

Structural and Functional Characterisation of Properdin and Properdin-binding Complement Inhibitors from Ticks

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Abstract

The complement system is a major effector of the immune response which can lead to opsonisation and inflammation, as well as the removal of immune complexes and damaged self-cells. The complement system requires many regulatory proteins to prevent inappropriate activation towards healthy self-cells and to only target invading pathogens. This thesis focuses on a complement regulator/activator called properdin, which has primarily been associated with the alternative pathway (AP). Properdin functions by stabilising the AP C3 convertase (C3bBb).

In this thesis, I investigated the molecular basis of AP inhibition by a novel set of properdin-binding complement inhibitors from the salivary glands of the tick *Rhipicephalus pulchellus*, CIRp-A1 and homologs. These proteins were recombinantly expressed in insect and bacterial systems for functional and structural characterisation. ELISA-based complement activation assays showed that CIRp-A1 specifically inhibits the AP and has an IC_{50} of 13 nM. AP haemolysis assays using different animal sera showed that CIRp-A1 and homologs have different inhibitory activity profiles in different animals. To understand the molecular basis of host specificity, crystal structures of CIRp-A1 and homologs were solved. The structures of these inhibitors, in combination with mutagenesis studies of CIRp-A1, suggested that a loop contributes to the inhibitory activity of CIRp-A1.

Pulldown assays using CIRp-A1 and mammalian cell-expressed recombinant properdin suggested that CIRp-A1 binds to the final domain of properdin (TSR6), which had been implicated in C3b-properdin interaction. Pulldown assays using C3b showed that CIRp-A1 inhibits the binding of properdin to C3b and displaces properdin already bound to C3b. By binding to TSR6, CIRp-A1 might sterically hinder the interaction between properdin and C3b, or change the conformation of properdin such that it can no longer interact with C3b. The exact molecular mechanism behind properdin inhibition by CIRp-A1 remains to be elucidated in future studies, particularly with structural studies of the properdin-CIRp-A1 complex.

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List of Abbreviations

AAA	Abdominal aortic aneurysm
aHUS	Atypical haemolytic uremic syndrome
AMD	Age-related macular degeneration
AP	Alternative pathway
APS	Ammonium persulfate
C1-INH	C1-inhibitor
C3G	C3 glomerulopathy
C4BP	C4-binding protein
CCP	Complement control protein
CD	Circular dichroism
CHO	Chinese hamster ovary
CFHR	Complement factor H-related protein
CP	Classical pathway
CPN	Carboxypeptidase N
CR1	Complement receptor type 1
CV	Column volume
CVF	Cobra venom factor
DAF	Decay accelerating factor
DDD	Dense deposit disease
DMEM	Dulbecco's Modified Eagle's Medium
EDC	1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscopy
EMDB	Electron Microscopy Data Bank
EST	Expressed sequence tag
FB	Factor B
FBS	Fetal bovine serum
FD	Factor D
FH	Factor H
FHL-1	Factor H-like protein 1
FI	Factor I
FT	Flow-through
GAG	Glycosaminoglycan
GPI	Glycophosphatidylinositol
HAE	Hereditary angioedema
HBP	Histamine-binding protein
HEK	Human embryonic kidney
HUVEC	Human umbilical vein endothelial cell
Hib	<i>Haemophilus influenzae</i> type b
HIC	Hydrophobic interaction chromatography
HPLC	High performance liquid chromatography
IC ₅₀	Half maximal inhibitory concentration

IgG	Immunoglobulin G
IgM	Immunoglobulin M
ILP	Isac-like protein
IR	Infrared
IRAC	<i>Ixodes ricinus</i> anti-complement protein
Isac	<i>Ixodes scalpularis</i> anti-complement protein
ITC	Isothermal titration calorimetry
LB	Luria Broth
Lili-Mip	Lipocalin-like milk protein
LLG	Log-likelihood gain
LOS	Lipooligosaccharide
LP	Lectin pathway
LPS	Lipopolysaccharide
MAC	Membrane attack complex
MALS	Multi-angle light scattering
MASP	MBL-associated serine protease
MBL	Mannose-binding lectin
MCP	Membrane cofactor protein
MEM-NEAA	Minimum Essential Medium Non-Essential Amino Acids
MPO	Myeloperoxidase
MR	Molecular replacement
MST	Micro-scale thermophoresis
MW	Molecular weight
NCS	Non-crystallographic symmetry
NHS	N-hydroxysuccinimide
OD ₆₀₀	Optical density at 600 nm
OmCI	<i>Ornithodoros moubata</i> complement inhibitor
OVA	Ovalbumin
P	Properdin
PBS	Phosphate-buffered saline
PBST	PBS with 0.1% (v/v) Tween 20
PCR	Polymerase chain reaction
PDB	Protein Data Bank
PEI	Polyethylenimine
PIGA	Phosphatidylinositol glycan class A
PNH	Paroxysmal nocturnal haemoglobinuria
RABP	Retinoic acid binding protein
RaCI	<i>Rhipicephalus appendiculatus</i> complement inhibitor
R _{eq}	Response at equilibrium
RFZ	Rotation Function Z-score
RU	Response unit
SCIN	Staphylococcal complement inhibitor
SCR	Short consensus repeat
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SEC	Size exclusion chromatography
SGE	Salivary gland extract
SLE	Systemic lupus erythematosus
SPR	Surface plasmon resonance
SSL7	Staphylococcal superantigen-like protein 7
T-Jump	Temperature Jump
TED	Thioester-containing domain
TEMED	Tetramethylethylenediamine
TFZ	Translation Function Z-score
TLR	Toll-like receptor
T _M	Melting temperature
TSR	Thrombospondin type 1 repeat
vWA	von Willebrand factor type-A
YCB	Yeast Carbon Base

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CHAPTER 1: INTRODUCTION

1.1 The complement system

1.1.1 Overview of complement activation

The complement system is an ancient and essential component of innate immunity, and functions as the first line of defence against invading pathogens. The activation of the complement system results in a pro-inflammatory response, leading to the recruitment and activation of immune cells (Heeger et al., 2005; Nataf et al., 1999). Although first described as the heat-sensitive component of human plasma that ‘complements’ the antibodies in killing bacteria (Kaufmann, 2008), other functions of complement have since been identified. The complement system is important for homeostasis through the clearance of immune complexes and apoptotic cells (Henderson et al., 2002; Taylor et al., 2000; Waxman et al., 1984). There is also increasing evidence of its role in cell metabolism (Denk et al., 2017; Kolev et al., 2015) and in the nervous system (Fraser et al., 2010; Stevens et al., 2007). The complement system has also been associated with other physiological networks, such as the coagulation system (Gulla et al., 2010; Huber-Lang et al., 2006; Krarup et al., 2007) and Toll-like receptors (TLRs) (Raby et al., 2011; Zhang et al., 2007).

The complement system, illustrated in **Figure 1.1**, consists of a cascade of proteolytic reactions involving a huge network of soluble and membrane-associated serum proteins (Sim and Tsiftoglou, 2004). The classical pathway (CP) is initiated when C1q of the C1 complex recognises immunoglobulin G (IgG) or immunoglobulin M (IgM) in surface-bound immune complexes (Hughes-Jones and Gardner, 1979; Nash et al., 2001), or surface-bound pentraxins (Nauta et al., 2003). C1q has also been shown to recognise non-immune ligands, such as DNA and calreticulin (Gaboriaud et al., 2012; Païdassi et al., 2008, 2011). The C1 complex also contains the serine proteases C1r and C1s. Upon ligand binding by C1q, C1r auto-activates and cleaves C1s; C1s then cleaves C4 into C4a and C4b (Kardos et al., 2001; Kerr et al., 2005). C2 then binds C4b and is cleaved by C1s to generate the CP C3 convertase C4b2a (Müller-Eberhard et al., 1967). The lectin pathway (LP) is initiated by the recognition of carbohydrates on pathogenic surfaces by

mannose-binding lectin (MBL), ficolins or collectins (Liu et al., 2005; Ma et al., 2013; Matsushita and Fujita, 1992; Matsushita et al., 2002). The MBL complex is formed by the LP pattern recognition molecules and MBL-associated serine proteases (MASPs). MASP-1 and MASP-2 of the LP are homologous to C1r and C1s of the CP, respectively. MASP-1 auto-activates and cleaves MASP-2 as well as C2; MASP-2 then cleaves C4 (Chen and Wallis, 2004; Matsushita et al., 2000; Rossi et al., 2001). The cleavage of C2 and C4 results in the formation of the LP C3 convertase (C4b2a), which is the same as the CP C3 convertase.

