NGTC	0.7631	(9/9)	+++
Sequence	159 NSTE	0.5087	(5/9)	+
Sequence	442 NDTC	0.5970	(7/9)	+
Sequence	472 NCSK	0.6403	(8/9)	+
Sequence	514 NVTY	0.5990	(9/9)	++

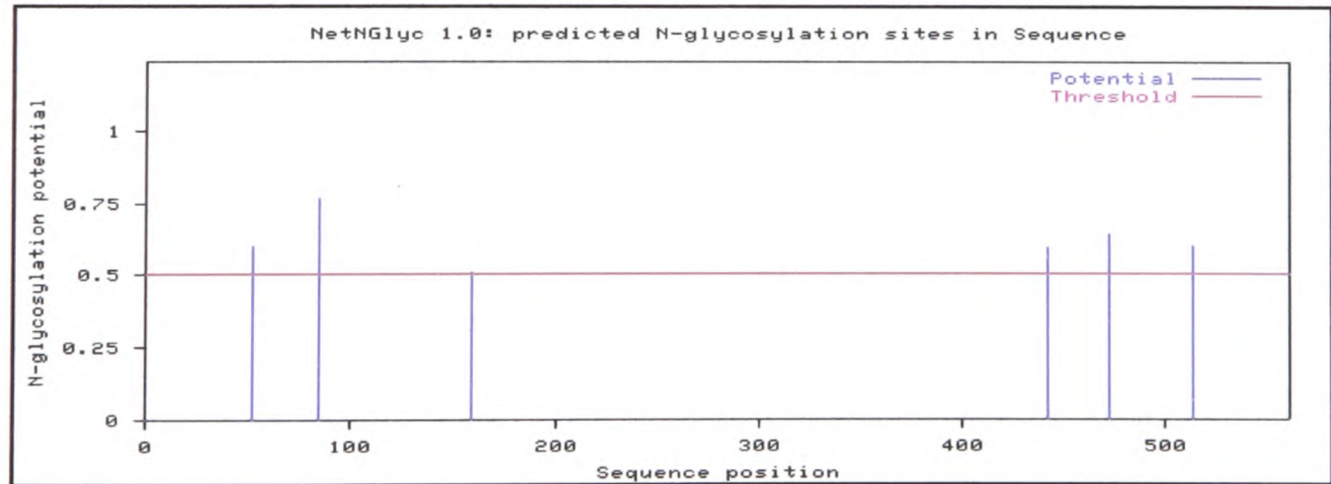


Fig. 55. Prediction of N-glycosylation sites in human complement factor I. The primary sequence of human complement fI was submitted for analysis to the NetNGlyc 1.0 server (<http://www.cbs.dtu.dk/services/NetNGlyc/>) for prediction of candidate sites for attachment of N-linked carbohydrates. As expected, six such sites were identified, three in each chain as previously reported (Fig. 9). On human fI all the six predicted sites are occupied (Goldberger et. al, 1984) (section 1.3.4). N-X-S/T repeats in the sequence output below are highlighted in blue, while the asparagines (N) that are predicted and determined to be N-glycosylated are highlighted in red. The tetrapeptide RRKR that separates the heavy from the light chain in the pro-active form was not included in the sequence analysed.

7.2 MATERIALS AND METHODS

7.2.1 Preparation of human factor I for *N*-glycan extraction

Human complement factor I purified to homogeneity (as described in Chapter 2) was prepared for the analysis of the heavy and light chain *N*-glycan pools. Two fl samples of 25 µg each (217.8 µl of 0.115 µg/µl in 25 mM HEPES, 145 mM NaCl, 0.5 mM EDTA, pH 7.4) were concentrated using 7 µl of Strataclean resin and then treated for analysis by SDS-PAGE. Both samples were reduced with 50 mM DTT at 70 °C for 10 min. and then alkylated with 10 mM iodoacetamide. The SDS-PAGE gel was prepared according to Küster et al. (Küster et al., 1997) and run using 500 V, 25 mA for 1 h. The heavy and the light chains of reduced fl were visualised with Coomassie blue staining and from both samples the bands corresponding to the two chains were excised carefully and pooled together into heavy and light chain pools. For control, a piece of the gel, similar in size, containing no protein was also excised. The bands of each pool were cut in finer pieces, frozen and then washed for three cycles, with each step taking 30 min. at 4 °C beginning with 1 ml of acetonitrile followed by 1 ml of 20 mM NaHCO₃, pH 7.0, ending with acetonitrile. This step was aimed at removing background contamination that interferes with the subsequent fluorescent labelling of the glycans. In addition, these washes remove any traces of reducing and alkylating agents as well as residual SDS. The washed gel fragments were dried in a vacuum centrifuge prior to the enzymatic removal of the *N*-glycans.

7.2.2 Removal of *N*-linked glycans and 2AB labelling

The in-gel *N*-linked glycan release was performed as described by Radcliffe et al. (Radcliffe et al., 2002). The dried gel pieces were incubated with 3 units of peptide-*N*-glycosidase F (Peptide-N⁴-(acetyl-beta-glucosaminyl)-asparagine amidase; PNGase F; EC 3.5.1.52; **Roche Diagnostics Ltd.**, East Sussex, UK) (1000U/ml) in 100 µl of 20 mM NaHCO₃, pH 7.0 for 16 hrs. at 37 °C. The *N*-linked glycans were extracted from the gel pieces by harvesting the supernatants with a series of gel incubations in a sonicating water bath for 30 min. (each incubation) at RT: first with 3 X 200 µl of water and then with 200 µl of acetonitrile, 200 µl of water and finally 200

µl of acetonitrile. The harvested supernatants were further concentrated by vacuum centrifugation to approximately 1000 µl. Each preparation was depleted of ions using 50 µl of H⁺ activated AG-50 X12 ion exchange resin by **Bio-Rad Laboratories Ltd.** [Hemel Hempstead Hertfordshire, UK] that was removed by filtering each preparation through a 0.45 µm **Millipore** LH filter. From each filtered preparation half was completely dried for 2-aminobenzamide (2AB) labelling. The labelling was carried out according to Bigge et al. (Bigge et al., 1995) using the Tag 2AB glycan labelling kit by **Ludger** [Oxford, UK]. Briefly, 5 µl of the 2AB labelling mixture was transferred to the tubes in which the glycans were dried, and following brief vortexing and centrifugation, the preparations were incubated at 65 ° C for 2 hours. The incubations were followed by short vortexing and spinning prior to the removal of the excess 2AB fluorophore from the reaction mixtures. The excess fluorophore was removed by ascending chromatography using 3 MM chromatography paper by **Whatman plc** [Brentford, Middlesex, UK] in acetonitrile. The labelled glycans were eventually collected in volumes of 100 µl of water ready for glycan analysis.

7.2.3 Separation of glycans

The two glycan pools from the heavy and the light chains were analysed by Normal Phase-HPLC (NP-HPLC) as described by Guile et al. (Guile et al., 1996) using a 4.6 x 250 mm TSK amide-80 column by **Anachem** [Luton, UK]. The separations were performed on a 2690 Alliance separation module [**Waters**, Milford, MA, USA] equipped with Waters temperature control modules and a Waters 474 fluorescence detector. The module system was controlled and operated via the Waters Millenium 32 computer software. A typical mixture would contain 80 µl of acetonitrile, 10 µl of water, 10 µl of sample. The separation was based on a linear gradient of 20-58% solvent A (50 mM formic acid adjusted to pH 4.4 with ammonium hydroxide) with solvent B (acetonitrile). The separation was monitored by fluorescence at 420 nm using excitation at 330 nm. The glycan profiles from the NP-HPLC were calibrated using as reference a dextran ladder prepared from hydrolysed and 2AB-labelled glucose oligomers (Guile et al., 1996). Glucose Units values (GU values) were assigned to the glycans, while the glycan structure and composition was predicted using a glycan database through the software PeakTime (E. Hart, R. A. Dwek, P. M.

Rudd [Glycobiology Institute, Department of Biochemistry, University of Oxford], unpublished work).

7.2.4 Analysis of glycans

In order to analyse the composition of both pools, digestion reactions were set up using a number of the following exoglycosidases supplied by **Glyco Inc** [Upper Heyford, UK]: *Streptococcus pneumonia* sialidase recombinant from *Escherichia coli* (NAN1, EC 3.2.1.18), *Arthrobacter urefaciens* sialidase (ABS, EC 3.2.1.18), bovine testis β -galactosidase (BTG, EC 3.2.1.23), bovine kidney fucosidase (BKF, EC 3.2.1.51) and β -*N*-acetylglucosaminidase (GuH, EC 3.2.1.30) cloned from *Streptococcus pneumonia* and expressed in *Escherichia coli*. The enzymes were used according to the manufacturer recommended concentrations and all the digestions were carried out using 50 mM sodium acetate, pH 5.5 as reaction buffer for 16 h at 37 °C. The reaction mixtures were separated using the NP-HPLC system and analysed using the methodology described in the previous section. Once all the profiles were collected and analysed, the construction of profiles for the N-glycan composition of each chain was carried out. The detailed interpretation of the glycan composition and calculation of the relative amounts (%) of the individual carbohydrate groups in each pool, were based on the collective comparison of the results derived from the analysis of all runs for each pool.

7.3 RESULTS

2AB-labeled glycans released from the heavy (Fig. 56) and the light (Fig. 57) chains were analysed separately and assigned structures from GU values and shifts with enzyme digest arrays. The structures and their amounts within the glycan pools of each chain in Table 23 correspond to the average population of fI in circulation. As fI is a circulating enzyme that is synthesized and secreted mainly from liver hepatocytes, it has been assumed that the majority of the enzyme population in serum will have a relatively uniform pattern of glycosylation.

The profiles of the undigested glycan pools of both chains were found to have a similar composition (Figs. 56, 57). For comparison purposes, both pools were analysed for their composition using the same combinations of digestions. No oligomannose structures were detected in either of the two chains with all the glycans found to terminate in galactose or sialic acids. The digestion with ABS indicated that both chains contain structures, comprising 95% and 96% of the heavy and the light chain glycans, with sialic acids. The disialylated (S_2) structures were found to be in excess over the monosialylated (S_1) ones: 55% over 40% for the heavy chain and 62% over 35% for the light chain (Table 23). Separate digestions of the glycan pools from both chains with NanI that removes only the non-reducing terminal $\alpha(2-3)$ linked sialic acids, refined the sialic acid linkage pattern for both chains and revealed that in both the mono- and di-sialylated structures, the non-reducing terminal sialic acids are mainly $\alpha(2-6)$ linked to galactose residues.

The subsequent digestions using combinations of ABS, BTG (removes galactose), BKF (removes core fucose) and GuH (removes terminal GlcNAc) revealed that both pools contained biantennary (A_2) and trinantennary (A_3) glycans with the biantennary structures being the predominant type in the heavy (86%) and light (94%) glycan pools (Figs. 56, 57) (Table 23). In quantitative aspects, the light chain glycan pool was found to contain 12% more biantennary glycans than the heavy chain (Table 23). The majority of the structures identified contained two galactose residues (G_2); 98% for the heavy chain and 99% for the light chain (Table 23). The dominant type of glycan identified in both chains was the $A_2G_2S_2$ [6,6] structure (Fig. 58), comprising

53% and 61% of the heavy and the light chain glycan pools (Table 23), respectively. The rest of the glycan structures were found to contain three galactose residues (G₃); approximately 2% for the heavy chain and 1% for the light chain (Table 23). Evidence for outer core fucosylation was not found, either for the heavy or for the light chain glycan pools.

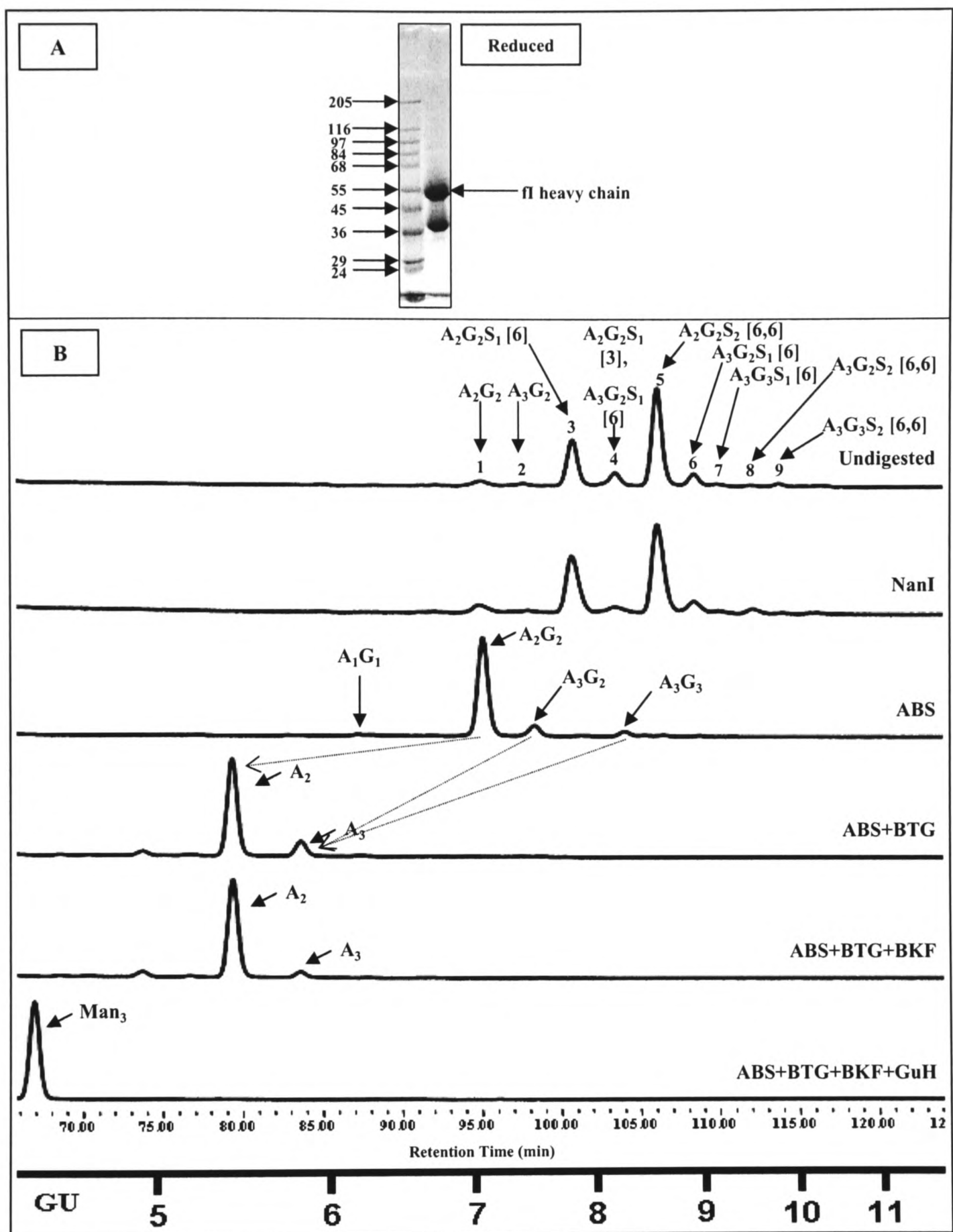


Fig. 56. N-linked glycan analysis for the heavy chain of human complement factor I. NP-HPLC exoglycosidase digestion profiles of the glycan pool from the heavy chain of human complement factor I. 2AB-labelled *N*-linked glycans were digested by exoglycosidases and analysed by NP-HPLC. Abbreviations of structures: all the *N*-glycans contain two core *N*-acetyl-glucosamines (GlcNAcs): Man_χ, number χ of mannose residues on core GlcNAcs, A_χ, number χ of antennae on the trimannosyl core: A₂, biantennary and A₃, triantennary; G_χ, number χ of galactose residues on antennae, S_χ, number χ of sialic acids on antennae. The profile indicates most notably the presence of core tri-mannose structures. Detailed information about percentage areas and Glucose Units (GU) is available in Table 23. The exoglycosidases used were: NanI (removes only non reducing terminal α(2-3) linked sialic acids), ABS (removes non reducing terminal α(2-3 & 2-6) sialic acids), BTG (removes β galactose), BKF (removes α core fucose) and GuH (removal of terminal β GlcNAcs). The diasylalted glycans are mainly α(2-6) linked, but may contain small amounts of α(2-3) which are masked in the analysis.

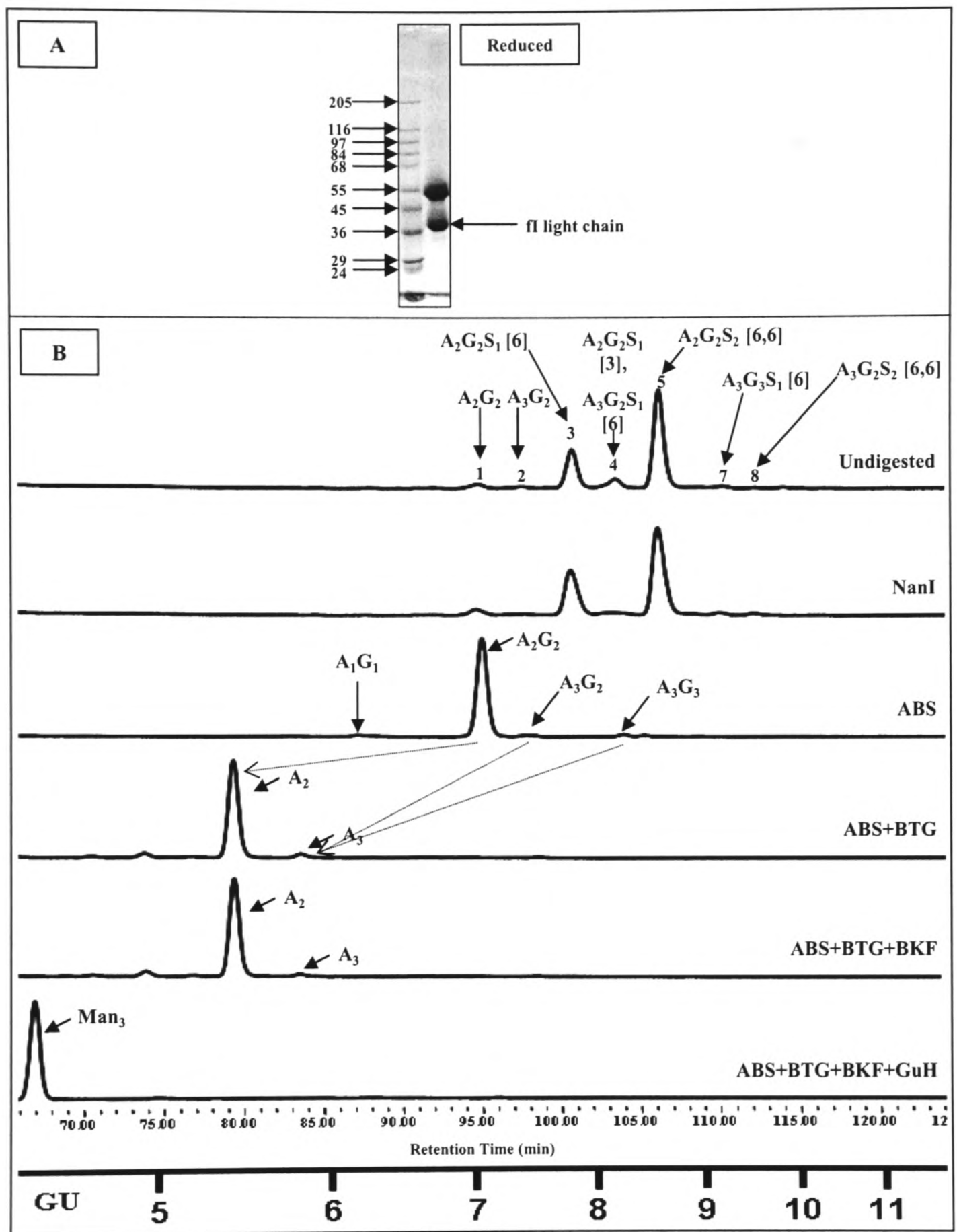


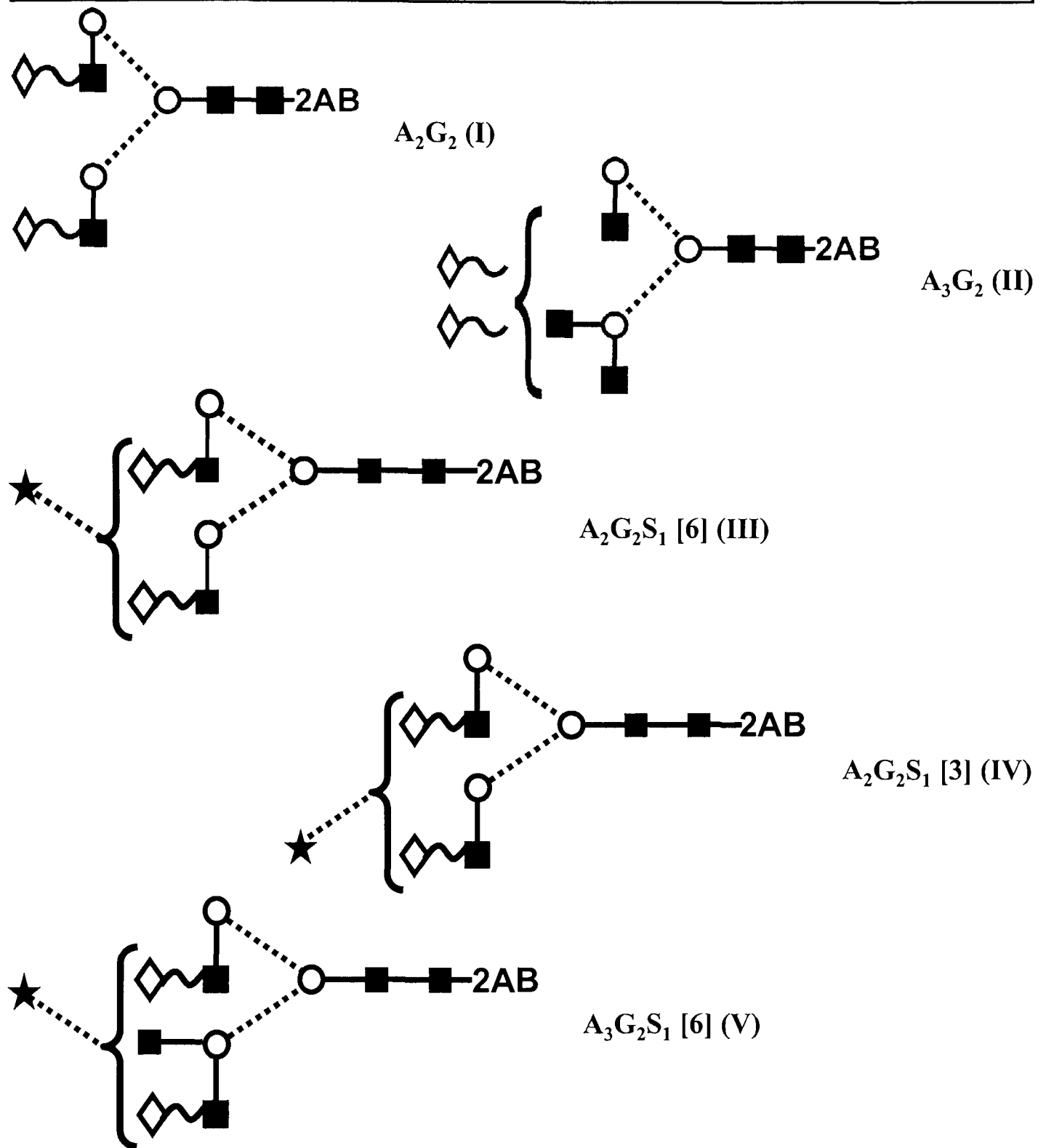
Fig. 57. N-linked glycan analysis for the light chain of human complement factor I. NP-HPLC exoglycosidase digestion profiles of the glycan pool from the light chain of human complement factor I. 2AB-labelled *N*-linked light chain glycans were digested by exoglycosidases and analysed by NP-HPLC as for the case of the heavy chain. For nomenclature see legend of Fig. 56.

Table 23. N-linked glycan analysis for human complement factor I

Peak No. ¹	Structure ²	Diagrammatical representation ³	Glucose Unit (GU) values		% Glycan pool	
			Heavy Chain	Light Chain	Heavy Chain	Light chain
1	A ₂ G ₂	I	7.1	7	3.9	3.5
2	A ₃ G ₂	II	7.4	7.3	1.4	1.5
3	A ₂ G ₂ S ₁ [6]	III	7.8	7.7	25.9	25.2
4	A ₂ G ₂ S ₁ [3], A ₃ G ₂ S ₁ [6]	IV V	8.2	8.1	4.0, 4.0	4.1, 4.1
5	A ₂ G ₂ S ₂ [6, 6]	VI	8.5	8.5	52.5	61.3
6	A ₃ G ₂ S ₁ [6]	VII	8.9	-	5.5	-
7	A ₃ G ₃ S ₁ [6]	VIII	9.2	9.1	0.6	1.3
8	A ₃ G ₂ S ₂ [6, 6]	IX	9.8	9.4	0.6	0.5
9	A ₃ G ₃ S ₂ [6,6]	X	10.2	-	1.4	-
<u>Heavy chain⁴</u>			<u>Light chain⁴</u>			
Biantennary-A ₂ : 86.3% Triantennary-A ₃ : 13.5% Total A: 99.8%			Biantennary-A ₂ : 94.1% Triantennary-A ₃ : 7.4% Total A: 101.4%			
G ₂ : 97.8% G ₃ : 2.0% Total G: 99.8%			G ₂ : 101.1% G ₃ : 1.3% Total G: 102.4%			
Monosialylated-S ₁ : 40.0% of which α(2-6) linked: 90% α(2-3) linked: 10% Total S: 94.5%	Disialylated-S ₂ : 54.5% mainly α(2-6) linked		Monosialylated-S ₁ : 34.7% of which α(2-6) linked: 90% α(2-3) linked: 10% Total S: 96.5%	Disialylated-S ₂ : 61.8% mainly α(2-6) linked		

1. Each number corresponds to a peak indicated in the panels of the glycan analysis.
2. Identified glycan structures are explained in Fig. 56.
3. Diagrammatical representations of the identified glycan structures are illustrated in the following pages.
4. The total glycan pool values (%) for the A, G and S units have been calculated separately for both chains.

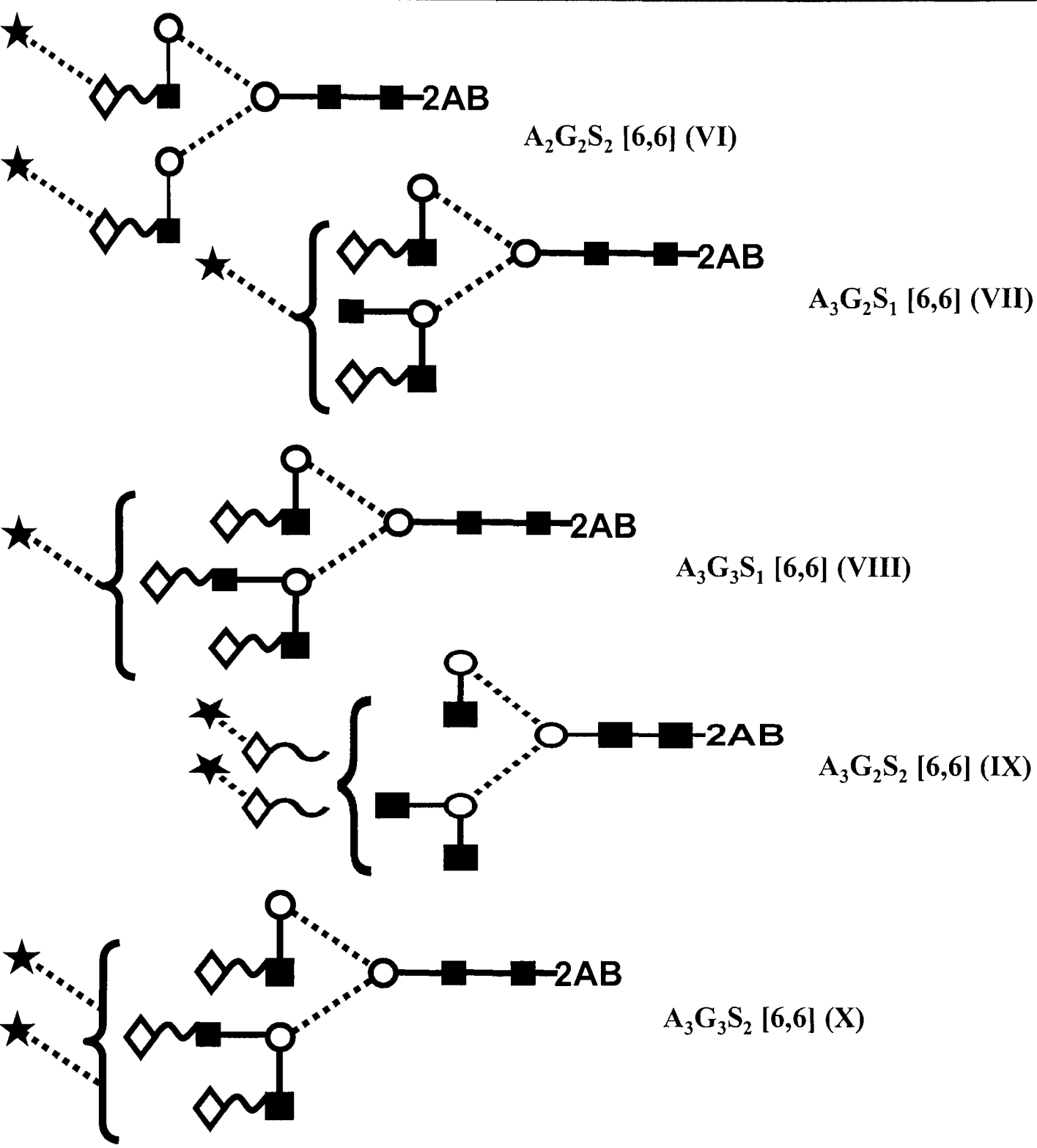
Diagrammatical representations of the glycan structures identified on human complement factor I (1)



Linkage position			
○	Mannose	◇	Galactose
■	GlcNAc	★	Sialic acid

- A. Each monosaccharide residue is symbolised by a distinct shape which is not related to its actual structure or size.
- B. The angle of each bond indicated the linkage position at the reducing terminus, while the type of the line corresponds to anomericity.
- C. Residues with N-acetylation are represented by filled shapes.

Diagrammatical representations of the glycan structures identified on human complement factor I (2)



Linkage position			
	Mannose		Galactose
	GlcNAc		Sialic acid
			β -linkage
			α -linkage
			Unknown β -linkage

- A. Each monosaccharide residue is symbolised by a distinct shape which is not related to its actual structure or size.
- B. The angle of each bond indicated the linkage position at the reducing terminus, while the type of the line corresponds to anomericity.
- C. Residues with N-acetylation are represented by filled shapes.