The alternative pathway is different from the classical and lectin pathways in that it is continuously active at a low level, and is rapidly amplified on activator surfaces. The low-level AP activity is due to the tick-over process which describes the spontaneous hydrolysis of an internal thioester bond in C3 (Pangburn et al., 1981). This reaction occurs continuously at a low rate and generates C3(H₂O) which is functionally similar to C3b. C3(H₂O) can interact with factor B (FB) to form C3(H₂O)B; FB is then cleaved by the serine protease factor D (FD) to form the fluid-phase AP C3 convertase C3(H₂O)Bb (Pangburn et al., 1981). Others have suggested that C3(H₂O) is generated when C3 comes into contact with biological or artificial surface interfaces, for example, gas bubbles, activated platelets and lipid particles (Andersson et al., 2002; Nilsson and Nilsson Ekdahl, 2012; Nilsson Ekdahl et al., 1992). Alternatively, C3 could be cleaved into C3a and C3b by non-specific proteases, such as trypsin, plasmin, thrombin and elastase (Amara et al., 2010; Claesson et al., 2010; Minta et al., 1977). Immune cells release proteases at sites of inflammation and infection, where C3 activation would be beneficial (Harrison, 2017; Heutink et al., 2010). C3b generated by proteolytic cleavage would result in the fluid-phase AP C3 convertase C3bBb. The fluid-phase AP C3 convertases (C3(H₂O)Bb or C3bBb) cleave C3 into C3a and C3b at a low rate (**Figure 1.1A**).

Proteolytic cleavage of C3 and release of C3a induces a conformational change which exposes a reactive thioester in C3b, previously protected and solvent inaccessible (Janssen et al., 2006; **Figure 1.2**). C3b can use this thioester group to covalently attach to nearby surfaces (Law et al., 1980). Surface-bound C3b interacts with FB to form C3bB; FB is then cleaved by FD to form C3bBb. Properdin (P) binds to and stabilises this C3bBb complex, forming the surface-bound AP C3 convertase C3bBbP (Farries et al., 1988a; Fearon and Austen, 1975). All three complement pathways converge at the point

of C3 cleavage into C3a and C3b by surface-bound C3 convertase complexes (C3bBbP or C4b2a) (**Figure 1.1B**). The CP and LP also result in C3b deposition on surfaces. C3b molecules generated by any of the three pathways can form new AP C3 convertases, which then generate even more C3b. Therefore, the alternative pathway provides a powerful amplification loop for all complement pathways via the generation of AP C3 convertases.

This amplification of C3 activation is rapidly down-regulated on host cells by complement regulators, but proceeds on pathogens and altered host cells lacking such regulators (discussed in **Section 1.1.2**). The deposition of C3b on pathogenic cells targets them for uptake by phagocytes (Helmy et al., 2006) or destruction by the lytic pathway (Müller-Eberhard, 1985). The binding of additional C3b molecules to surface-bound C3 convertases leads to the formation of C5 convertases (C3bBbC3b or C4bC2aC3b) (Kinoshita et al., 1988; Takata et al., 1987), which cleave C5 into C5a and C5b (**Figure 1.1C**). The anaphylotoxins C3a and C5a promote local inflammation and chemotaxis (Ward, 2009; Zhou, 2012). C5b can initiate the terminal/lytic pathway by recruiting C6, C7, C8 and C9, forming the membrane attack complex (MAC) (Morgan et al., 2016). The MAC forms a pore in target membranes, leading to membrane disruption and cell lysis (Müller-Eberhard, 1985) (**Figure 1.1D**).

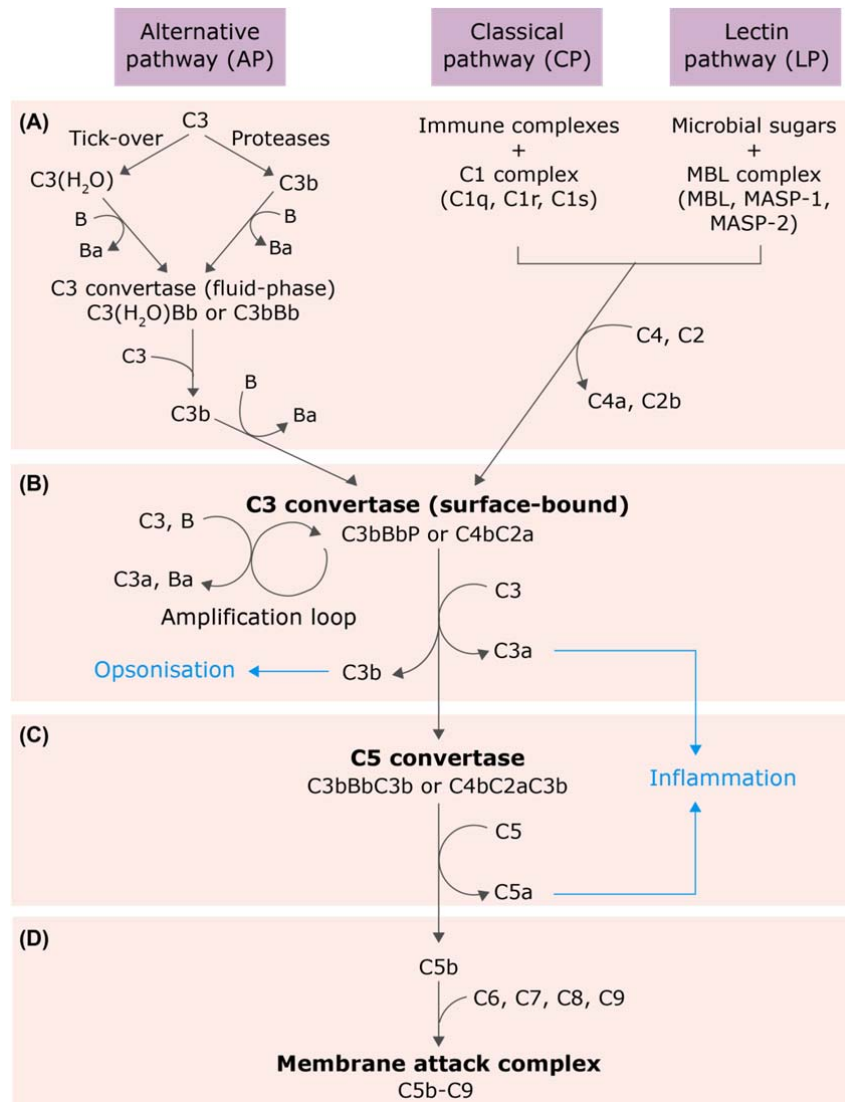


Figure 1.1. The complement cascade. A schematic overview of the complement system. (A) The complement cascade can be activated via the alternative (AP), classical (CP) or lectin (LP) pathways. The AP is thought to be initiated by the spontaneous hydrolysis of a thioester bond within C3 which results in the activated form C3(H₂O), or by non-specific proteolytic cleavage of C3 which results in C3b. C3(H₂O) or C3b can then form fluid-phase C3 convertases. The CP is activated by the recognition of immune complexes by a molecule called C1q. The LP is activated by the recognition of sugar molecules on pathogenic surfaces by mannose-binding lectins (MBL). (B) All three pathways converge into the formation of surface-bound C3 convertases (C3bBbP or C4bC2a). Properdin (P) is shown here as a part of the surface-bound AP C3 convertase. These enzyme complexes cleave C3 to generate the anaphylatoxin C3a and the opsonin C3b. The deposition of C3b on a nearby surface initiates an amplification loop which generates additional AP C3 convertase complexes. (C) The binding of additional C3b molecules to C3 convertases leads to the formation of C5 convertases (C3bBbC3b or C4bC2aC3b), which cleave C5 into C5a and C5b. (D) C5b can initiate the terminal pathway by recruiting C6–C9, which leads to the formation of the membrane attack complex (MAC). The MAC is inserted into target membranes and causes cell lysis.

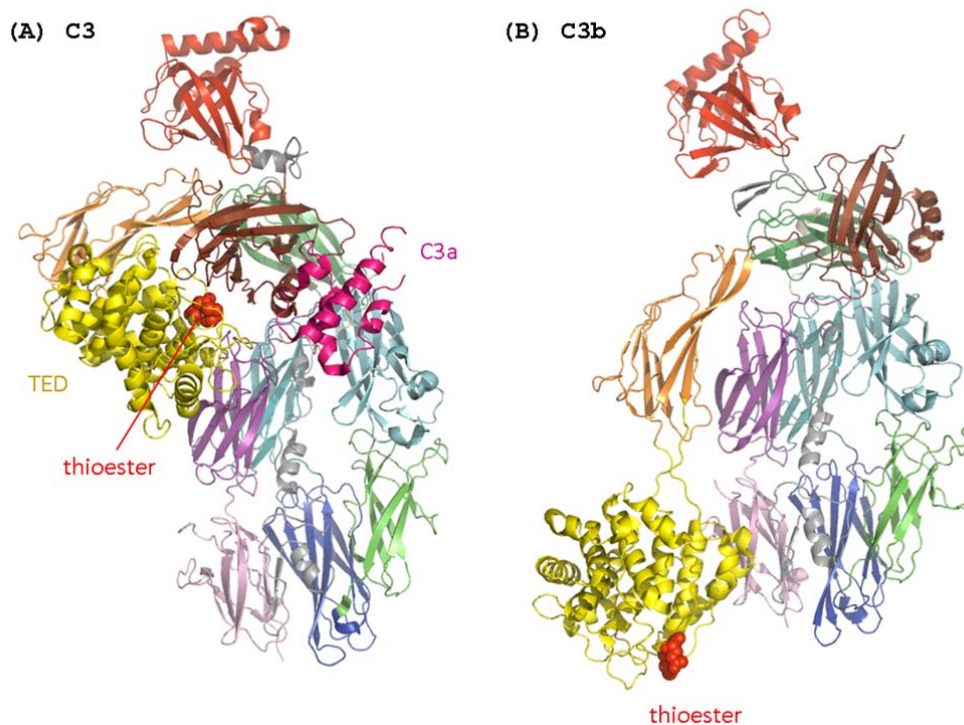


Figure 1.2. Conformational change in C3b upon C3 cleavage. Crystal structures of C3 (left: PDB 2A73, Janssen et al., 2005) and C3b (right: PDB 2I07, Janssen et al., 2006) were compared. C3 cleavage and release of the anaphylatoxin domain (C3a, magenta) induces domain rearrangement. This conformational change exposes a thioester group (red, surface representation) within the thioester-containing domain (TED, yellow) that can be used to covalently attach to surfaces in close proximity. Images were produced with PyMOL (Version 2.1, Schrödinger, LLC).

1.1.2 Complement regulation

The proteolytic cascade of complement must be tightly regulated to avoid activation against healthy host cells and to ensure targeted activation against invading pathogens or modified host cells. The alternative pathway, in particular, must be tightly regulated as C3b deposition and the amplification loop via the AP are non-specific, and could lead to damage to host cells. Activator and non-activator surfaces are subjected to different mechanisms of complement regulation. Complement regulators can either be soluble or membrane-bound.

C1-inhibitor (C1-INH) is a serine protease inhibitor which irreversibly binds to and inactivates C1r and C1s of the CP C1 complex, and MASP-1 and MASP-2 of the LP MBL complex (Davis et al., 2008). Many regulators act on the C3 convertases, as these

enzyme complexes are central components of the complement system. One mechanism of regulation is the promotion of the dissociation of these complexes, known as decay acceleration. The surface-bound decay accelerating factor (DAF, also known as CD55) and complement receptor type 1 (CR1, also known as CD35) bind to C3b and C4b to dissociate the C3 convertases (Fujita et al., 1987; Krych-Goldberg and Atkinson, 2001). Another mechanism of regulation of the C3 convertases is the enzymatic inactivation of complement proteins. Factor I (FI) is a serine protease that cleaves C3b and C4b in the presence of cofactors such as factor H (FH), CR1 and membrane cofactor protein (MCP, also known as CD46) (Masaki et al., 1992). FI cleavage of C3b results in breakdown products such as iC3b, C3c and C3d, which are unable to form C3 convertases.

The soluble regulator FH is a key negative regulator of the alternative pathway. It binds to C3b and dissociates the AP C3 convertase (Wu et al., 2009). FH binds to host surfaces by interacting with host-specific glycans, such as sulphated glycosaminoglycans (GAGs), sialic acid and heparin sulphate (Langford-Smith et al., 2015). FH consists of 20 complement control protein (CCP) repeats, each about 60 amino acids long (Makou et al., 2013). Mutagenesis studies identified CCP1-4 to be important for the decay acceleration function of FH (Kühn and Zipfel, 1996). The crystal structure of FH CCP1-4 in complex with C3b showed that FH and Bb compete for the same binding site on C3b (**Figure 1.3**; Wu et al., 2009); however, it remains unclear how FH dissociates Bb from pre-formed AP C3 convertase and leads to decay acceleration. As well as CCP1-4, CCP19-20 also binds C3b at a different site, leading to an enhanced avidity of binding of FH to C3b (Kajander et al., 2011; Morgan et al., 2011; Sharma and Pangburn, 1996). The C-terminal end of FH has been shown to interact with host markers such as sialic acid and heparin (Blackmore et al., 1998; Jokiranta et al., 2005; Manuelian et al., 2003; Prodinger et al., 1998; Ram et al., 1998), and CCP19-20 has been shown to be critical for self-discrimination (Ferreira et al., 2006). It has been suggested that FH has a higher binding avidity for C3b bound to a non-activator surface, as a result of binding to both C3b and surface markers (Meri and Pangburn, 1990). On the other hand, C3b bound to an activator surface may be protected from FH due to weaker binding.

There are other complement regulatory proteins in the FH protein family, such as FH-like protein 1 (FHL-1) and complement FH-related proteins (CFHRs) (Józsi et al., 2015). These proteins consist of different numbers of CCP repeats. FHL-1 functions similarly to

FH, and has both decay acceleration and FI cofactor activities (Zipfel and Skerka, 1999). CFHR1, CFHR2 and CFHR5 were shown to compete with FH for binding to C3b, and therefore, modulate FH activity (Goicoechea de Jorge et al., 2013). These proteins can form homo- or hetero-dimers which increase binding to their ligands via avidity effect. CFHR3 and CFHR4 might function in a similar manner (Fritsche et al., 2010; Hebecker and Józsi, 2012). By functioning as competitive antagonists of FH, CFHRs could enable fine-tuning of complement regulation by FH depending on the surface (Goicoechea de Jorge et al., 2013). C4-binding protein (C4BP) is a soluble regulator of the CP and LP which functions similarly to FH. It has decay accelerating activity which dissociates the CP/LP C3 convertases and cofactor activity which promotes FI-mediated cleavage of C4b (Ermert and Blom, 2016). C4BP is a large glycoprotein (500 kDa) with seven α -chains and one β -chain, both consisting of CCP repeats.

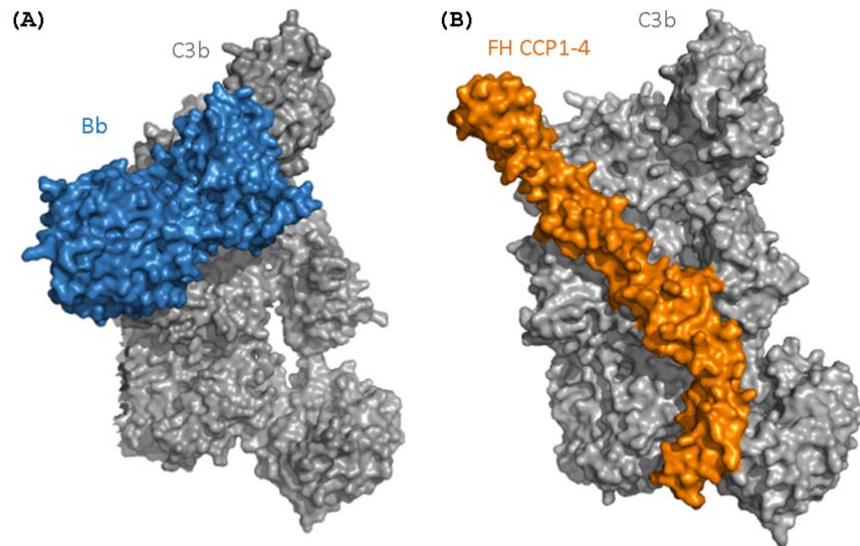


Figure 1.3. C3b binding site of Bb versus FH. Comparison of crystal structures of C3bBb (PDB 2WIN, Rooijakkers et al., 2009) and C3b in complex with FH CCP1-4 (PDB 2WII, Wu et al., 2009). Images were generated with PyMOL (Version 2.1, Schrödinger, LLC).

As described above, most complement regulators function by down-regulating complement activation on non-activator surfaces. Properdin is the only positive regulator of complement, and it functions by stabilising the AP C3 convertase (Farries et al., 1988a; Fearon and Austen, 1975). Thus, properdin and FH have opposing regulatory functions

on the alternative pathway. The absence of negative regulators leads to the recruitment of properdin and AP amplification. Structural data suggests that properdin binding to the AP C3 convertase results in the rearrangement of C3b TED domain that limits regulation by FH (Alcorlo et al., 2013). Therefore, the activation of the alternative pathway depends on the surface, and the relative recruitment of properdin and FH to that surface.

There are also regulators of the terminal pathway. Clusterin and vitronectin are soluble regulators of the terminal pathway. Clusterin inhibits C9 binding to the C5b-8 complex and vitronectin inhibits the association of C5b-7 with membranes (Podack and Müller-Eberhard, 1979; Su, 1996; Tschopp and French, 1994). CD59 is a small membrane-bound protein that inhibits C9 incorporation into C5b-8 during MAC formation (Farkas et al., 2002). Additionally, carboxypeptidase N (CPN) cleaves and inactivates anaphylatoxins C3a and C5a (Skidgel and Erdös, 2007). Complement regulators are summarised in **Figure 1.4** and **Table 1.1**.