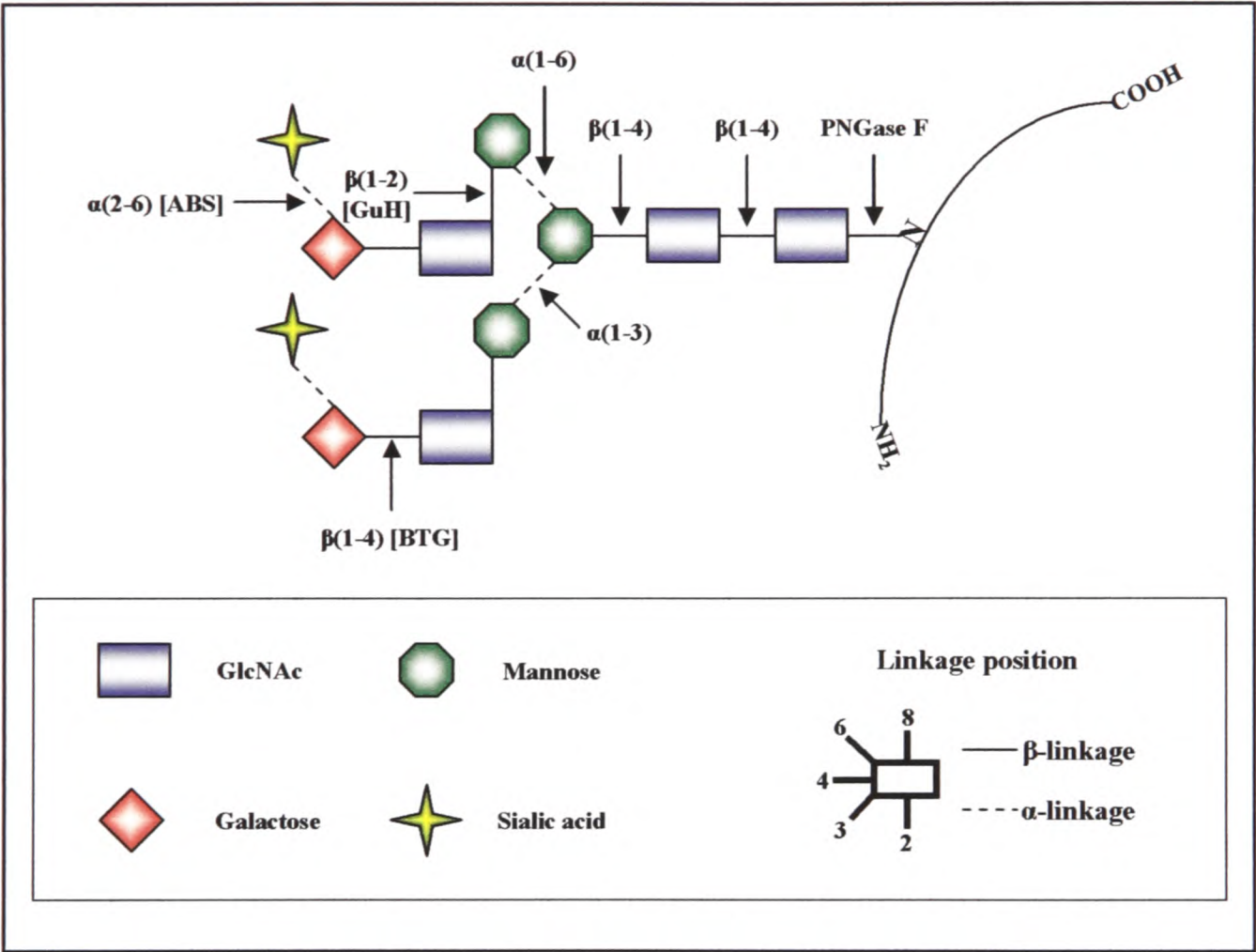


Fig 58. Diagrammatical representation of the A₂G₂S₂ glycan structure. Composition analysis of the total *N*-linked glycan pools derived from the heavy and light chains of human fI, indicated that the A₂G₂S₂ structure is the dominant type (Table 23). *N*-linked oligosaccharide units in glycoproteins consist of a common core of three mannose and two *N*-acetylglucosamine (GlcNAc) residues. Additional sugar residues are added to give rise to a broad range of glycan structures. The arrows indicate the types of linkages that connect the various residues within the glycan, while in square brackets, next to each bond name, the initials correspond to the exoglycosidase [section 7.2.4] that cleaves the particular bond.

7.4 DISCUSSION

The results from the current analysis of the heavy and light chain glycans of human complement factor I are in agreement with the earlier report which indicated that human fI contains predominantly complex biantennary glycans the majority of which are disialylated (Ritchie et al., 2002). The analysis described in this chapter refined the earlier findings further by assessing the composition of the glycan pool of each chain separately;

Overall, this study shows that both chains contain complex glycans as opposed to oligomannose glycans. This suggests that all the precursor glycans of the structure $\text{GlcNAc}_2\text{Man}_9\text{Glc}_3$ that are initially attached to the fI polypeptide (Nilsson and von Heijne, 1993) in the endoplasmic reticulum (ER) of the hepatocyte cells, do undergo, in both the ER and the Golgi complex organelles, further processing towards the various complex glycan structures detected. An individual protein may interact with the glycosylation machinery in the Golgi complex to develop certain motifs, like the addition of charged groups (e.g. sialic acids), that are important for its specialised recognition function (Rudd and Dwek, 1997). As fI is post-translationally activated within the Golgi complex (section 1.3.4), any variability among the glycan structures identified, could be linked to the conformational changes that the cleavage of the $\text{R}^{321}\text{-I}^{322}$ induces. The particular cleavage is of high importance not only for the SP domain, but also for the heavy chain as the fI molecule adopts the mature catalytic bilobal conformation (section 1.6). The processing of a glycan on a glycoprotein by glycosidases in the post-translational phase is a dynamic process, the extent and patterns of which are affected by the level of accessibility of the particular substrate glycan in the various stages of the enzyme cascade editing. As the degree of glycan accessibility is governed by the conformational status of the glycoprotein in the various stages, it appears that the processing of the glycan(s) post-translationally is facilitated significantly by the conformational status of the polypeptide backbone.

As the fI molecules enter the Golgi complex from the ER, the activation of the zymogen induces changes in conformation that may alter the positioning of the loops that bear the glycans and together their accessibility to the glycosidases (remove

mannose residues) and glycosyl transferases (attach carbohydrate groups) (e.g. steric hindrance). As the various glycosidases have distinct narrow specificities, the processing of a glycoprotein through various complex glycosidation cascade pathways depends both on the recognition and accessibility of the glycans by the glycosidases and glycosyl transferases in various stages of the editing. If some glycans are more accessible than others on the same glycoprotein, they are likely to be processed more extensively as is the case for the fI glycans. The processing occurs in sequential steps in which the product of a glycosylation event will provide the substrate for the next enzyme in the pathway. The fact that both chains of fI share to a large degree similar glycan structures suggests common processing events, while differences are consistent with a role for the local 3D structure of the protein in guiding the processing.

In aspects of glycan structure, the biantennary glycans are in both chains in large excess compared to triantennary, while it is of interest to note the difference (8%) in the excess of biantennary structures in the light chain glycan pool compared to the one of the heavy chain (Table 23). Although there is no detailed information about the role of the fI glycans in the interactions with the substrate and the cofactor to allow assessment of the importance of the different glycan structures in these interactions, the fact that these interactions are ionic in nature may allow a role for the sialic acid groups on both chains. At physiological pH the sialic acids are negatively charged groups that could affect the interactions of fI with the cofactor and the substrate; the interactions within the C3b-fH-fI complex have been experimentally demonstrated to be ionic strength-dependent (sections 1.5.4 and 1.5.5). At physiological pH, any one fI molecule may carry as many as 12 negative charges due to sialic acid groups alone (disialylated glycans), or may have 6 or less such charges. Further detailed experimental deglycosylation work is necessary to establish whether the fI glycans do have a role in the interactions as has already been proposed (Ullman et al., 1998). In addition, at the structural level, modelling work could shed light towards the interaction of fI glycans with known sites of interaction on C3b and fH.

Chapter Eight

CHAPTER 8: TOWARDS THE ATOMIC STRUCTURE OF HUMAN COMPLEMENT FACTOR I

The experimental work described in the chapter was carried out mainly in the Laboratory of Molecular Biophysics, Department of Biochemistry, University of Oxford in collaboration with Dr. Pietro Roversi who was exclusively in charge of the crystallization trials, the analysis of the diffraction data and developed the partial deglycosylation protocol for fI. The author contributed in the purification of fI as well as in other aspects of protein chemistry, namely the generation of deglycosylated fI and functional assays for the activity of enzyme preparations from dissolved crystals. The author wishes to acknowledge Dr. Susan M. Lea for her guidance and use of experimental facilities, as well as Mr. Max D. Crispin from the Institute of Glycobiology, Department of Biochemistry, University of Oxford for his advice on the design of a partial deglycosylation strategy for human fI.

8.1 INTRODUCTION

The most detailed model of fI to date is based on X-ray and neutron low angle scattering and homology modelling (Chamberlain et al., 1998) (Fig. 17) (section 1.6). This low resolution model proposes fI as a V-shaped molecule with the first three N-terminal domains of the heavy chain forming a compact triangular arrangement, where the first and third domain are linked by the disulphide bond formed between C¹⁵ and C²³⁷. Together with the SP domain, this defined two globular entities shaping a bilobal structure in factor I as originally observed using transmission electron microscopy (DiScipio, 1992). The two pairs C¹⁵ and C²³⁷ and C³⁰⁹ and C⁴³⁵ form inter-domain disulphide bridges that support the bilobal backbone of the enzyme (Fig. 17).

This chapter summarises the results of the crystallisation trials so far, which have led to crystal needles of human fI diffracting to 4 Å. Dissolved crystals were found to contain proteolytically active enzyme. Partially deglycosylated human fI yielded

crystals of similar needle morphology to the native ones, although their diffraction has not been examined yet.

8.2 MATERIALS AND METHODS

8.2.1 Sample preparation

8.2.1.1 Native human factor I

Native human complement factor I was purified to homogeneity as described in detail in section 2.3.2. The purified enzyme was dialysed against a desired buffer and concentrated (section 2.4.1) further; the preparations were used either directly for the crystallisation screens, or were further processed prior to plating.

In independent experiments, the composition of the purified native human fI population was also examined using anion exchange and IsoElectricFocusing (IEF); these experiments examined the certain degree of charge heterogeneity of the material tested in the crystallization trials. Highly pure protein preparations isolated from natural sources, can contain, at the molecular level, a mixture of individuals varying in primary sequence (polymorphic variants) (section 1.3.5) and/or in glycosylation (Chapter 7). For the crystallisation of protein macromolecules, a high degree of sample homogeneity is needed for regular packing of the target protein into crystals.

The anion exchange was originally tested for the removal of contaminants that co-eluted with fI in size exclusion chromatography during the purification of batch 3 (Table 24). Factor I (10 mg) was dialysed extensively against 25 mM Tris-HCl, 70 mM NaCl, pH 7.4 (Buffer A) and then loaded on a Mono-Q column (HR 5/5, 5 mm diam x 5 cm) pre-equilibrated for 25 CVs with the same buffer on the FPLC system. Using a continuous flow rate of 1ml/min, the column was washed extensively for 25 CVs for the removal of any unbound material and then using 25 mM Tris-HCl, 1 M NaCl, pH 7.4 as buffer B, the column was eluted with a 20 ml linear gradient from 70 to 1000 mM NaCl. Fractions of 1 ml were collected. Upon completion of the run,

selected fractions were analysed by SDS-10% PAGE to assess the quality of the unbound and eluate material.

Factor I from the same batch that was subjected to anion exchange (batch 3), was purified to homogeneity and then subjected to IEF using the PhastSystem apparatus **Amersham Biosciences UK Limited** [Chalfont St Giles, Bucks, UK]. The analysis was carried out according to the PhastSystem separation methodology:[http://www1.amershambiosciences.com/aptrix/upp00919.nsf/\(FileDownload\)?OpenAgent&docid=6A40D56559A0E8ABC1256EB40044A906&file=80132015.pdf](http://www1.amershambiosciences.com/aptrix/upp00919.nsf/(FileDownload)?OpenAgent&docid=6A40D56559A0E8ABC1256EB40044A906&file=80132015.pdf). A typical experimental set up of the PhastSystem is illustrated step by step in <http://www1.amershambiosciences.com/pdfs/phastsys/phguide.pdf>. Briefly, 8 µl of fI (7.6 µg/µl) was analysed via SDS-PAGE under non-reducing conditions on an IEF 5-8 PhastGel in parallel with another sample containing pI standards. The gel was run in 3 successive steps; step 1: 2000 V, 0.20 mA, 3.5 W at 15 ° C for 75 Vh, step 2: 200 V, 0.2 mA, 3.5 W at 15 ° C for 15 Vh and step 3: 2000 V, 5 mA, 3.5 W at 15 ° C for 510 Vh. Upon completion of the run, the gel was fixed for 5 min at 20 ° C, washed for 2 min at 20 ° C, Coomassie blue stained for 10 min at 50 ° C and then washed again for 10 min at 50 ° C.

8.2.1.2 Deglycosylation of human complement factor I

During the crystallisation trials, native human fI (purified as described in section 2.3.2) was also subjected to complete or partial deglycosylation. The deglycosylation was undertaken to reduce the heterogeneity of the protein sample and improve the quality of the initial native protein crystals. The natural glycosylation introduces heterogeneity in the native human serum population of fI molecules and thus can prevent crystal growth; moreover, reducing the length of the glycans should ease the formation of crystal contacts involving them. The deglycosylation removes the molecular heterogeneity that the variability in the glycan composition introduces and contributes to the reduction of the total molecular weight of the target protein. The removal of molecular heterogeneity ensures, from batch to batch, a more uniform and controlled behaviour of the protein in the trials. Two protocols for the complete and partial deglycosylation of fI have recently been established. From the two

Table 24. Factor I preparations used in crystallization trials

Batch No.	Preparation Date	Buffer ¹	Concentration	Plates
I	Feb 2003	15 mM HEPES, 135 mM NaCl, 0.5 mM EDTA, pH 7.2	10 mg/ml	1-8
II	Dec 2003	25 mM HEPES, 140 mM NaCl, 0.5 mM EDTA, pH 7.4	3.2 mg/ml	9-23
III	Mar 2004	25 mM Tris-HCl, 140 mM NaCl, pH 7.4	2.5 mg/ml	24-33
			7.6 mg/ml	34-73
IV ²	May 2004	25 mM Tris-HCl, 140 mM NaCl, 0.025% OG ³ , pH 7.4	0.06 mg/ml	43, 44, 45, 55, 56, 57B
V ⁴	Feb 2005	20 mM MES, 100 mM NaCl, pH 5.0	0.8 mg/ml	74-77

1. Different buffers were used from batch to batch in an effort to optimise the best conditions for crystallisation.
2. This batch of fI was subjected to complete deglycosylation using PNGase F.
3. OG: Octyl- β -D-glucopyranoside
4. This batch of factor I was subjected to partial deglycosylations using ABS, BTG and GuH.

deglycosylated fI forms generated, mainly the partially deglycosylated form was produced in higher amounts and used in the most recent screenings. Native human fI had mainly been used in the preliminary screenings (Table 24).

Based on the existing (Goldberger et al., 1984) and recently gathered information about the glycosylation of human fI (Chapter 7), for its complete deglycosylation, a preliminary digestion timecourse of fI with PNGase F was initially set up. PNGase F is an amidase that cleaves between the innermost GlcNAc and Asparagine (N) residue of high mannose, hybrid, and complex oligosaccharides from glycoproteins (Fig. 58). A mastermix containing 35 µg of human fI (stock solution of 3.18 µg/µl in 25 mM HEPES, 145 mM NaCl, 0.5 mM EDTA, pH 7.4 (HBS)) and 1.12 Units of PNGase F (diluted 25 X from stock to prepare a working solution of 0.04 U/ µl) was made up using HBS buffer to 105 µl with a final concentration of 0.01% SDS and incubated at 37 ° C for intervals of 0, 1, 2, 4, 8 and 16 h. At the end of each time interval two aliquots of 7.5 µl each were removed and kept aside in sample buffer for SDS-PAGE analysis. The SDS-PAGE analysis results from the preliminary digestion timecourse were used for the setting up of a batch deglycosylation reaction. A mixture containing 2.5 mg of human fI (stock solution of 0.833 µg/µl in 25 mM HEPES, 145 mM NaCl, 0.5 mM EDTA, pH 7.4) and 80.0 Units of PNGase F (diluted 25 X from stock to prepare a working solution of 0.04 U/µl) was made up using HBS buffer to 7,500 µl with a final concentration of 0.01% SDS and incubated at 37 ° C for 24 hours. Upon completion of the incubation, the mixture was dialysed extensively against PBS, 0.5 mM EDTA, pH 7.4 in the presence of another dialysis sac containing 2 ml of packed volume of H⁺ activated De-Acidite FF (SRA 69) anion exchange resin [**The Permutit company Ltd.**, London, UK]. The dialysis was selected as the most efficient method of ensuring the complete removal of the anionic SDS detergent from the reaction mixture. After dialysis, the deglycosylated enzyme was purified using size exclusion chromatography. The reaction mix (7.5 ml) of a concentration of 0.33 µg/µl, was loaded on a Superdex S75 (26/60, 26 mm diam x 60 cm) column pre-equilibrated with the running buffer 25 mM Tris-HCl, 140 mM NaCl, pH 7.4. Fractions of 2 ml were collected. Upon completion of the run, selected fractions were analysed by SDS-10% PAGE to assess the quality of the eluate.

Partial deglycosylation was carried out as an alternative to the complete deglycosylation. As a strategy it is milder for fI, since the complete removal of glycans from a protein or enzyme may irreversibly affect the structural stability and conformation of the molecule. The partial deglycosylation used for fI is based on the use of a combination of the three exoglycosidases ABS (Sialidase), BTG, (Galactosidase) and GuH (*N*-acetylglucosaminidase) that were also employed in the *N*-linked glycan analysis of human complement fI (Chapter 7). These enzymes remove sialic acid, galactose and *N*-acetylglucosamine residues from the reducing end of the *N*-glycans, leaving fI with homogeneous glycans containing only the tri-core mannose and the two GlcNAc residues (the Man₃GlcNAc₂ core) (Fig. 58). A mixture containing 1.9 mg (approximately 0.021 μ moles) of human fI (5.53 μ g/ μ l in 20 mM MES, 100 mM NaCl, pH 5.0), 0.02 Units of ABS (stock of 0.02 U/ μ l), 0.02 Units of BTG (stock of 0.005 U/ μ l) and 0.02 Units of GuH (stock of 0.04 U/ μ l) was made up to 564.5 μ l with a final concentration of 95 mM sodium acetate, pH 5.0. The reaction mixture was incubated at 37 ° C for 3 hours and then transferred to 4 ° C to quench the reactions. The deglycosylated fI was re-purified for further studies by size-exclusion chromatography using a Superdex 200 16/60 (16 mm diam x 16 cm column) as described before (section 2.3.2.4). The sample was diluted to 2 ml using 20 mM MES, 100 mM NaCl, pH 5.0 and run down the column that was pre-equilibrated in the same buffer. Upon completion of the purification, selected fractions were pooled and concentrated to 0.8 μ g/ μ l using a Vivaspin ultracentrifugal filter concentrator [10,000 MWCO] (section 2.4.1).

8.2.1.3 Complexes of human factor I with inhibitors

For some of the crystallisation trials, the native and partially deglycosylated forms of fI were mixed with selected additives prior to plating. The selected compounds were Pefabloc SC and Suramin that had been shown to strongly inhibit both the proteolytic and the amidolytic activities of fI (Chapter 3). To date, Pefabloc SC has only been plated with native fI; briefly, 5.4 mg of Pefabloc SC (MW 240) was dissolved by gentle mixing in 208 μ l of MQ H₂O to prepare a Pefabloc SC stock solution of 10 mM; two μ l of this stock solution was immediately added to 50 μ l of 28

μ M fI protein solution to obtain a final inhibitor concentration of 0.4 mM. Native fI with Pefabloc SC was tested for crystallisation in plates 32, 53, 54, 69 and 70. For the case of Suramin, 57 mg of the compound was dissolved in 200 μ l of 20 mM MES, 100 mM NaCl, pH 5.0 to prepare a stock solution of 200 mM; two μ l of this stock solution was added to 50 μ l of the native fI protein sample. Similarly, for the partially deglycosylated fI, an 18 mM Suramin stock solution was prepared in 25 mM Tris, 100 mM NaCl, pH 7.3, while 2 μ l of this stock solution was added to 50 μ l of deglycosylated fI at 0.7 μ g/ μ l (approximately 10 μ M) to give a final concentration of Suramin of 0.66 mM. Factor I with Suramin was tested for crystallisation in plates 71 (native fI) and C8-13/14, 76, 77, 78 (partially deglycosylated fI).

8.2.2 Operation of the crystallisation robot

All the crystal screens were prepared using the protein crystallisation robot Tecan Genesis 150 [Tecan, Theale, UK] (Fig. 59) which has been configured for semi and complete automation of protein crystallisation methods. It facilitates efficient screening of large numbers of potential crystallisation conditions with very small protein sample volumes (as little as 0.2 μ l of sample per drop). The system automates complete set-up of sitting drops increasing both productivity and analytical precision. The robot automates plate assembly, transferring buffers, detergents and other additives from tubes, vials, or microplates to crystallography plates, thus ensuring that they are mixed well.

Typically, 96-well plates (CrystalQuick low profile plates) [Greiner Bio-One Ltd, Stonehouse, Gloucestershire, UK] were used. During the process of preparing the crystallization trays (plating), the protein sample was split in four 650 μ l Eppendorf tubes that were cooled by a metal block immersed in ice. Four robot tips were used for all the pipetting. About 50 μ l of protein solution is enough for screening 96 conditions in a plate using 0.2+0.2 μ l drops. All water used for the buffer solutions was deionised and degassed. A 1:1 isopropanol:water mix was used to clean the robot dispensing tips.



Fig 59. The Tecan Genesis 150 robot used for the preparation of the crystal screens.

Picture taken from: <http://biop.ox.ac.uk/oxford/s9home/facilities.html>

Standard screens were available as a starting point, but it was also possible to set up a range of custom grid screens, through the operative Gemini version 4.00 [1998-2002, **Tecan Software GmbH**, Theale, UK] computer software, to optimise promising crystallisation conditions. The user specifies the chemicals to be mixed and their quantities *via* scripts that can be adapted to the specific screen at hand. Plate sealing was performed by hand using Starseal Advanced Polyolefin film [**Star Lab**, Ahrensburg, Germany]. The plates are incubated in a controlled temperature environment (ranging from 4 to 20 °C) and inspected by eye using an MZ8 or MZ12 Leica microscope [**Leica Microsystems Ltd.**, Milton Keynes, Buckinghamshire, UK]. Images of all the drops in a plate can be acquired with Leica software (Leica QWin) interfaced to a semi-automatically operated camera mounted on an MZ16 Leica microscope (Fig. 60), analysed and stored electronically.

8.2.3 Crystallisation trials

The concentration of the protein used in the crystallization trials varied between 0.06 and 10 mg/ml depending on the batch. A summary of the five batches used, their buffers and protein concentration is presented in Table 24. Initial crystallisation trials were carried out using sparse-matrix (Jancarik and Kim, 1991) and reverse screening (Stura, 1999; Stura et al., 1992; Stura et al., 1994) methodologies. The sparse matrix approach enabled the sampling of a large number of combinations of various pH, precipitants and additives; the reverse-screening approach facilitated the characterisation of the protein solubility at various pH values using a few typical PEG and salt precipitants. In the initial crystallisation trials, more than 600 different conditions have been tried using commercially available screens (Table 25) as well as custom prepared ones. Table 26 summarizes the conditions that yielded crystals and were pursued further for optimization. All screens were plated as sitting drops equilibrated against mother liquor using the vapor diffusion technique in sitting drops; all solutions were plated using the Tecan Genesis 150 robot. For each condition, 0.2 µl of protein solution was mixed with the same volume of the mother liquor solution



Fig 60. The Leica MZ16 microscope system used for the recording of images of selected drops during the crystal screen trials.

Picture taken from: <http://biop.ox.ac.uk/oxford/s9home/facilities.html>

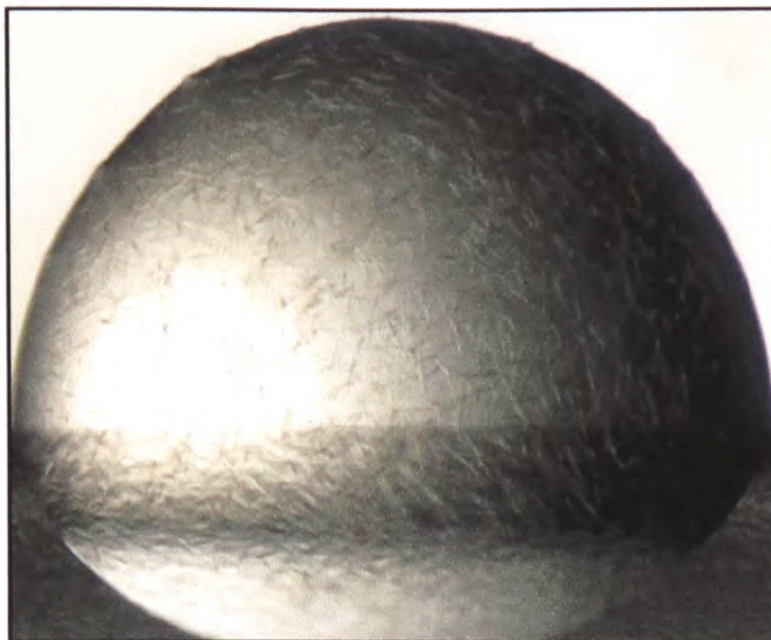
Table 25. Summary of commercially available kits used for the initial crystallisation screenings of human complement factor I

Screens	Manufacturer	No. conditions
Structure Screens I & II	Molecular Dimensions Ltd. [Soham, Cambridgeshire, UK]	96
Stura Footprint I & II	As above	96
Wizard Screens I & II	Emerald BioSystems, Inc. [Bainbridge Island, WA, USA]	96
Natrix	Hampton Research [Aliso Viejo, CA, USA]	48
Crystal Screen Cryo	As above	48
Crystal Screen Lite	As above	48
MembFac	As above	48
NaCl	As above	24
Ammonium Sulphate	As above	24
PEG/LiCl	As above	24
PEG 6000	As above	24
MPD	As above	24
Quick Screen	As above	24

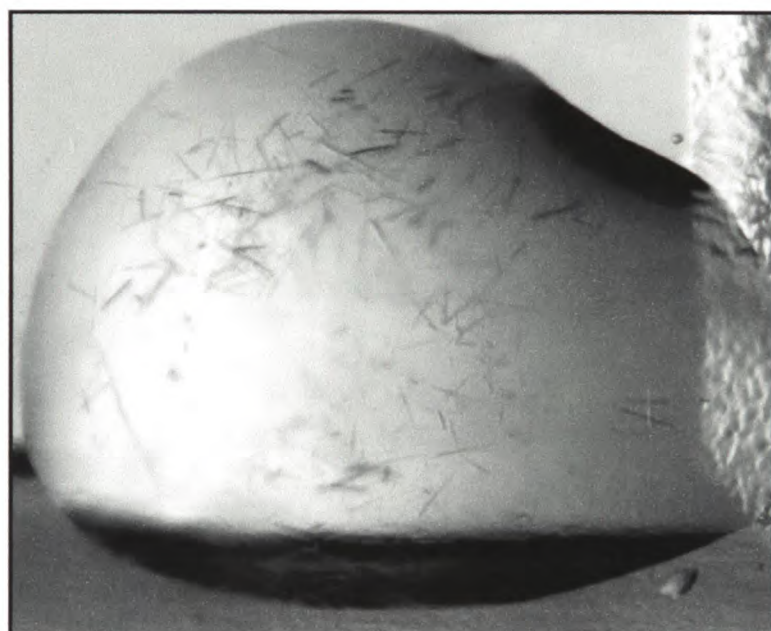
Table 26. Summary of the conditions that yielded crystals of human complement factor I¹

Condition No.	Plate & Drop	Screen & Condition	Image ²
1	Plate 20 Drop A3	Stura Footprint Screen Condition I-4 33% PEG 600, 0.2 M Imidazole Malate pH 5.5	A
2	Plate 23 drop B10	Screen around condition 1 29.2% PEG 600 ³ , Imidazole Malate 10 mM pH 4.3	B
3	Plate 26 drop A3	Screen around condition 1 28.7% PEG 600, Imidazole malate 0.075 M pH 4.2	C
4	Plate 30 Drop G12	Screen around condition 1 30% PEG 600, 2.65% glycerol Imidazole Malate 10 mM pH 4.5	D
5	Plate 31 Drop A9	Screen around condition 1 28.64% PEG 600, Imidazole Malate 10 mM pH 4.3, 2% isopropanol	E
6	Plate 32 Drop A9	Screen around condition 1 28.6% PEG 600, Imidazole Malate 10 mM pH 4.3 fI 2.45 mg/ml+PEFABLOC 1mM	F The crystal had an approximate size of 150x20x20 µm and was shot at the ESRF 11 March 2005.
7	Plate 74 Drop H3	Stura Footprint Screen Condition II-16 30% PEG 4000, 0.2 M Imidazole Malate pH 6.0 Partially deglycosylated fI:0.7 mg/ml	G
8	Plate C8-13 Drop H11	Molecular Dimensions Structure Screen I-45 Sodium Formate 4.0 M, Suramin 0.66 mM Partially deglycosylated fI at 0.7 mg/ml Grown at 12 ° C	H These crystals are about 20x20x20 µm and have not been tested for diffraction to date

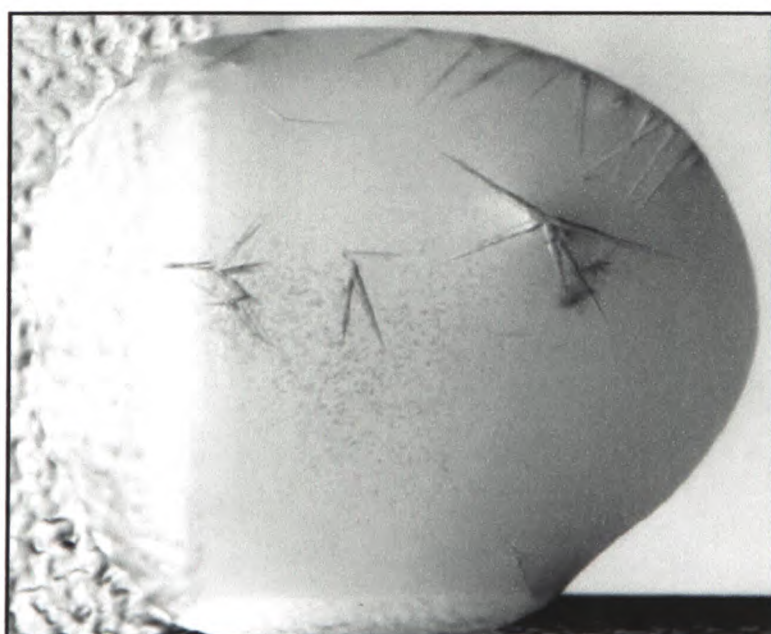
1. All drops were 0.2 µl protein solution + 0.2 µl screen solution. The protein concentration was 2.45 mg/ml and the crystals were grown at 16 ° C unless otherwise stated.
2. **ALL IMAGES ARE ILLUSTRATED IN THE FOLLOWING PAGES.**
3. 28-30% PEG600 provided cryoprotection