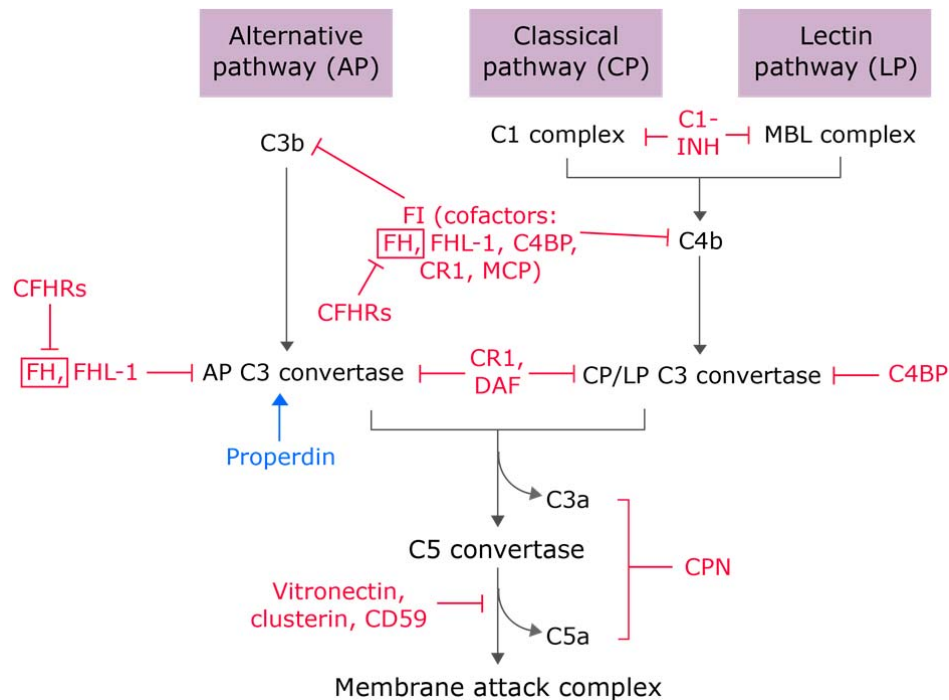


Figure 1.4. Schematic of the complement cascade with regulators. Figure 1.1 was simplified to show the points of action of complement regulators in Table 1.1. Positive and negative regulators are in blue and red, respectively.

Table 1.1. Regulators of the complement system. Complement regulators can be soluble or membrane-bound. The pathway(s) that the regulator is involved with is shown.

Regulator	Pathway(s)	Function
Soluble regulators		
Factor H	Alternative	Decay acceleration of AP C3 convertase; cofactor for FI
FHL-1	Alternative	Decay acceleration of AP C3 convertase; cofactor for FI
CFHR1,2,5	Alternative	Modulate FH activity
Factor I	Alternative, Classical, Lectin	Serine protease; inactivates C3b and C4b
Properdin	Alternative	Stabilises surface-bound AP C3 convertase
C1-INH	Classical, Lectin	Serine protease; inactivates C1r, C1s, MASP-1 and MASP-2
C4BP	Classical, Lectin	Decay acceleration of CP/LP C3 convertases; cofactor for FI
Vitronectin	Terminal	Inhibits MAC formation
Clusterin	Terminal	Inhibits MAC formation
CPN	N/A	Inactivates C3a and C5a
Membrane-bound regulators		
CR1/CD35	Alternative, Classical, Lectin	Decay acceleration of AP/CP/LP C3 convertases, cofactor for FI
DAF/CD55	Alternative, Classical, Lectin	Decay acceleration of AP/CP/LP C3 convertases
MCP/CD46	Alternative, Classical, Lectin	Cofactor for FI
CD59	Terminal	Blocks C9 incorporation into the MAC

Many complement regulators also function outside of the complement system and interconnect with other immune/inflammatory response pathways (Zipfel and Skerka, 2009). For example, in addition to its complement inhibitory function, C1-INH also inhibits Factor XIIa and kallikrein of the contact activation system, Factor XIa and thrombin of the coagulation system, and plasmin and tissue plasminogen activator of the fibrinolytic system (Davis et al., 2008). On the other hand, C4BP has been shown to bind plasminogen and enhance its activation by urokinase-type plasminogen activator (Agarwal et al., 2015).

1.1.3 Complement in diseases and complement therapeutics

Complement-related diseases

Dysregulation of complement, resulting in inefficient or excessive complement activation, has been linked with many inflammatory and autoimmune diseases (McGeer et al., 2017; Ricklin and Lambris, 2013). There is also increasing evidence for the role of complement in metabolic and neurodegenerative disorders (Bonifati and Kishore, 2007; Phielers et al.,

2013). As the focus of this thesis is properdin of the alternative pathway, complement-related diseases for which the alternative pathway has been primarily implicated will be discussed here (summarised in **Table 1.2**).

Table 1.2. Diseases associated with the complement alternative pathway.

Disease	AP components affected
Atypical haemolytic uremic syndrome (aHUS)	C3, FB, FH, MCP, FI
Dense deposit disease (DDD)	C3, FH, FI, MCP, FB
Paroxysmal nocturnal haemoglobinuria (PNH)	DAF, CD59
Age-related macular degeneration (AMD)	FH, C3, FI, FB
Systemic lupus erythematosus (SLE)	MCP, FH
Rheumatoid arthritis	C3, FB
Alzheimer's disease	FH, CR1, CD59

Complement dysregulation can affect the ability to discriminate self from non-self resulting in self-attack, inflammation and lysis. Atypical haemolytic uremic syndrome (aHUS) is a disease characterised by low levels of circulating red blood cells due to their destruction (haemolytic anaemia) and low blood platelet count (thrombocytopenia), and can lead to acute kidney failure. aHUS is associated with mutations in one or more of complement proteins of the alternative pathway, such as C3, FB, FH, MCP and FI, and neutralising autoantibodies against complement proteins, such as anti-FH antibodies (Bajic et al., 2015; Roumenina et al., 2011). Structural and functional analyses of FH and aHUS-associated FH mutants showed that the mutations impair the binding of FH to C3b, as well as the binding of FH to GAGs and sialic acid glycans which are host markers (Blaum et al., 2014; Manuelian et al., 2003; Morgan et al., 2011). Similarly, aHUS-associated mutations in C3 resulted in reduced binding of C3 to complement regulators FH and MCP (Roumenina et al., 2012; Schramm et al., 2015). As a consequence of these mutations, complement dysregulation leads to the lysis of red blood cells and glomerular endothelial cells.

Other diseases which affect self-discrimination of the alternative pathway include dense deposit disease (DDD) and paroxysmal nocturnal haemoglobinuria (PNH). DDD is a rare renal disease and is a type of C3 glomerulopathy (C3G). Mutations in FH and C3 have been associated with increased risk of DDD, as well as FI and MCP (Barbour et al., 2013; Servais et al., 2012). Additionally, autoantibodies against the AP C3 convertase (C3 nephritic factors), which stabilise the enzyme complex, are associated with DDD, as well

as anti-FB and anti-FH autoantibodies (Strobel et al., 2010; Zhang et al., 2012). Compromised AP regulation results in increased C3b deposition and excessive accumulation of complement activation products in the glomerular basement membrane of the kidney, called dense deposits (Smith et al., 2007), ultimately leading to kidney malfunction. PNH is a rare disease characterised by haemolytic anaemia, blood clots (thrombosis) and bone marrow failure. PNH is associated with X-linked genetic mutations in phosphatidylinositol glycan class A (*PIGA*) gene, which is critical for the biosynthesis of glycosphosphatidylinositol (GPI) anchors. *PIGA* mutations are somatically acquired by hematopoietic stem cells (Luzzatto, 2006) and red blood cells from PNH patients lack GPI-anchored proteins DAF and CD59. The absence of these two complement regulators results in dysregulation of alternative pathway amplification and MAC formation, leading to haemolysis (Brodsky, 2014).

Complement dysregulation can also affect the ability to properly clear cellular debris, resulting in debris accumulation and inflammation. Age-related macular degeneration (AMD) is a leading cause of vision loss among people over the age of 50. It affects a part of the retina called the macula and causes impairment in central vision (Mitchell et al., 2018). The impairment in central vision is associated with the degradation of retinal pigment epithelial cells and the loss of photoreceptors. An early clinical sign of AMD is the excessive accumulation of extracellular deposits and debris, called drusen, which then leads to localised inflammation (Tan et al., 2016). In healthy individuals, FH binds to oxidised lipids in drusen, and prevents complement activation. Individuals with the FH variant Y402H are at higher risk of AMD; this FH variant has reduced binding to oxidised lipids and therefore, complement activation occurs on the retinal pigment epithelium (Shaw et al., 2012; Weismann et al., 2011). Mutants of other components of the alternative pathway have also been shown to confer higher risk for AMD, including C3, FI and FB (Tan et al., 2016). Polymorphisms in these genes also lead to complement dysregulation, ultimately resulting in increased complement activation (Heurich et al., 2011; Seddon et al., 2013).