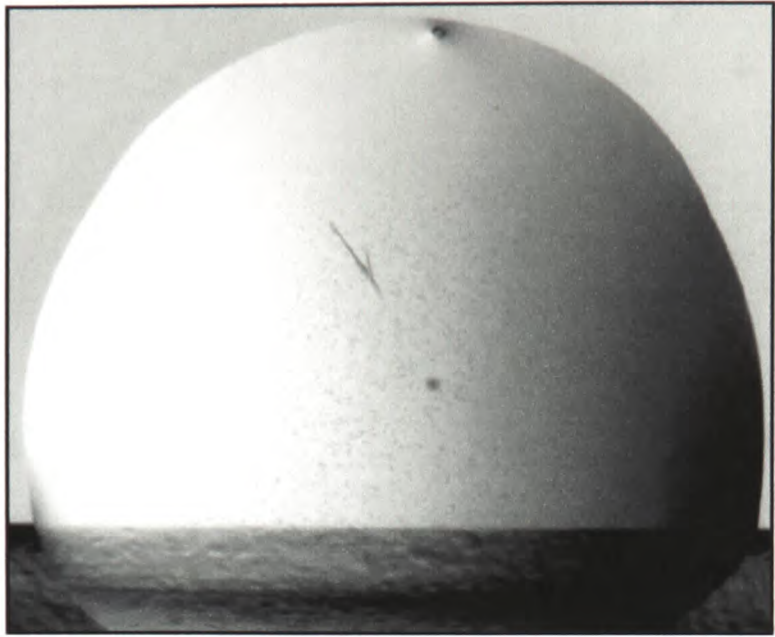
A



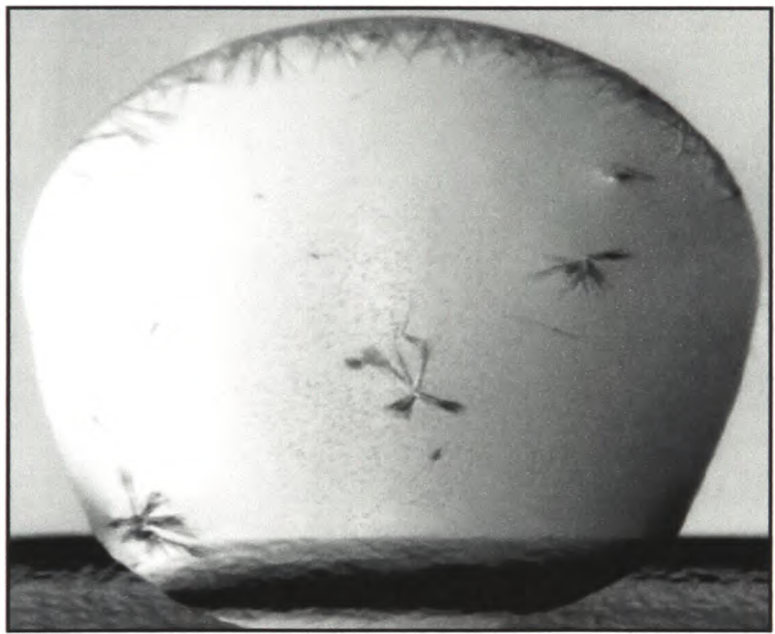
B



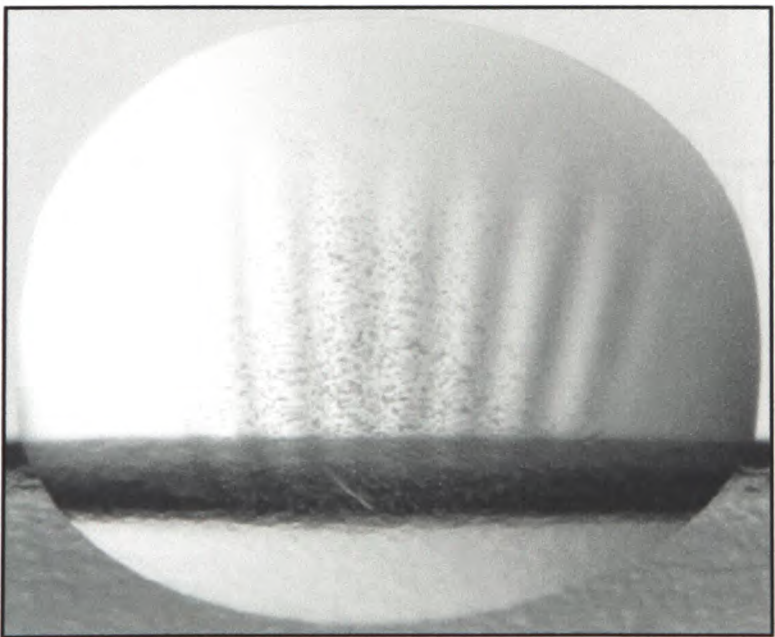
C



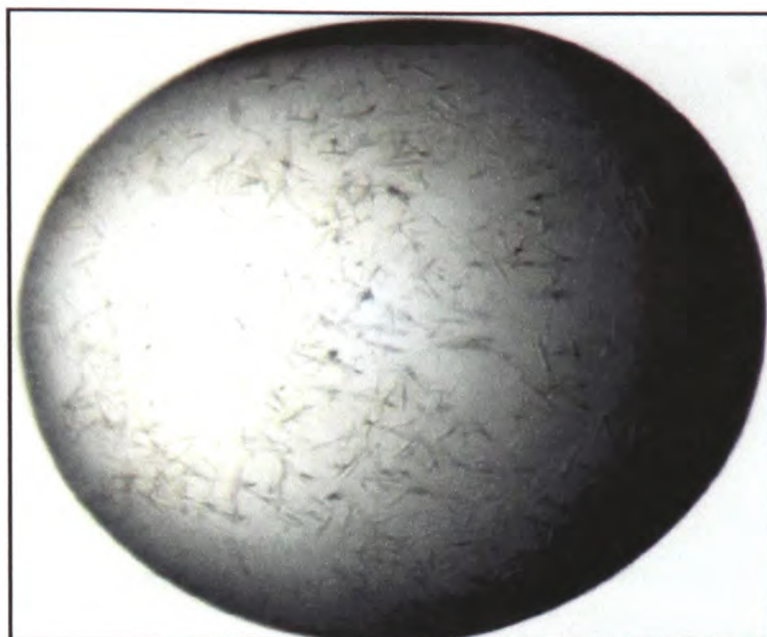
D



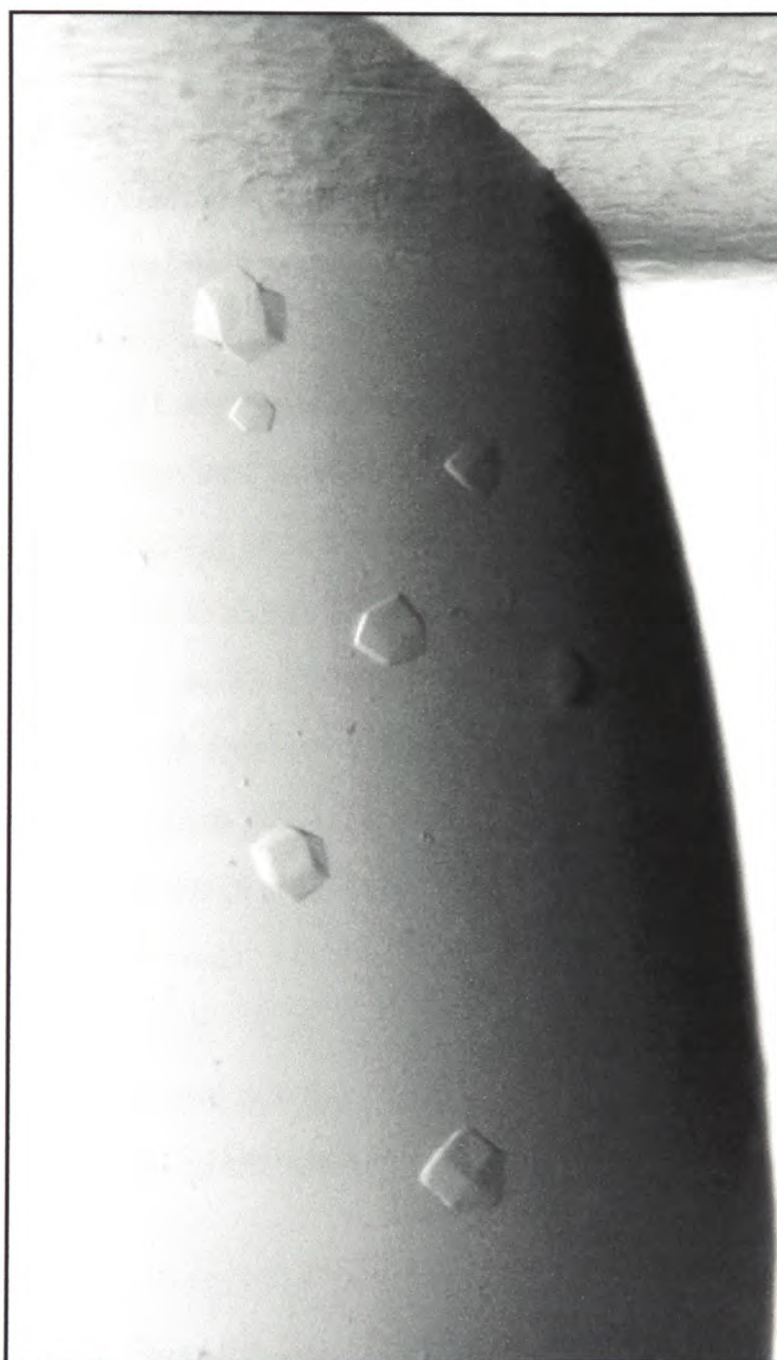
E



F



G



H

to yield a final drop size of 0.4 μ l. The plates were eventually stored at temperatures ranging from 4 to 20 ° C.

8.2.4 Functional assay

To examine whether the needle-like shaped crystals formed in the early crystallisation trials were composed of protein, crystals were removed from their sitting drops for further analysis. The removal of the crystals was carried out via pipetting with care to avoid disturbing the balance of the plate and/or any other neighbouring wells; all crystals were repeatedly washed in 0.6 ml Eppendorf-type tubes using fresh stocks of the original mother liquor. Eventually, the washed needles were harvested by mild-centrifugation and the extra supernatant liquor was removed by pipetting. All the needles were dissolved in 10mM potassium phosphate, 0.5mM EDTA, 0.1 % Tween 20, pH 6.2 buffer. The OD_{280nm} of the solution was measured and found to be 0.263, suggesting that the crystals contained protein (0.175 μ g/ μ l). The quality of the dissolved needles was analysed by SDS-10% PAGE both under reducing and non reducing conditions using native human fI as reference. Once the analysis confirmed that the crystals contained fI, the dissolved enzyme was further tested in the proteolytic assay using a timecourse of incubation at 37 ° C from 0 up to 480 min; seven identical reaction mixtures were prepared, each containing dissolved fI at a final concentration of 1.25 μ M (0.525 μ g in each reaction mixture). The composition of the mixtures was as described in section 3.2.1. Untreated fI was also used at an equimolar final concentration as a positive control. At the end of the incubation, the reaction mixtures were analysed by SDS-8.5% PAGE and autoradiography. The total activity and chemical behaviour of the dissolved enzyme was compared with that of the untreated fI.

8.2.5 Cryoprotection and diffraction

About 20 crystal needles were mounted on nylon loops by scooping them out of the drop, and flash-frozen by plunging them in liquid nitrogen without the need for cryoprotectant (28-30% PEG 600 provides cryoprotection). The crystals were stored

at 100 K and transferred to the synchrotron sources in Daresbury (SRS) and Grenoble (ESRF) for examination of the diffraction patterns. Table 27 summarizes the diffraction experiments.

Table 27. Summary of diffraction experiments on human complement factor I crystals

Date	Beamline (Location)	Crystals	Diffraction
30.05.2004	SRS 9.6 (Daresbury, UK)	T26-G12	None observed
		T26-G5	
		T11-E11	
		T31-D6	
		T26-C8	
		T24-C10	
		T26-C5	
		T32-G3	
31.07.2004	ESRF ID14-4 (Grenoble, Fr.)	T28-A4	None observed
		T24-B10	As above
		T61-E5	Weak diffraction
		T24-C9	Some images
		T32-F10	Weak diffraction
		T40-F5	Tiny cubes-no diffraction
		T32-E10	Protein
		T23-B10	None observed
		T32-G3	Diffraction
		T18-B10	None observed
04.12.2004	ESRF ID14-3 (Grenoble, Fr.)	T26-C10	4 Å diffraction
		T26-C1	
16.02.2005	ESRF ID29 (Grenoble, Fr.)	T26-G11	Poor diffraction
		T30-E3	4 Å diffraction
10.03.2005	ESRF ID29 (Grenoble, Fr.)	T30-E4	4 Å diffraction
		T26-H7	Iced
		T26-A3	Low Res Diffr.
		T23-B10	Low Res Diffr.
		T32-A9	9 Å diffraction

8.3 RESULTS AND DISCUSSION

8.3.1 Native human factor I variants

The anion exchange, originally employed for the removal of plasma contaminants from the fI preparation during the purification of batch 3, was successful. The major contaminant removed was eluted in peak 4 (Fig. 61) and identified later via N-terminal sequencing as prothrombin. The SDS-10% PAGE analysis (Fig. 62) confirmed that the majority of the fI present in the start material did not bind to the resin at pH 7.4. A small proportion however did bind, indicating that within the native enzyme population, a number of variants with more negative surface charges compared to the average exist. Prothrombin has a theoretical pI value of 5.24 and at the physiological pH of 7.4, it is more negatively charged than fI; the stronger negative charge explains the elution of prothrombin in higher NaCl concentration compared to fI which eluted in peaks 1-3 within the 200-300 mM NaCl range; Although this run did not originally aim in separating any natural occurring native fI variants, it was clear that there were at least two more fI variants present in peaks 1-3. The presence of differently charged species *in vivo* was decided to be further investigated using IEF.

The IsoElectricFocusing (IEF) carried out after the purification of fI from batch 3, indicated the presence of at least four differently charged species in the native fI population (Fig. 63). This preliminary experiment confirmed the early observations from the anion exchange experiment which showed the existence of differently charged fI species in serum. For the case of fI, this difference in the negative charge must be due either to natural sequence variation (polymorphic variants, section 1.3.5) and/or to variations in the sialylation of the attached carbohydrates (Table 23) and/or due to partially cleaved fI species created through natural proteolysis and/or due to the partial removal of the four basic residues R³¹⁸RKR from the C-terminal of the fI heavy chain following the activation of the enzyme by the cleavage of the R³²¹-I³²² bond (Fig. 9 & section 1.3.4). The removal of such basic residues occurs in circulation by enzymes such as carboxypeptidase B. Any partial removal suggests that the native

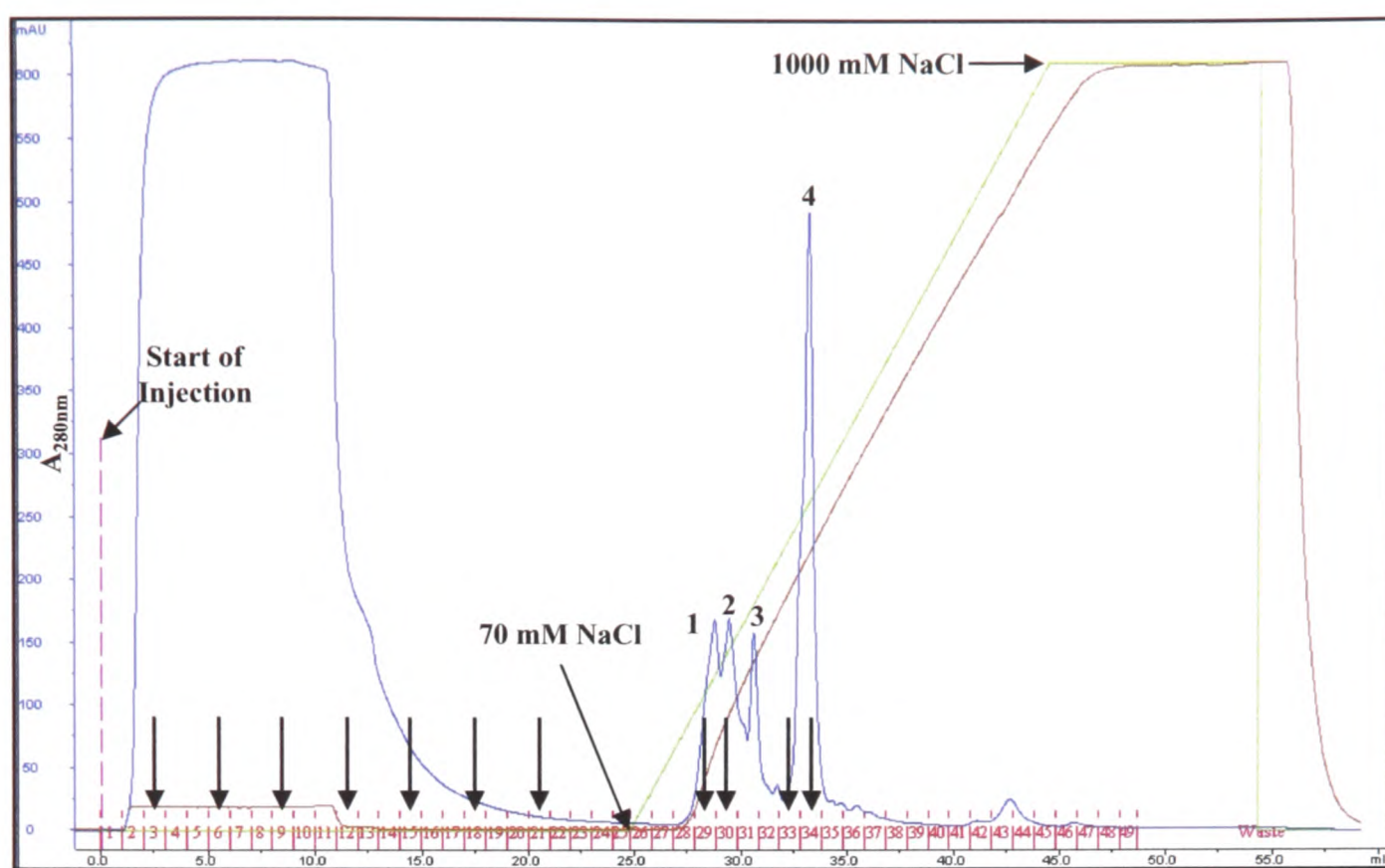


Fig. 61 Anion exchange chromatography for the removal of contaminants from a preparation of human complement factor I. During a fl purification (Batch III, Table 24), anion exchange chromatography was selected as an additional step for the removal of a major contaminant species that co-eluted with fl during the size exclusion chromatography. The anion exchange was carried out using a Mono-Q column (HR 5/5, 5 mm diam x 5 cm) column as described in the materials and methods section of this chapter. The vertical arrows indicate selected fractions that were analysed by SDS-10% PAGE.

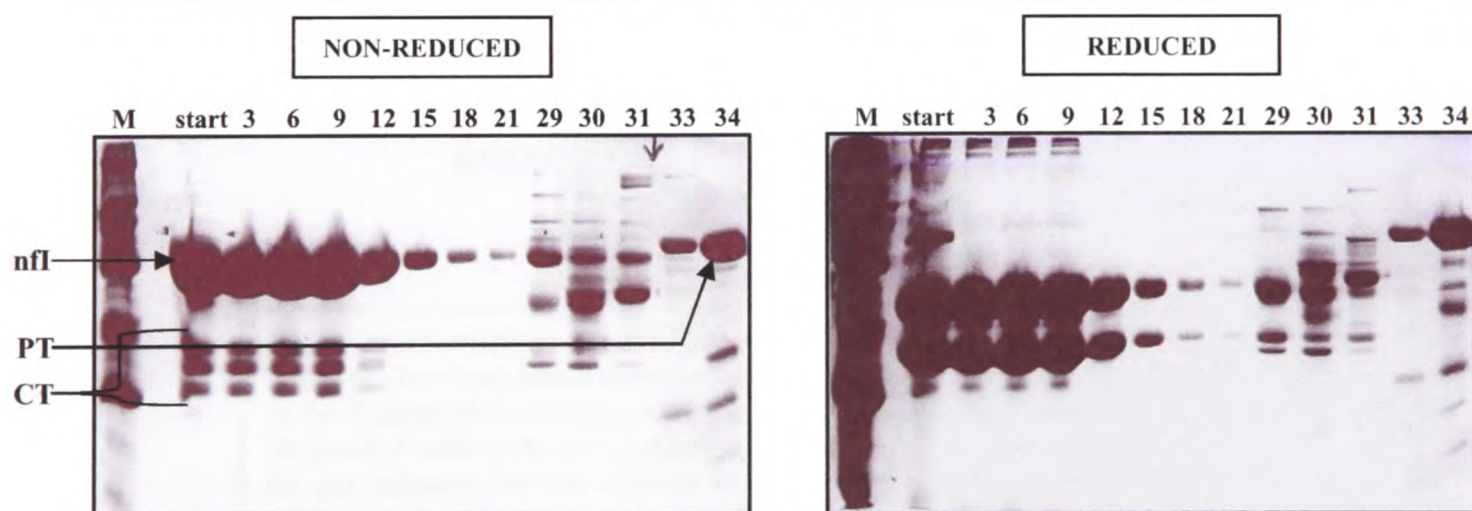


Fig. 62. SDS-10% PAGE analysis. For the analysis, 25 μ l from the selected 1 ml fractions were analysed under non-reduced and reduced conditions. The analysis showed that the major contaminant was removed successfully at peak 4 (Fig. 61, fractions 33 and 34); this species was subsequently identified as prothrombin (PT) (theoretical pI=5.24) through N-terminal sequence analysis. The majority of fl did not bind to the column (fractions 2-25); The minor peaks (1-3) which in the early stage of the salt gradient were found to contain fl. Further investigation of the charge heterogeneity of native fl molecules was carried out using Isoelectric Focusing. The fl preparation also contained some other contaminants (CT) that were subsequently removed by size exclusion chromatography; only a small proportion of these species bound to the column in the physiological pH used for the anion exchange.

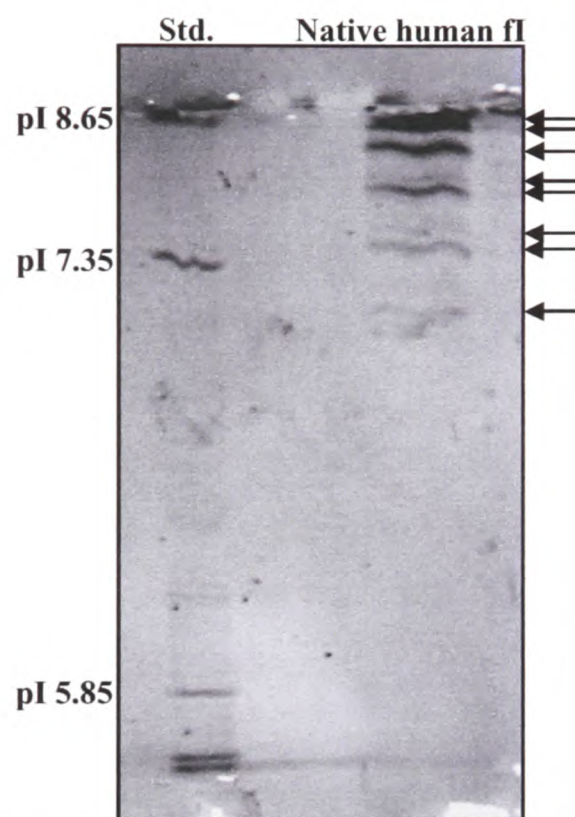


Fig. 63. Isoelectric focusing (IEF) of native human complement factor I. Following the observations about the existence of differently charged species in the fI population of serum, it was decided to analyse the composition of the purified native fI by IEF as described in section 8.2.1.1. The standards of the gel indicated that the detected multiple bands (arrows) correspond to differently charged species with pI values ranging from alkaline (8.65) to close to physiological (7.2).

fI population can contain enzyme molecules the heavy chain of which is composed of 317-321 amino acids.

8.3.2 Deglycosylation of human factor I

The SDS-PAGE analysis of the preliminary digestion timecourse of fI with PNGase F showed that PNGase F can remove efficiently all of six *N*-linked glycans of fI in the presence of 0.01% SDS (Fig. 64). The use of a low amount of SDS was originally indicated by previous preliminary tests done by Xuehui Chen and eventually employed in the mixture as a means of achieving an increased efficiency of deglycosylation. In the mastermix under analysis, the deglycosylation was found to be progressive over the 16 hr time period reaching completion after approximately 10-12 hours. The samples collected within the first 2 hours of incubation and examined under reducing conditions, indicated that the glycans of the light chain were removed faster than the ones of the heavy chain; this suggested a preference of the amidase for the glycans of the light chain. Under the denaturing conditions created by the presence of SDS, structurally the glycans of the light chain must have been more easily accessible than the ones of the heavy chain.

Regarding the purification of fI from the deglycosylation batch reaction via size exclusion, the elution profile contained two main peaks (Fig. 65); The SDS-PAGE analysis (Fig. 66) showed that both peaks, the first eluting around the void volume of the column and the second later, contain deglycosylated fI indicating that a proportion of the deglycosylated enzyme had aggregated (non-covalent interactions) probably due to limited solubility. To examine whether the complete deglycosylation causes a loss of solubility for fI, the solubility properties of the deglycosylated fI were subsequently examined; the test was based on concentrating a sample from fraction 71, which contained non-aggregated material, on a ultracentrifugal filter concentrator device (section 2.4.1) and measuring its concentration as the volume decreases. After concentrating the sample by approximately 7 fold, the OD₂₈₀ that is representative of the concentration increased only by 1.75 fold from 0.08 to 0.14; this indicated that the deglycosylated fI forms aggregates, which above a threshold point that appears concentration dependent, precipitate. The most likely explanation for the aggregation

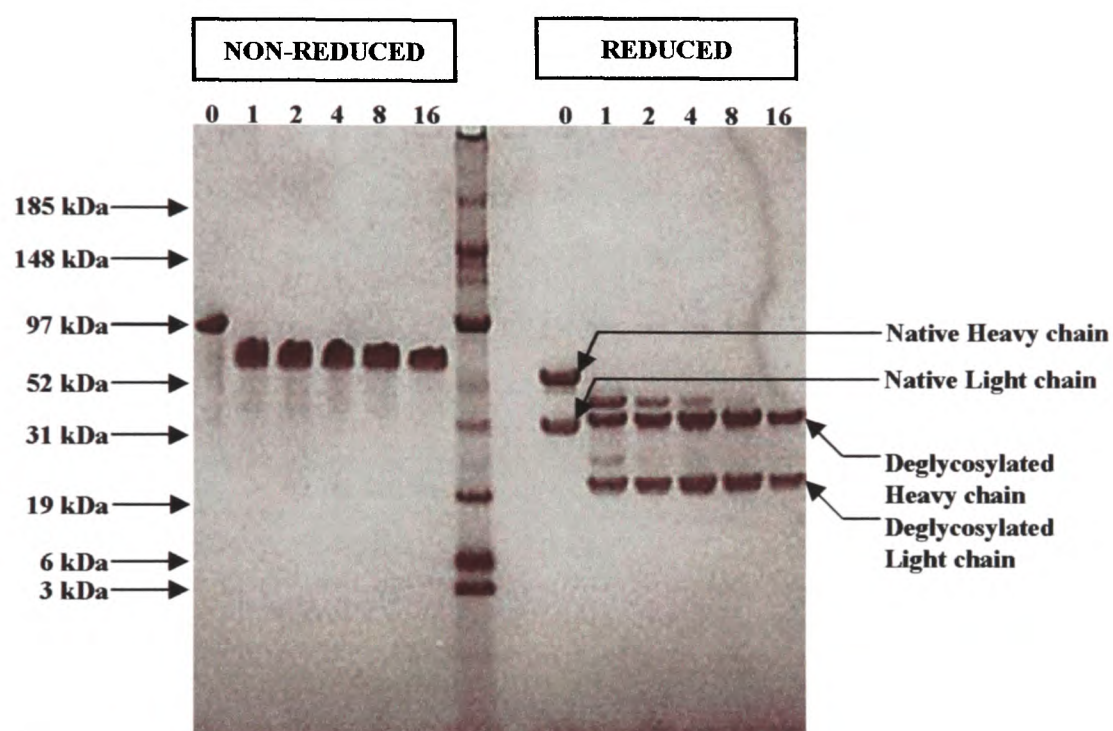


Fig 64. Timecourse of the *N*-linked deglycosylation of human complement factor I by PNGase F. During the human fI crystallisation trials a protocol for the complete deglycosylation of human fI was optimised. The SDS-PAGE analysis showed, under both reduced and non-reduced conditions, that the activity of PNGase F leads eventually to the complete removal of all six *N*-linked glycans (as determined by the MW difference between reduced samples 0 and 16) present on human fI which occupy all the potential *N*-linked glycan attachment sites. The laddering pattern observed under the reduced conditions shows the progressive deglycosylation of both the heavy and the light chains. The bands between the native and deglycosylated forms of the two chains represent partially deglycosylated species of the heavy and light chains. The glycans of the light chain appear to be cleaved faster compared to the ones of the heavy chain. In the absence of any glycans on the heavy or the light chains, the calculated molecular weights for the heavy and the light chains are 35.3 and 27.6 kDa, respectively; the observed molecular weights for the heavy and the light chains of native fI are 50 and 38 kDa, respectively.

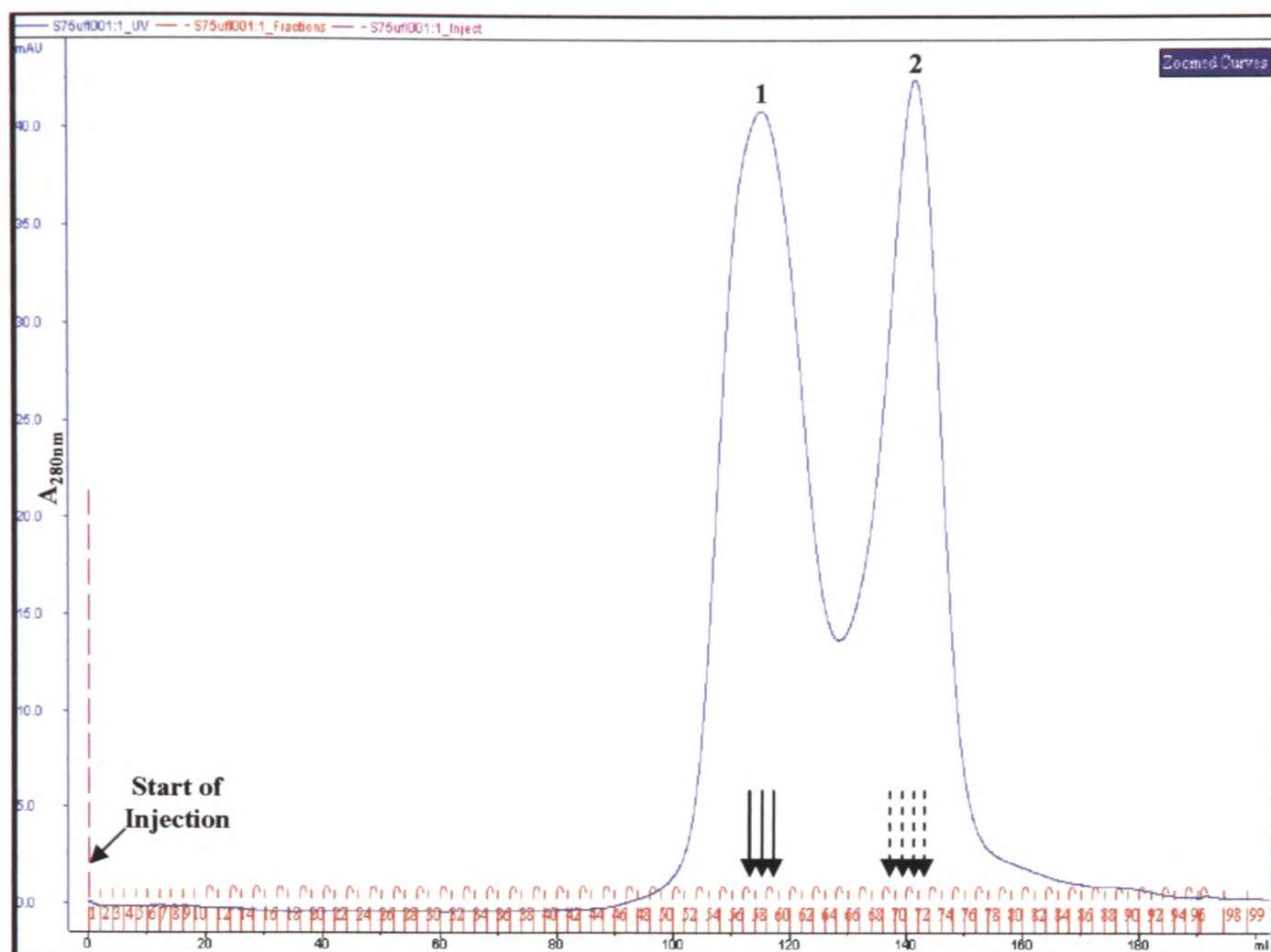


Fig. 65. Size exclusion chromatography for the purification of deglycosylated human complement factor I. 7.5 ml of the dialysed mixture containing 0.33 mg/ml of deglycosylated fI (degfI) were loaded and run on a Superdex S75 (26/60, 26 mm diam x 60 cm) column pre-equilibrated with the running buffer 25 mM Tris-HCl, 140 mM NaCl, pH 7.4; 2 ml fractions were collected. The vertical arrows indicate fractions that were selected for analysis by SDS-PAGE.