Other autoimmune diseases have also been associated with the alternative pathway. Systemic lupus erythematosus (SLE) is a chronic inflammatory disease which is caused by impaired clearance of apoptotic cells and immune complexes, resulting in tissue damage in many organs, including skin and joints. Although SLE is strongly linked with

genetic deficiencies in the components of the classical pathway (C1, C2, C4) (Truedsson et al., 2007), mutations in MCP or FH could cause early onset of nephritis in patients with SLE (Jönsen et al., 2011). The alternative pathway has also been linked with pathogenesis of rheumatoid arthritis and joint destruction (Holers and Banda, 2018), and with chronic inflammatory response to apoptotic cells and amyloid beta peptides in Alzheimer's disease (Crehan et al., 2012). In summary, the association of the alternative pathway with a wide range of diseases highlights the importance of achieving the balance between activation and regulation to avoid tissue damage and maintain homeostasis (Holers, 2008).

Complement therapeutics

Improved understanding of the role of complement in various diseases has resulted in increased efforts to develop complement-targeting therapeutics. As well as inflammatory and immune diseases discussed above, complement therapeutics could improve tolerance and compatibility of transplants (Sacks and Zhou, 2012; Stites et al., 2015). Since complement is a major component of the innate immunity, complement therapeutics could impact complement-mediated defence against infections; additionally, the role of complement in the clearance of immune complexes and apoptotic cells needs to be preserved (Wagner and Frank, 2010). Due to the difficulty of achieving the balance between activation and regulation, only a couple of complement therapeutics are in use clinically (Ricklin et al., 2018).

Four different C1-INH concentrates have been approved by the US and/or the European Union for the treatment of hereditary angioedema (HAE), caused by C1-INH deficiency (Feussner et al., 2014). They are human plasma-derived C1-INH preparations Cinryze (Viro Pharma) (Aygören-Pürsün et al., 2017), Ceter (Sanquin) (Hofstra et al., 2012), and Berinert (CSL Behring) (Riedl et al., 2016), and recombinant C1-INH Ruconest (Pharming) (Varga and Farkas, 2011).

Eculizumab (Alexion Pharmaceuticals) is the first and only complement-targeting drug approved for clinical use. Eculizumab is a humanised anti-C5 antibody, which binds to C5 and inhibits C5 cleavage by preventing the interaction of C5 with the C5 convertase (Rother et al., 2007; Schatz-Jakobsen et al., 2016). By inhibiting C5 cleavage, it ultimately inhibits the generation of C5a and the formation of the MAC. Eculizumab is

currently used clinically for the treatment of aHUS and PNH; clinical trials are ongoing for other diseases (Hillmen et al., 2006; Legendre et al., 2013). Patients being treated with Eculizumab might be at higher risk of *Neisseria meningitidis* infection due to inhibition of MAC generation, so they are provided with vaccination and antibiotics before starting treatment and are carefully monitored (Rother et al., 2007). However, there is evidence that Eculizumab-treated patients remain at risk despite vaccination due to Eculizumab blocking complement-mediated lysis of *N. meningitidis* (Konar and Granoff, 2017).

The success of Eculizumab demonstrates the therapeutic value of complement inhibitors. However, treatment with Eculizumab costs £340,200 per person per year, making it one of most expensive drugs in the world (Morgan and Harris, 2015). Therefore, there is a need to develop novel complement therapeutics. Since Eculizumab targets the terminal complement pathway, there is an ongoing effort to develop novel inhibitors that target different components and regulators of the complement system, for example C3 and the C3 convertases (Merle et al., 2015). Several drug candidates are currently being evaluated in clinical trials (**Table 1.3**).

Despite the current complement-targeting drug discovery landscape with many therapeutics in the pipeline, many challenges remain. There is the aforementioned increased susceptibility to bacterial infections. Studies of complement-deficient individuals has indicated that deficiencies in terminal pathway components results in increased susceptibility to *Neisseria* infections, whereas deficiencies in C3 or components upstream of C3 cleavage leads to increased susceptibility to a broader range of bacterial infections (Audemard-Verger et al., 2016; Pettigrew et al., 2009). Infection risk can be mitigated by prophylactic antibiotics and/or vaccination, although vaccination did not confer full protection against meningococcal infection in complement-deficient individuals and patients being treated with Eculizumab (Konar and Granoff, 2017; Platonov, 2003). APL-2 (Apellis Pharmaceuticals), developed from the C3-binding cyclic peptide compstatin (Mastellos et al., 2015), is currently undergoing phase I clinical trials for the treatment of PNH (ClinicalTrials.gov Identifier NCT02588833). Trial participants had to show evidence for or were given vaccinations against *Neisseria meningitidis* and *Haemophilus influenzae* type b (Hib). The results of these clinical trials will be informative on whether increased infection risk can be managed when targeting C3.

High target concentration and high turnover present additional challenges to the development of complement-targeting drugs, as these impact drug dosing. For example, C3 plasma concentration is 1.2 mg/ml, and 10-20% of plasma C3 is consumed and replaced daily (Morgan and Harris, 2015). C5 plasma concentration is 75 µg/ml, and C5-targeting Eculizumab is administered to patients being treated for aHUS via intravenous infusion at 900 mg once a week for 4 weeks, and then 1.2 g in the fifth week, followed by 1.2 g every 12-16 days (from the National Institute for Health and Care Excellence website). Complement proteins are also produced locally at sites of inflammation, which may further increase their concentrations (Daha and van Kooten, 2000; Lubbers et al., 2017). To this end, various strategies are being developed to reduce dose, such as engineering antibodies and targeting neopeptides on complement proteins (Harris, 2018). Alexion Pharmaceuticals has generated ALXN1210 by modifying Eculizumab to increase recycling efficiency and duration of action (Sheridan et al., 2018). It is currently undergoing phase III clinical trials for the treatment of PNH, and is administered every 8 weeks following initial dose (ClinicalTrials.gov Identifier NCT02946463). The alternative approach to reducing dose is to target neopeptides on complement proteins which are only present when activated, and therefore, are present at lower concentrations in plasma (Harris, 2018). For example, antibodies have been developed that bind specifically to activated C3b, but not C3, but these are yet to be evaluated in clinical trials (Katschke et al., 2009; Paixão-Cavalcante et al., 2014).

Table 1.3. Complement-targeting therapeutics in clinical trials. This table was modified from (Ricklin et al., 2018). The table in the reference showed ongoing, completed and terminated trials from 2012 onwards. The table here shows ongoing clinical trials at the time of writing (July 2019). The trial number is the identifier on ClinicalTrials.gov website.

Target	Drug candidate (Company)	Trial number	Phase	Disease(s)
MASP-2	Narsoplimab/OMS721 (Omeros Corporation)	NCT02682407	II	IgA nephropathy, lupus nephritis, membranous nephropathy, C3G
		NCT02222545	II	Thrombotic microangiopathies
		NCT03205995	III	aHUS
		NCT03608033	III	IgA nephropathy
C3	APL-2 (Apellis Pharmaceuticals)	NCT02588833	I	PNH
		NCT02264639	I	PNH (as add-on therapy)
		NCT03777332	I	Geographic atrophy
		NCT03465709	I,II	AMD
		NCT03593200	II	PNH
		NCT03226678	II	Warm autoimmune haemolytic anaemia, cold agglutinin disease
FB	IONIS-FB-LRx (Ionis Pharmaceuticals)	NCT03815825	II	Geographic atrophy
C5	Ravulizumab/ALXN1210 (Alexion Pharmaceuticals)	NCT02598583	I,II	PNH (dose escalation study)
		NCT02605993	II	PNH (multiple ascending dose study)
		NCT02946463	III	PNH (comparison to Eculizumab)
		NCT03056040	III	PNH (with patients currently treated with Eculizumab)
C5	Tesidolumab/LFG316 (Novartis Pharmaceuticals)	NCT02949128	III	aHUS
		NCT02534909	II	PNH
C5	Zimura (Ophthotech Corporation)	NCT02686658	II	AMD
C5	Coversin/rVA576 (AKARI Therapeutics)	NCT03588026	III	PNH
		NCT03829449	III	PNH, aHUS (long term safety)
C5aR1	Avacopan/CCX168 (ChemoCentryx)	NCT02994927	III	Anti-neutrophil cytoplasmic-antibody-associated vasculitis

1.2 The role of properdin in complement regulation

1.2.1 Properdin domain structure and oligomerisation

Human properdin is a glycoprotein which is 442 amino acids long after signal peptide removal. Properdin is composed of thrombospondin type 1 repeat (TSR) domains, connected head to tail. Initially, properdin was annotated as containing five and a half TSR domains (Nolan et al., 1992), based on the consensus TSR sequence of six cysteine, three tryptophan and two arginine residues. Properdin was later expanded to six TSR domains, with TSR6 containing a large insertion (Higgins et al., 1995), and then the N-terminal region was re-defined as an additional TSR domain, named TSR0 (Sun et al., 2004) (**Figure 1.6A**). TSR domains also exist in thrombospondins, extracellular matrix proteins (mindin, F-spondin) and other complement proteins (C6, C7, C8, C9). When this thesis was started, the atomic structure of properdin had not been solved; however, crystal structures of TSR domains from other proteins were available. The crystal structure of two TSR domains of thrombospondin-1 shows the stacking of cysteine, tryptophan and arginine side chains forming a CWR-layered motif (Tan et al., 2002) (**Figure 1.5A**). Three strands form the core of the structure, of which the N-terminal strand contains the tryptophan residues and the second strand contains the layered arginine residues. The TSR domains are connected by a five-residue linker between the C-terminal cysteine residue in the preceding TSR domain and the first tryptophan residue of the CWR-layered motif in the following TSR domain (Tan et al., 2002) (**Figure 1.5A**).