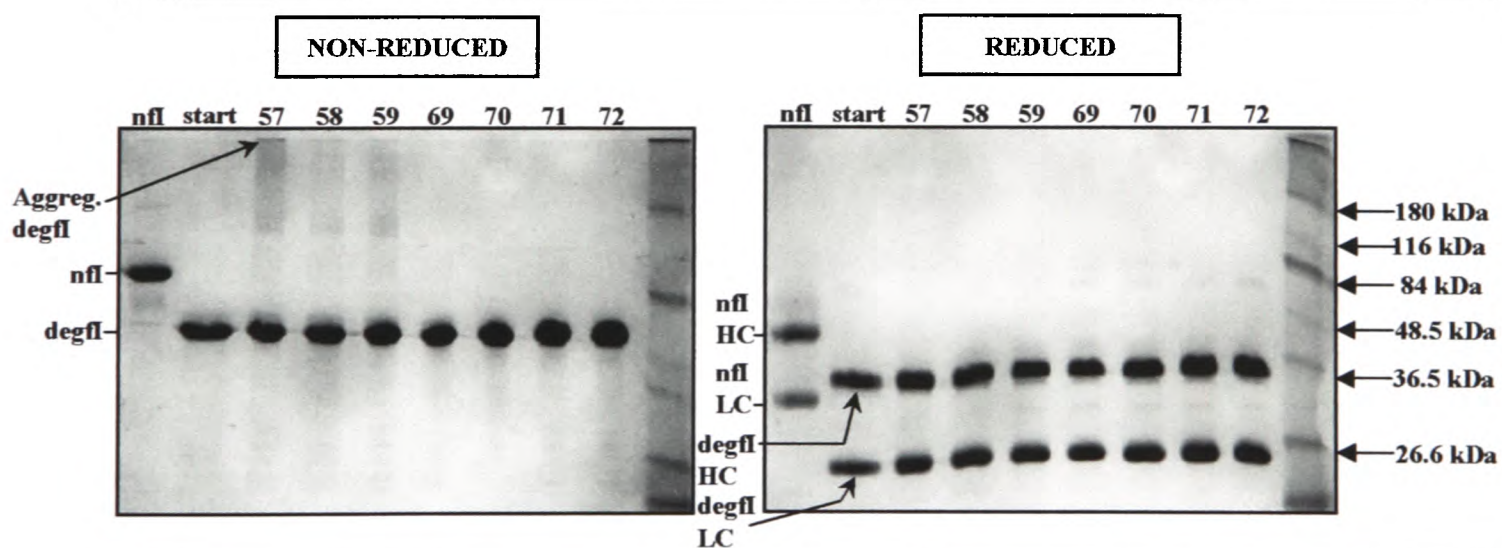


Fig. 66. SDS-10% PAGE analysis of the eluate from the Superdex 75 size exclusion chromatography. For the analysis, 150 μ l from the selected 2 ml fractions were analysed under non-reduced and reduced conditions, using as references native fI (nfl) and start material from the deglycosylation reaction mixture. In the absence of any *N*-linked glycans, the heavy and the light chains have calculated molecular weights of 35.3 and 27.6 kDa, respectively. The analysis showed that both peaks 1 and 2 contained deglycosylated fI. This clearly indicated that peak 1 contained aggregated deglycosylated fI (aggreg. degfI) that was eluting around the void volume of the column, while peak 2 contained monomeric deglycosylated fI (degfI). The solubility properties of the deglycosylated fI were subsequently examined using a sample from fraction 71, from the middle of peak 2, which contained non-aggregated material. Further studies using the non-polar detergent Octyl- β -D-glucopyranoside showed that the presence of detergent in solution can prevent the aggregation and subsequent precipitation of the monomeric deglycosylated fI.

effect is that polar or hydrophobic patches on the surface of the enzyme become exposed upon the removal of the *N*-linked glycans and are involved in polar or hydrophobic intermolecular interactions. The level of aggregation is dependent on the concentration of protein in a given buffer system. At the early stages of concentrating, some aggregation seems to occur and as the volume is reduced, more material tends to form aggregates and/or bind to the formed aggregates. Above a certain threshold limit, large aggregates lose their solubility and eventually precipitate.

To test the latter hypothesis we proceeded to examine the solubility properties of the deglycosylated fI sample in the presence of a non-polar detergent, Octyl- β -D-glucopyranoside (OG) (MW=292.38 g/mole) [Fluka, Sigma [St. Louis, MO, USA]]. For this purpose a 2.5% w/v detergent stock solution was prepared (by dissolving 1.1 mg of detergent in 4.4 ml of MQ H₂O) and was used to prepare two solutions with deglycosylated and non-aggregated fI sample (fraction 71) from the S75 column; a 0.025% w/v OG solution (50 μ l OG 2.5% w/v stock in 4950 μ l deglycosylated fI) and a 0.01% w/V OG solution (20 μ l OG 2.5% w/v stock in 4980 μ l deglycosylated fI). The critical micelle concentration of OG is 20-25 mM (0.58-0.73%) so these two solutions would be free from micelles formed by detergent molecules. Similarly, as described above, the solubility properties of the deglycosylated fI in the presence of detergent were examined by concentrating. For the case of the 0.025% OG solution, upon a 44 fold concentration from 5,000 to 115 μ l, the OD₂₈₀ increased by a factor of 19 from 0.038 to 0.732, while for the 0.01% OG solution, upon a 91 fold concentration from 5,000 to 55 μ l, the OD₂₈₀ increased by a factor of 15 times from 0.036 to 0.548. The results from both experiments confirmed that the complete deglycosylation of fI decreases the enzyme solubility dramatically (native fI stays monomeric in solution at least up to 10 mg/ml at physiological conditions), while the addition of a mild, non-polar detergent like OG in solution can prevent the aggregation and subsequent precipitation of the monomeric deglycosylated fI. Based on these tests, the aggregated deglycosylated fI from the S75 elution fractions 49-67 was made 0.025% w/v in OG and incubated for 24 hrs at 4 ° C with continuous stirring. The monomers contained in the mixture were eventually separated from multimers by size exclusion chromatography and the fractions containing monomeric deglycosylated fI were plated for crystallisation trials.

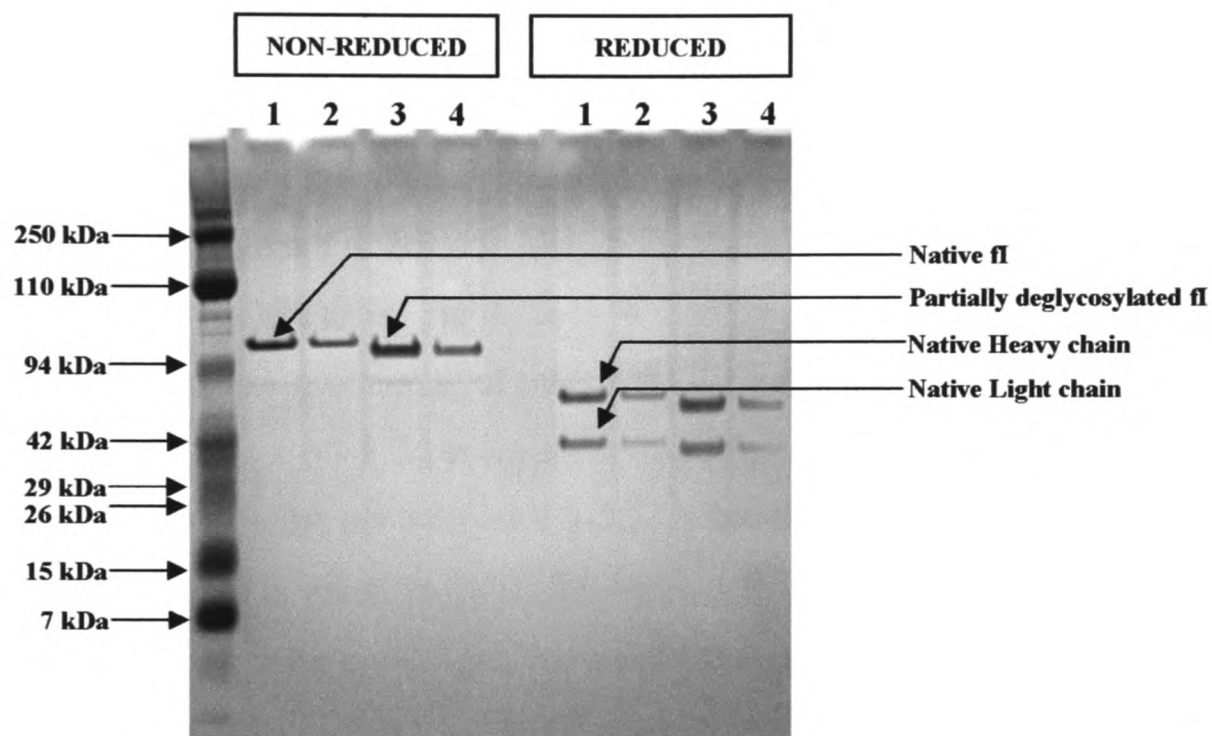


Fig. 67. SDS-PAGE analysis of the partial *N*-linked deglycosylation of human complement factor I. The detailed characterisation of the composition of the *N*-linked glycans of human complement factor I allowed the design of a strategy for the partial deglycosylation of the enzyme for the crystallisation screens (section 8.2.1.2). The deglycosylation was based on the use of an exoglycosidase cocktail containing the ABS (Sialidase), BTG (Galactosidase) and GuH (*N*-acetylglucosaminidase) enzymes that remove sialic acid, galactose and glucosamine residues, respectively, from the reducing end of the *N*-glycans. Removal of these residues leaves fI with homogeneous glycans, containing only the tri-core mannose and the two GlcNAC residues (Fig. 58). The particular strategy is suitable for the generation of material for crystallisation studies as it removes the molecular heterogeneity that the variability in the glycan compositions introduces and contributes to the decrease of the total molecular weight of the target enzyme. In addition, the complete deglycosylation of human fI had been found to cause a large loss of solubility for the enzyme, indicating that the glycans are important for the hydration of the molecule in solution. Upon completion of the deglycosylation, the enzyme was re-purified for plating and its quality was examined by SDS-PAGE using native fI as the standard. The figure above corresponds to a NuPAGE 4-12% gradient gel run using MES buffer and stained using Coomassie Blue. Samples 1 and 2: 1.25 and 0.625 μg of native human fI; Samples 3 and 4: 1.25 and 0.625 μg of partially deglycosylated human fI.

8.3.3 Functional properties of dissolved crystals

The SDS-PAGE analysis indicated that the dissolved crystals contained native human fI (Fig. 68). The dissolved enzyme was found to be proteolytically active cleaving ^{125}I -C3(NH₃) similarly as native fI (Fig. 69).

8.3.4 Crystallisation trials

An initial hit was obtained in condition three of the Stura Footprint I screen, 33 % PEG600, 0.2 Imidazole Malate pH 5.5: these needle-like microcrystals were approximately 10-15 μm in length, took 4 days to appear, and are pictured in Image A, Table 26. Further refinement of this condition was carried out. Needle-like crystals of dimensions 200 x 30 x 30 microns could be grown reproducibly around 28-30% PEG600 and within the pH interval 4.5-5.5. A summary of the conditions that yielded crystals of interest is given in Table 26. Images B-D correspond to crystals that have grown around the same conditions and exhibited a similar needle-like morphology. A few other conditions (Table 26, Images E-G) that also gave needle-like microcrystals were followed for optimisation, but have not yielded diffraction-quality crystals to date.

The best results to date were obtained with crystals that diffracted up to about 4 Å resolution (Table 27), but so far it has not been possible to integrate a full dataset, due to the difficulties in indexing the images. The crystals examined however were found to have a high degree of mosaicity. Measuring the cell repeats directly from the spots values led to the construction of a primitive lattice compatible with a trigonal or hexagonal arrangement, with a unit cell shaped resembling a flat tile, approximately $a=b=194$ Å (Fig. 71), $c=41$ Å (Fig. 72). The short axis c is in the direction of the long axis of the needle.

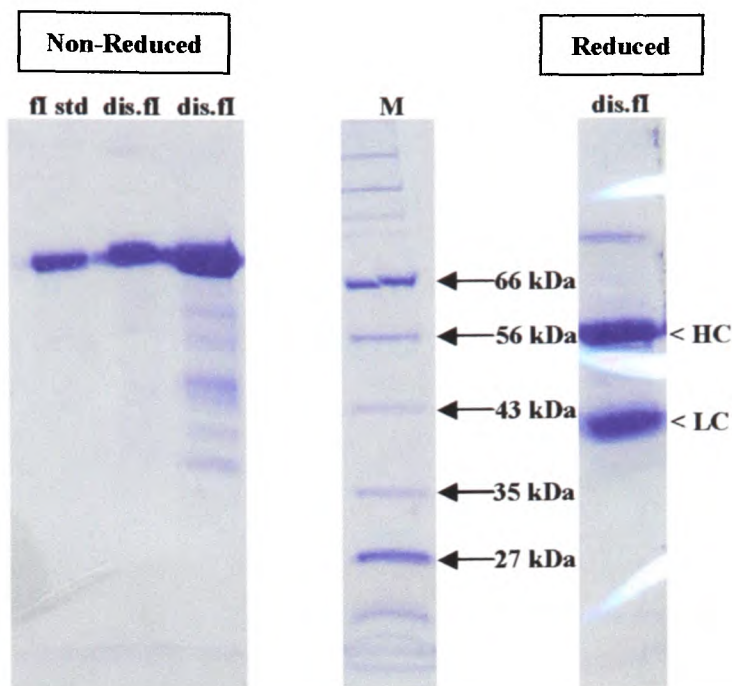


Fig. 68. SDS-10% PAGE analysis of dissolved crystals of native fI. fIstd: reference fI, dis.fI: fI from dissolved crystals, M: Marker

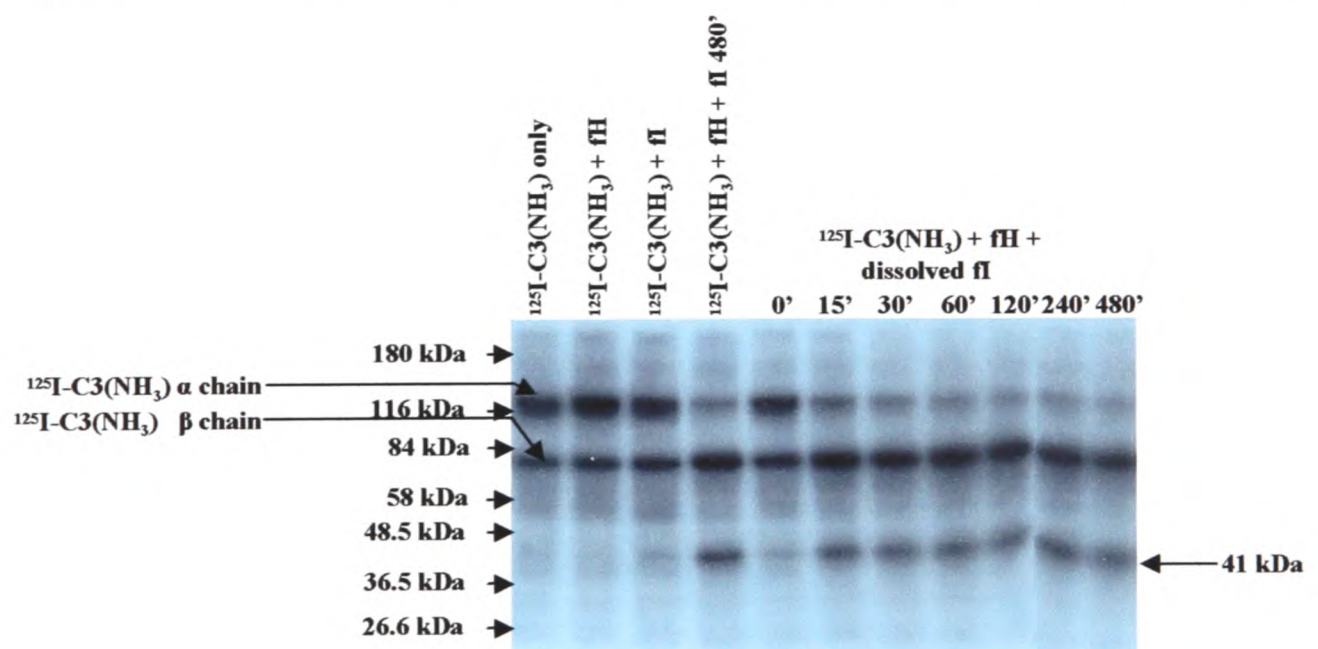


Fig. 69. Proteolytic activity of fI from dissolved crystals. The autoradiograph of an SDS-8.5% PAGE gel shows the cleavage of $^{125}\text{I-C3(NH}_3\text{)}$ by fI from dissolved crystals in the presence of cofactor H within a 480 min timecourse. $^{125}\text{I-C3(NH}_3\text{)}$ is seen as a two-band pattern, the highly labelled 114 kDa α chain and the β chain of 75 kDa. fI can cleave the substrate $^{125}\text{I-C3(NH}_3\text{)}$ only in the presence of fH. On incubation with fI and fH, the α chain is cleaved into three fragments, one (~70 kDa) running just above the β chain (on the autoradiograph it appears to co-run with the β chain), another of 41 kDa and a small fragment (C3f, 2 kDa) which is not visible on the autoradiograph. Lanes 1-4: controls, endpoint t=480 min.; the fI from the dissolved crystals was found to be proteolytically active cleaving $^{125}\text{I-C3(NH}_3\text{)}$ similarly as the native intact form.