The sequence alignment of TSR domains of human properdin with TSR domains of human thrombospondin-1 shows that the CWR-layered motif residues are highly conserved, with the exception of TSR4, which only has two tryptophan and one arginine residues (**Figure 1.5B**). TSR1 and TSR2 both have an extra cysteine residue, which may form an inter-domain disulphide bond. While TSR0 contains six cysteine residues, the spacing is inconsistent with a TSR fold and the domain also lacks the conserved tryptophan and arginine residues (**Figure 1.5B**); therefore, TSR0 is unlikely to have the protein fold containing a CWR-layered motif.

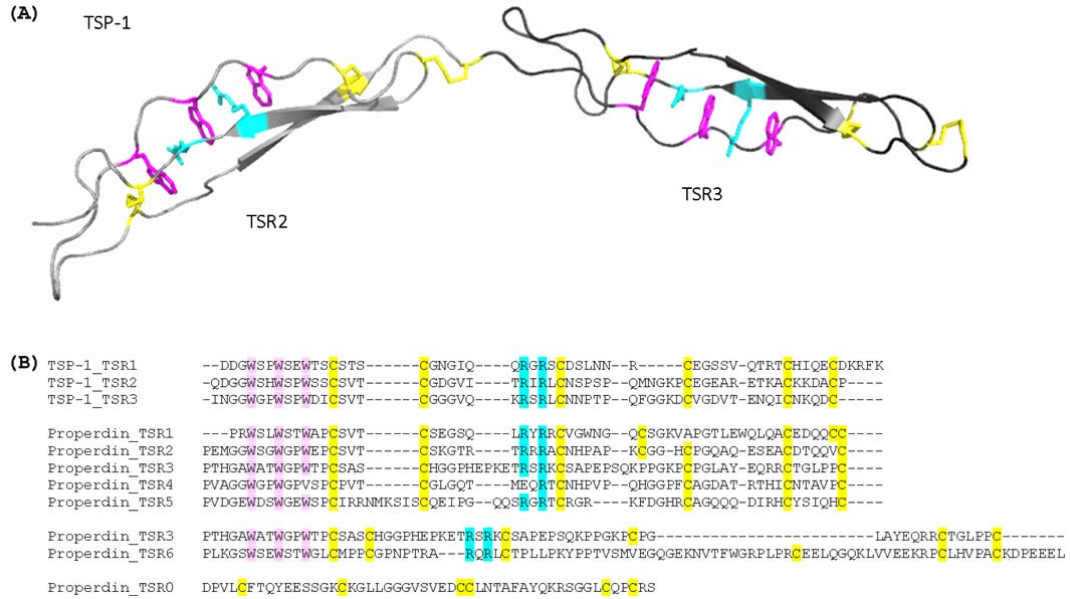


Figure 1.5. TSR domains of thrombospondin-1 and properdin. (A) The crystal structure of TSR2 and TSR3 domains of thrombospondin-1 (PDB 1LSL) shows the stacking of cysteine (yellow), tryptophan (magenta) and arginine (cyan) residues of the CWR-layered motif. Image was produced with PyMOL (Version 2.1, Schrödinger, LLC). (C) The sequence alignment of TSR domains of human thrombospondin-1 and human properdin shows that residues of the CWR-layered motif are highly conserved in TSR1 to TSR5. TSR0 does not contain the conserved tryptophan or arginine residues, whereas TSR6 contains a large insertion.

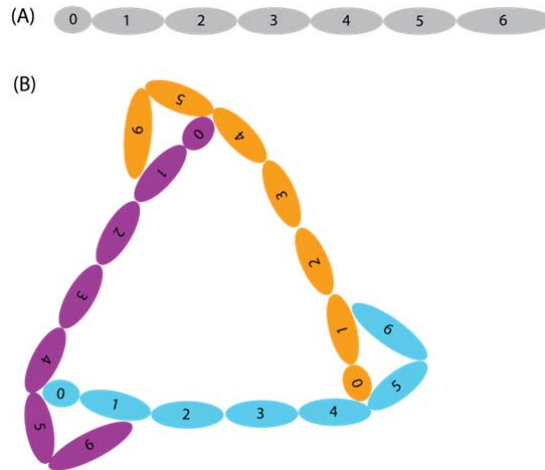


Figure 1.6. Properdin domain arrangement. (A) Model of the structure of properdin monomer, consisting of seven TSR domains. (B) Schematic cartoon of the proposed TSR domain arrangement in properdin trimer. Each properdin molecule is coloured differently.

Post-translational modifications of properdin include N-linked glycosylation, O-linked glycosylation and the relatively unusual C-mannosylation (Hartmann and Hofsteenge, 2000; Higgins et al., 1995; Yang et al., 2016). The N-glycosylation on an asparagine residue in TSR6 was shown not to be essential for properdin function (Higgins et al., 1995). Properdin contains C-mannosylated tryptophan residues (Hartmann and Hofsteenge, 2000). Recent native mass spectrometry analysis of human properdin showed that there is extensive heterogeneity in the occupancy of C-mannosylation sites on properdin (Yang et al., 2016). All 17 tryptophan residues within the CWR-layered motifs of TSR1 to TSR6 could be modified by C-mannosylation (Yang et al., 2016). This is an unusual type of glycosylation as it involves a C-C bond linkage. The functional role of C-mannosylation in proteins containing TSR domains is unknown. The hydrophilic mannose groups are predicted to be exposed on the surface (Furmanek and Hofsteenge, 2000; Tan et al., 2002). In properdin, it has been suggested to affect the flexibility of TSR domains, properdin oligomerisation or properdin ligand binding (Harrison, 2017).

Human plasma contains a mixture of various oligomeric forms of properdin, mainly dimers, trimers and tetramers in a 1:2:1 ratio (Pangburn, 1989; Smith et al., 1984). Higher oligomers were shown to have a higher affinity for C3b, C3bB and C3bBb (Pangburn, 1989). The increased affinity is likely due to the avidity effect as properdin oligomers have multiple binding sites for C3 convertases. Structural studies had suggested that oligomerisation occurs via interactions between the two N-terminal domains of one properdin molecule and the two C-terminal domains of the next (Alcorlo et al., 2013; Sun et al., 2004) (**Figure 1.6B**). This was later confirmed by the low resolution crystal structure of properdin bound to C3b (Pedersen et al., 2017). The functional roles of the different TSR domains were elucidated via mutagenesis studies (Higgins et al., 1995). Recombinant properdin lacking TSR6 could not form oligomers; properdin lacking TSR5 or TSR6 could not bind C3b; and properdin lacking TSR4, TSR5 or TSR6 could not stabilise the AP C3 convertase (Higgins et al., 1995). Structural studies have shown that TSR4 and TSR5 could be responsible for properdin-C3 convertase interaction (Alcorlo et al., 2013; Pedersen et al., 2017); these structures will be discussed later in this chapter.

1.2.2 Properdin function

When properdin was discovered, it was proposed to initiate the alternative pathway by acting as a pattern recognition molecule (Pillemer et al., 1954). In this model, the AP is activated in a similar manner to the CP and the LP. This view was later challenged and replaced with the well-established function of properdin as a positive regulator of the AP (Nelson, 1958). Properdin up-regulates the alternative pathway by stabilising the AP C3 convertase (C3bBb); the half-life of the C3 convertase is increased by up to 10-fold by properdin (Fearon and Austen, 1975; Pangburn and Müller-Eberhard, 1986). Properdin binds more favourably to surface-bound C3bBb, C3bB and C3b than their fluid-phase counterparts, which suggests that amplification of the AP by properdin occurs on an activator surface, but not during tick-over in the fluid phase (Farries et al., 1988a).