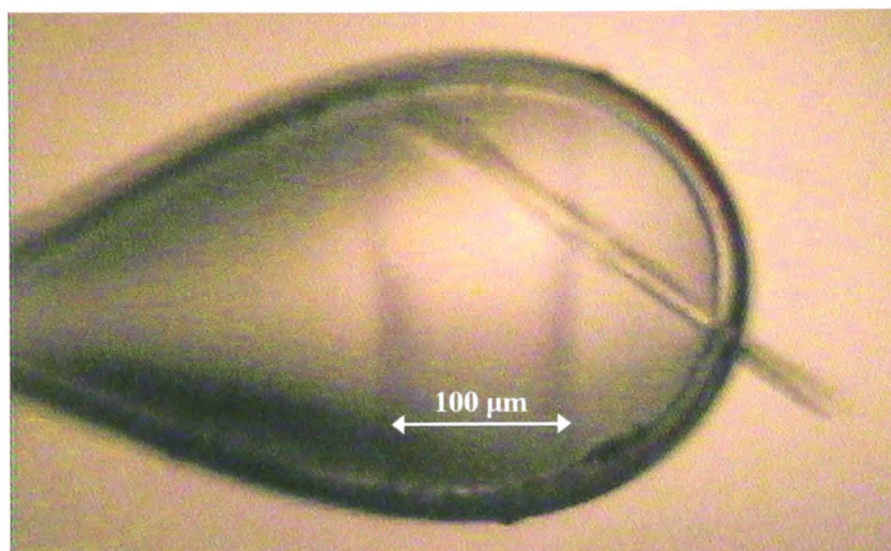


Fig. 70. A needle crystal of approximately $20 \times 20 \times 300 \mu\text{m}^3$ from Tray 28 drop C1 was mounted on a nylon loop and frozen at approximately $T=100 \text{ K}$ using the cryoprotectant buffer 25% PEG600, 0.1 M Imidazole Malate pH 4.5. The crystal was exposed to the ID14-3, [ESRF, Grenoble] $100 \times 100 \mu\text{m}^2$ beam for a total of 45 min. The discoloration, visible as a vertical band in the film of mother liquor inside the loop, is due to the extended exposure to the X-rays and provides an indication of the beam width.

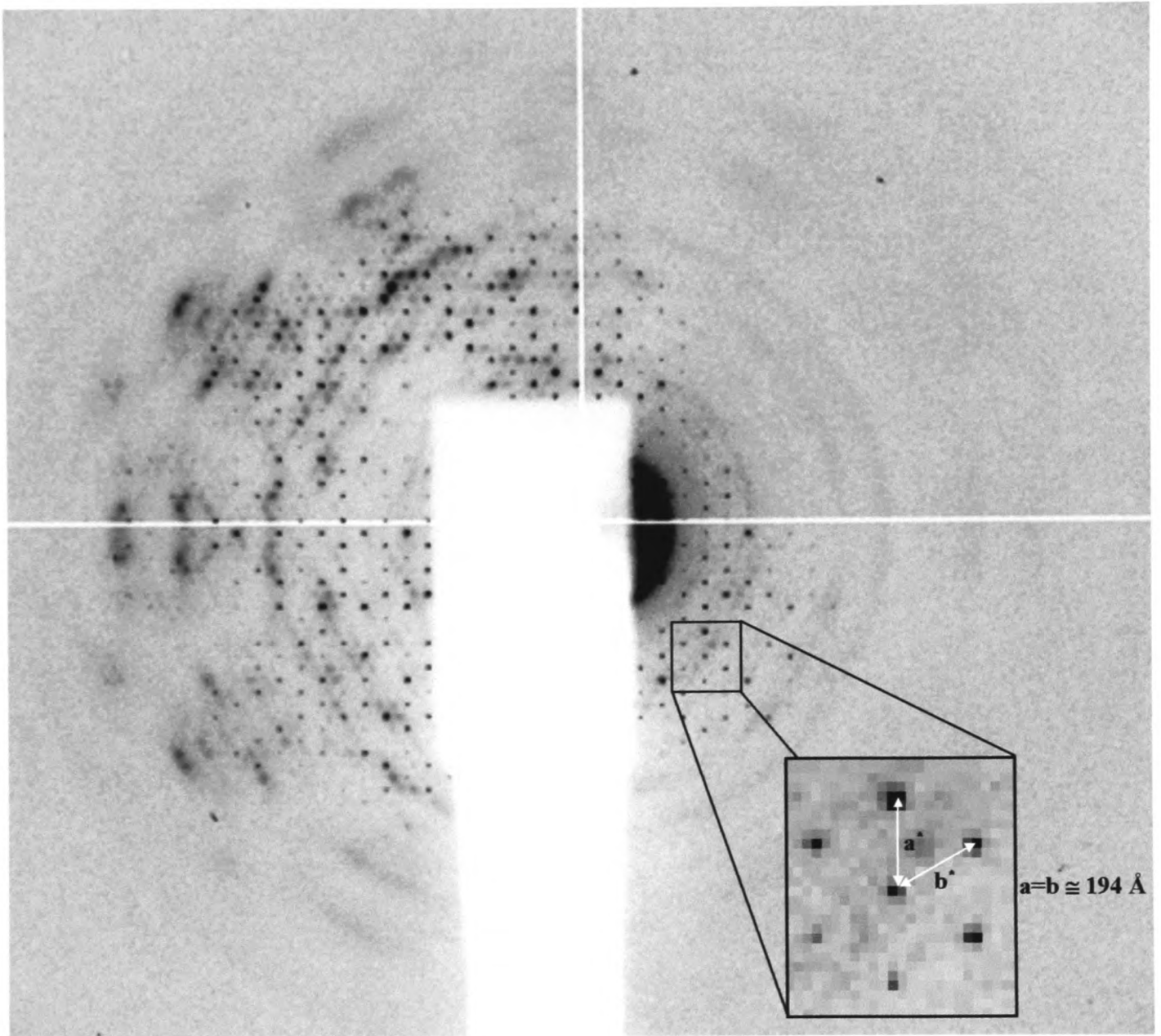


Fig. 71. The frame was collected at ID29 [ESRF, Grenoble] on 11 March 2005. $\lambda = 0.9765 \text{ \AA}$. The crystal from plate 32 drop A9 (VI) was exposed for 2 seconds using half a degree of oscillation. The crystal needle was oriented with its long axis approximately parallel to the X-ray beam. Due to low image resolution, high crystal mosaicity and suboptimal crystal orientation, it was not possible to index the spots. However, the distances (a^* and b^*) between the neighbouring spots of layer $hk0$ were measured from the frame and cell edges a and b were calculated to have an approximate length of $194 \text{ \AA}$. Plate distance (distance of mounted crystal from detector plate): 525 mm (approximately $8 \text{ \AA}$ at the edge).

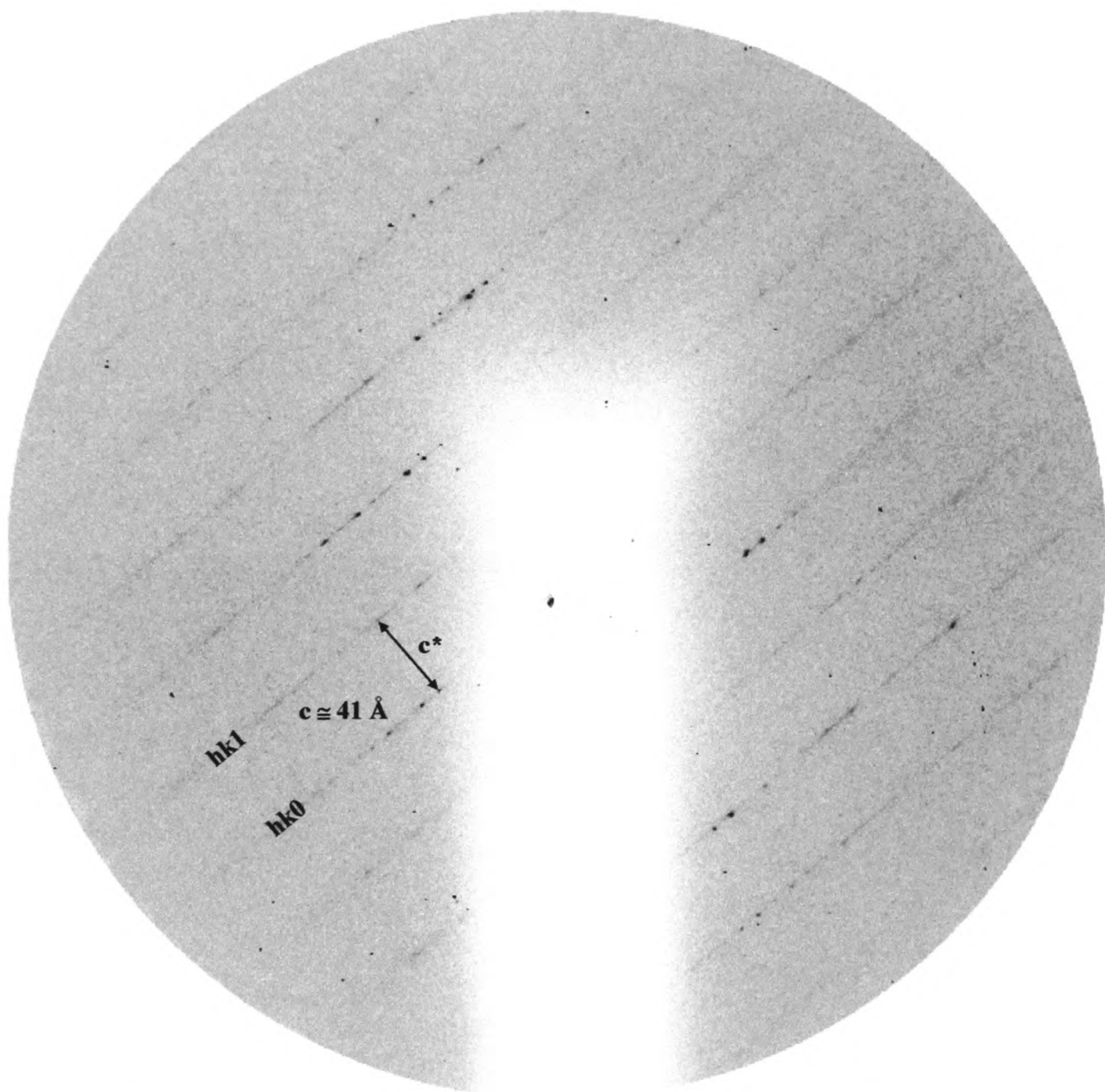


Fig. 72. The frame was collected at ID14-3 [ESRF, Grenoble] on 05 December 2004. $\lambda = 0.9310 \text{ \AA}$. The crystal from plate 26 drop C1 was exposed for 60 seconds using half a degree of oscillation. The crystal needle was oriented with its long axis approximately orthogonal to the X-ray beam. Due to low image resolution, high crystal mosaicity and suboptimal crystal orientation, it was not possible to index the spots. However, the distance (c^*) between the layers $hk0$ and $hk1$ was measured from the frame and cell edge c was calculated to have an approximate length of $41 \text{ \AA}$. Plate distance (distance of mounted crystal from detector plate): 600 mm ($6.6 \text{ \AA}$ at the edge).

Chapter Nine

CHAPTER 9: FUTURE PERSPECTIVES

Although the primary structure, physiological role and interactions of fI with other complement components were established prior to the start of the work described in this thesis, little was known about how the molecular structure of the enzyme affects its physiological function. In the recent years, two major areas of research on fI have attracted considerable attention; these include the nature and mechanisms of molecular interactions of the enzyme with its native substrates and cofactors, as well as how the molecular architecture of fI supports the narrow catalytic properties of the enzyme.

The work presented in this thesis represents a systematic analysis of the structural and functional properties of human complement factor I. Overall, the results described thoroughly through the text shed light primarily on the catalytic specificity of the SP domain, the role of the non-catalytic subunit domains in the physiological function of the enzyme and the detailed composition of the *N*-linked carbohydrates; in addition, they supported the identification of synthetic substrates and the experimental characterisation of the interchain disulphide bridge between C³⁰⁹ and C⁴³⁵. The effort to obtain an atomic structure for fI was also initiated through the collaborative work described in chapter 8.

The work described in chapters 3-8 provided several answers concerning the relationship between structure and function. In structural aspects, in contrast to what was earlier believed, it is now clear that the circulating fI has a catalytic SP domain subunit that has a natural conformation which can accommodate substrate recognition and cleavage. The SP domain can cleave synthetic substrates in the absence of cofactor(s), in contrast to the cleavage of natural substrates, with the optimum cleavage reaction buffer conditions varying for the natural and the synthetic substrates; the variations reported can be explained as already discussed on the basis of molecular protein-protein interactions that occur during the complex formation between the natural substrate, cofactor and enzyme. The active site of the domain was shown to have a molecular architecture that supports the cleavage of a narrow range of arginyl substrates; the findings on the specificity of the SP domain confirmed

earlier hypotheses based on the observations of the natural cleavage sites. Some sequence differences that were found between the cleaved synthetic substrates and sites of cleavage on natural substrates, can be explained by molecular interactions that occur during the complex formation downstream and/or upstream of the cleaved peptide bond.

The approach to examine the properties of fI and its catalytic subunit using two independent assays to test activity under different conditions was advantageous and certainly supported several constructive comparisons; such comparisons led eventually to the detailed characterisation of the catalytic properties of fI. It was of great interest to discover that the non-catalytic heavy chain of fI has a pivotal role in determining the association of fI within the C3b-fH-fI complex. Although sites of interaction for the case of this complex had been reported earlier, the activity of the isolated SP domain had not been previously tested using the proteolytic assay. The different properties of the SP domain suggested that the heavy chain supports anchoring of fI within the complex and provides sites of critical protein-protein interactions with the other components. The similar levels of amidolytic activity of both the intact fI and the SP domain, indicated that the Kazal-like domain embedded within the FIMAC heavy chain does not inhibit the activity of the SP domain in the absence of a cofactor and/or the substrate; these findings in total suggest that the molecular architecture of fI has evolved to physiologically deliver very specific reactions; a number of multiple interactions must develop in a predetermined and ordered manner triggered by the initial events of the C3b-fH-fI complex formation in fluid phase or on a cell surface. The interactions must develop in a series of events which may or may not be accompanied by structural rearrangements; these events eventually lead to the positioning of the target bonds ready for cleavage.

The interactions of fI with cofactor fH and C3b/C3(NH₃) are worth further additional detailed structural studies to reveal the events that occur during the conversion of C3b to iC3b. For example, now that it has been shown that the heavy chain participates in critical molecular interactions for the cleavage of a natural substrate, an interesting approach to examine this issue in more detail, would be the engineering and expression in a standard mammalian expression system (such as a CHO cell line) of recombinant N-terminal deletion mutant forms of fI lacking one,

more than one or even all of the domains of the heavy chain. Such an expression system would support proper molecular folding and post-translational processing like the addition of glycans; glycosylation has been shown to be important for the solubility of fI. Accessing the catalytic properties of each one of the deletion mutants in the proteolytic assay could delineate which of the heavy chain domain(s) is critical for the molecular interactions with the substrate and/or cofactor.

Regarding structural studies, the characterisation of the composition of the fI *N*-linked carbohydrates, will complement the completion of the development of an atomic model for fI. The existence of such a model could provide the basis for the understanding of all these molecular interactions discussed thus far and it would form a model for other non-characterised serine proteases of similar molecular organisation; in parallel, it would also be of great clinical value since it could eventually lead to the development of novel inhibitor compound(s). The high specificity of fI for its substrates makes this enzyme a potential molecular target for pharmacotherapy designed for clinical conditions concerning diseases such as rheumatoid arthritis and multiple sclerosis, where the depletion of complement via inhibiting fI is desirable. Based on information derived from an atomic structure, a novel highly selective inhibitor compound could be synthesized to specifically target the active site of the SP domain. The use of the two optimised independent assays (proteolytic and amidolytic) would be applied to testing and evaluating the potency of such inhibitory compound(s) prior to clinical trials.

Another interesting issue that deserves attention is derived from the experimental work for the characterisation of fI variants in various individuals. In the light of earlier evidence as well as information recently collected, it appears quite important to understand the basis of such variations at the genetic level of fI. The presence of single nucleotide polymorphisms (SNPs) in parts of the human fI alleles that encode for regions which participate in molecular interactions or in the SP domain, may be crucial for the activity of fI variants in individuals that affect the physiological levels of C3 and C4 in circulation and hence the complement response. Knowledge of these sites of polymorphism at specific points on the fI alleles could assist in the identification of individuals with abnormal levels of complement homeostasis. In

pharmacogenomics sense that would be beneficial as it could lead to the design of personalised fI inhibitory therapy.

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