In the past two decades, the pattern recognition model of properdin has re-surfaced, but this function still remains controversial in the field. In surface plasmon resonance experiments, a properdin-coated surface was shown to recruit C3b and FB, and serve as a platform for *in situ* AP C3 convertase assembly (Hourcade, 2006). Properdin has since been proposed to bind to a variety of surfaces and increase C3b deposition, including microorganisms and cells, such as *Neisseria gonorrhoeae* (Spitzer et al., 2007), *Chlamydia pneumoniae* (Cortes et al., 2011), neutrophils (Camous et al., 2011), proximal tubular epithelial cells (Gaarkeuken et al., 2008), apoptotic and necrotic cells (Ferreira et al., 2010; Kemper et al., 2008; Xu et al., 2008), and activated platelets (Saggu et al., 2013). Properdin has also been shown to bind to biological substrates, such as myeloperoxidase (MPO) (O'Flynn et al., 2014) and zymosan (Ferreira et al., 2010).

However, caution must be taken when interpreting the results from properdin binding studies. Unfractionated properdin contains non-physiological higher order aggregates of properdin, as a result of long-term storage and freeze-thawing (Pangburn, 1989). These higher order oligomers must be separated from physiological forms of properdin (dimer, trimer, tetramer), as they have been shown to bind non-specifically to surfaces. For example, unfractionated properdin could bind to different strains of *N. gonorrhoeae* and *Neisseria meningitidis*, but native forms of properdin, fractionated by size exclusion chromatography, could not bind directly to any of the bacterial strains (Agarwal et al., 2010). Unfractionated properdin could account for non-specific interactions in some of

the binding studies mentioned above (Camous et al., 2011; Gaarkeuken et al., 2008; Hourcade, 2006; Kemper et al., 2008; Spitzer et al., 2007; Xu et al., 2008).

Properdin is a highly positively charged protein ($pI > 9.5$; Pangburn, 1989) and it has been shown to interact with negatively charged surface glycosaminoglycans (GAGs) and sulphated glycoconjugates (Kemper et al., 2008; Yu et al., 2005). The CWR-layered motif of TSR domains is thought to constitute a heparin, heparan sulphate or GAG-binding site (Olsen and Kragelund, 2014; Tan et al., 2002; Tossavainen et al., 2006). Since many complement regulators interact with sugar molecules on the surfaces of host and pathogen, it is possible that properdin also modulates the alternative pathway in a similar manner (Langford-Smith et al., 2015). Properdin may also interact with bacterial lipopolysaccharide (LPS) and lipooligosaccharide (LOS) on microbial surfaces (Kimura et al., 2008) via charge-charge interactions, as LPS and LOS are also negatively charged molecules.

(Kimura et al., 2008) showed that there were major differences in the requirement of properdin and AP activity depending on the activators used in complement activation assays. In their work, properdin deficiency caused a higher reduction in LOS-induced complement activation than in LPS-induced complement activation. Additionally, properdin was shown not to be essential in zymosan-induced or cobra venom factor (CVF)-induced AP complement activation, and anti-OVA (ovalbumin)/OVA immune complex-induced CP activation (Kimura et al., 2008). Their work highlights that careful consideration should be given to the design of complement activation assays using surface activators, due to the surface-dependent balance of regulation between properdin and FH.

More recent studies have demonstrated the requirement of initial C3 activation for properdin recruitment. The use of compstatin, a peptide inhibitor of C3 proteolytic activation (Sahu et al., 1996), showed that C3 activation was required for properdin binding to zymosan, MPO, human umbilical vein endothelial cells (HUVECs) and *N. meningitidis* (Harboe et al., 2012, 2017). The requirement for C3 activation for properdin recruitment had also been shown using C3-deficient serum (Agarwal et al., 2011; Junker et al., 1998). These studies suggest that C3b deposition or C3 convertase formation occurs before properdin is recruited to the surface, supporting the role of properdin as a positive

regulator of the AP C3 convertase. Many of the properdin binding studies mentioned earlier were performed under conditions which allow C3 activation and C3b deposition, which makes it difficult to interpret whether properdin is acting as a pattern recognition molecule or is binding to C3b on the surface (Harboe et al., 2017). When investigating the role of properdin as an initiator of the alternative pathway, it is important to rule out initial C3b generation and deposition (Harrison, 2017).

1.2.3 Properdin sources

Properdin concentration in plasma is around 5 µg/ml (Pangburn, 1989). Unlike other complement proteins which are predominantly synthesised by hepatocytes in the liver (Lubbers et al., 2017), properdin is primarily produced by leukocytes, including T cells (Schwaeble et al., 1993), dendritic cells (Li et al., 2011) and monocytes (Schwaeble et al., 1994). Neutrophils do not constitutively secrete properdin but store it in secondary granules, which can be secreted in response to inflammatory agonists, such as C5a, TNF- α , IL-8 and fMLP (Camous et al., 2011; Wirthmueller et al., 1997). The release of properdin in response to inflammatory stimuli could result in higher local concentrations of properdin around activated neutrophils and amplify local AP activation at sites of inflammation (Cortes et al., 2013).

Additionally, properdin circulating in blood and properdin released from neutrophils might have different activity levels. Properdin released from activated neutrophils could directly bind to apoptotic T cells, whereas serum properdin could not (Kemper et al., 2008). Properdin from activated neutrophil supernatants could bind directly to activated platelets; however, physiological oligomers of properdin from serum could also bind to activated platelets (Saggu et al., 2013). A direct comparison of equal amounts of serum properdin and neutrophil-derived properdin needs to be conducted to investigate the functional differences between properdin from different sources (Blatt et al., 2016a). It is unknown whether neutrophil-derived properdin exists in the same oligomeric ratio as serum properdin.

1.2.4 Properdin deficiencies

Properdin deficiency is a recessive X-linked genetic disorder and is associated with increased susceptibility to infection with *N. meningitidis* (Braconier et al., 1983; Linton and Morgan, 1999). Properdin-deficient individuals have also been shown to be at higher risk of recurrent otitis media and pneumonia (Schejbel et al., 2009).

Properdin deficiency has been divided into three main categories. Type I deficiency is associated with the complete absence of circulating properdin in plasma. Type I mutations result in premature stop codons and truncated gene products (Helminen et al., 2012; Späth et al., 1999; Westberg et al., 1995), which are likely degraded intracellularly. There are also mutations that change the conformation of properdin such that it can no longer be secreted (van den Bogaard et al., 2000). These mutations are in highly conserved amino acid residues of TSR domains, suggesting that they could be essential for the structure of properdin (van den Bogaard et al., 2000; Fijen et al., 1999). Type II properdin deficiency is characterised by low concentrations of properdin in plasma (less than 10% of normal) (Nielsen and Koch, 1987; Westberg et al., 1995). Oligomerisation of properdin is thought to be affected, with a lower proportion of trimers and tetramers (Fredrikson et al., 1998; Pedersen et al., 2017; Sjöholm et al., 1988). The low concentrations of properdin could be a result of rapid degradation of improperly folded properdin and/or lower oligomeric forms of properdin. Type III properdin deficiency is associated with dysfunctional properdin, despite normal plasma levels of properdin and oligomerisation pattern. Type III deficiency probably affects the binding of properdin to C3b (Fredrikson et al., 1996).

1.2.5 Structural studies of C3 convertase-properdin

Recent low resolution structural studies have elucidated how properdin stabilises the AP C3 convertase (Alcorlo et al., 2013; Pedersen et al., 2017). The two studies used different techniques – electron microscopy (EM) and X-ray crystallography. The first is a 23 Å EM structure of the C3 convertase-properdin complex (**Figure 1.7A**; Alcorlo et al., 2013), where the C3 convertase was stabilised using a FB mutant (D279G) (Goicoechea de Jorge et al., 2007). The second is a 6 Å crystal structure of the C3 convertase-properdin complex (**Figure 1.7B**; Pedersen et al., 2017), where the C3 convertase was stabilised by

staphylococcal complement inhibitor (SCIN) (Rooijakkers et al., 2005). In both structures, an atomic model of properdin could not be constructed into the density due to low resolution. Nonetheless, they both provide insights into how properdin binds to and stabilises the C3 convertase.

Both structural models show that the C3bBb convertase complex interacts with a vertex of a properdin oligomer (Alcorlo et al., 2013; Pedersen et al., 2017). Both models suggest that properdin stabilises the C3 convertase by interacting with the C345C domain of C3b and the von Willebrand factor type-A (vWA) domain of Bb. When comparing the two models, the EM structure suggests a more extensive interaction with the Bb vWA domain compared to the crystal structure. Both models suggest a rotation or displacement of the C3b C345C domain relative to Bb, when compared to the crystal structure of SCIN-stabilised C3bBb complex (PDB 2WIN, Rooijakkers et al., 2009). The authors of the crystal structure model of properdin-C3bBb complex suggested that properdin TSR5 domain interacts with the C3b C345C domain; the putative binding site on C345C domain contains negatively charged acidic residues which might interact with a positively charged TSR domain (Pedersen et al., 2017). TSR4 domain of properdin might be involved in the interaction with Bb vWA domain (Pedersen et al., 2017).

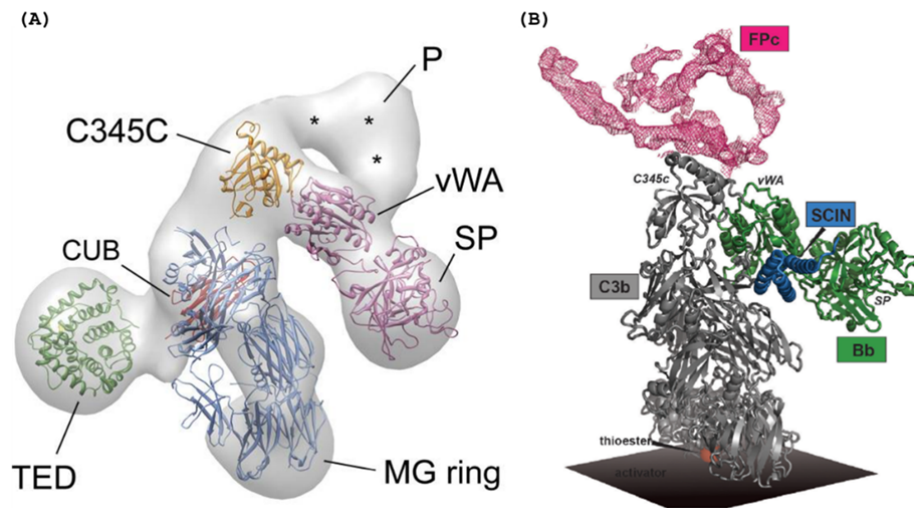


Figure 1.7. Structural studies of C3 convertase-properdin complex. (A) 23 Å EM structure of properdin bound to C3 convertase. The image was taken from the study (EMDB 2403, Alcorlo et al., 2013). (B) 6 Å crystal structure of pseudo-monomeric properdin (FPc) bound to SCIN-stabilised C3 convertase. The image was taken from the study (PDB 5M6W, Pedersen et al., 2017).

The main difference between the two models is the position of the TED domain of C3b. In the crystal structure model, the TED domain is in a position which agrees with the crystal structures of C3b and C3bBb (Janssen et al., 2006; Pedersen et al., 2017; Rooijakkers et al., 2009). In the EM structure model, however, only a third of the C3bBb-properdin complexes showed the TED domain in this position. The others showed rearrangements of the TED domain and also the CUB domain to which it is tethered (**Figure 1.7A**; Alcorlo et al., 2013). The authors suggest that the rearrangements of the TED and CUB domains upon properdin binding might interfere with the interaction of C3b with MCP and FI, respectively.

1.2.6 Properdin as a therapeutic target

Properdin is an attractive target for therapeutic inhibition since it is specific to the alternative pathway. Due to its function in the positive feedback amplification loop, inhibition of properdin could be effective in diseases caused by the over-activation of complement on host tissue, especially those specific to the alternative pathway, such as aHUS (Chen et al., 2018). In comparison to C3- or C5-targeting therapeutics, properdin inhibition would still preserve complement activation via the classical and lectin pathways (Smith-Jackson and Marchbank, 2018). As well as reduced MAC formation and C5a generation, properdin inhibition would also limit inflammatory responses mediated by upstream products C3a and C3b generated via the AP. Additionally, the concentration of properdin in serum (5 µg/ml) is much lower than that of C3 (1.2 mg/ml) and C5 (75 µg/ml), and therefore, properdin might be more feasible to inhibit using lower doses of drugs (Michels et al., 2019).

In vivo mouse disease models have shown promising results of inhibiting properdin in inflammatory diseases, such as renal ischemia-reperfusion injury (Miao et al., 2014; Miwa et al., 2013), allergic airway inflammation (Wang et al., 2015), abdominal aortic aneurysm (AAA) (Zhou et al., 2012, 2013), arthritis (Dimitrova et al., 2010, 2012; Kimura et al., 2010), intravascular and extravascular haemolysis (Gullipalli et al., 2018), and aHUS (Ueda et al., 2018). In general, the absence of properdin in properdin-deficient mice or in mice treated with anti-properdin antibodies has been found to be beneficial in mouse disease models in which complement activation is wrongly directed to the host surface (Michels et al., 2019). However, properdin inhibition has been shown to adversely

affect the progress of C3G (Leshner et al., 2013; Ruseva et al., 2013). These two studies showed that the absence of properdin converts mild C3G to lethal C3G in FH-deficient mice (Leshner et al., 2013; Ruseva et al., 2013). Therefore, properdin-targeting therapeutics might not be suitable for all AP-related complement disorders and the role of properdin in these disorders needs to be further studied.

Antibodies or fragments of properdin have been identified in many laboratories (Blatt et al., 2016b; Kouser et al., 2016; Pauly et al., 2014), as well as properdin-binding proteins from blood-sucking arthropods (Mendes-Sousa et al., 2017; Silva et al., 2016; Tyson et al., 2008). These molecules have potential to be developed as properdin-targeting therapeutics. Novartis introduced a fully human anti-properdin antibody (CLG561) which has completed phase II clinical trials (ClinicalTrials.gov Identifier NCT02515942) as monotherapy or in combination with human anti-C5 antibody (Tesidolumab) for geographic atrophy, an advanced form of AMD (Park et al., 2019). The results from the clinical trials remain unpublished. Novemed Therapeutics has developed an anti-properdin antibody (NM9401) and a small-molecule properdin antagonist for the treatment of PNH and rheumatoid arthritis, respectively (Ricklin and Lambris, 2016), but it is yet to be evaluated in clinical trials.

1.3 Complement evasion by pathogens

1.3.1 Mechanisms of complement evasion

Evasion of the host immune system is crucial for the survival of pathogens within their hosts. Therefore, pathogens have developed multiple mechanisms for attenuating or escaping host immune attacks. Since the complement system functions as the first line of defence against pathogens, as well as the mediator between the innate and adaptive immune systems, many serum-exposed pathogens have developed ways to modify or block complement (Lambris et al., 2008). Complement evasion by pathogens has been described for different levels of the complement cascade (Zipfel et al., 2013). Complement evasion strategies by pathogens can be divided into three categories (Lambris et al., 2008): inactivation of host complement proteins by enzymatic degradation, recruitment or mimicry of host complement regulators; and expression of complement modulatory or inhibitory molecules (**Table 1.4**).

One method of complement evasion by bacterial pathogens is the expression and secretion of proteases which can cleave and inactivate host complement proteins. For example, *Pseudomonas aeruginosa* secretes elastase (PaE) which cleaves C1q and C3 (Hong and Ghebrehiwet, 1992) and alkaline protease (PaAP) which cleaves C2 (Laarman et al., 2012), enabling it to attenuate complement activation via the three pathways. Another example is *Staphylococcus aureus*, which secretes a metalloprotease called aureolysin to cleave C3 (Laarman et al., 2011). Many other pathogens use proteolytic inactivation as an evasion strategy, and many also target C3 which is a central component of the complement cascade (Potempa and Potempa, 2012). Alternatively, some bacterial species express plasminogen activators and/or receptors on the surface, such as *S. aureus*, *Streptococcus pyogenes* and *Borrelia burgdorferi* (Bhattacharya et al., 2012). They are able to recruit and convert the host plasminogen into active plasmin, which then degrades IgG and C3 on bacterial surfaces.

Table 1.4. Examples of complement evasion strategies used by pathogens.

Pathogen	Protein	Host target
Enzymatic degradation of host complement proteins		
<i>P. aeruginosa</i>	PaE	C1q, C3
<i>P. aeruginosa</i>	PaAP	C2
<i>S. aureus</i>	Aureolysin	C3
<i>S. aureus</i>	Plasminogen activator	C3, IgG
<i>S. pyrogenes</i>	Plasminogen activator	C3, IgG
<i>B. burgdorferi</i>	Plasminogen receptor	C3, IgG
Recruitment or mimicry of host complement regulators		
<i>B. burgdorferi</i>	CD59-like protein	MAC
<i>B. burgdorferi</i>	CRASPs	FH, FHL-1, CFHRs
<i>N. meningitidis</i>	NspA	FH
<i>N. meningitidis</i>	PorA	C4BP
<i>N. meningitidis</i>	Opc, Msf	Vitronectin
<i>S. pneumoniae</i>	LytA	C4BP
<i>S. pneumoniae</i>	PspC, Hic	Vitronectin
<i>B. recurrentis</i>	CihC	C1-INH
<i>H. influenzae</i>	Hsf	Vitronectin
<i>P. aeruginosa</i>	Porin D, Lpd	Vitronectin
Expression of complement modulatory or inhibitory molecules		
<i>S. aureus</i>	Efb	C3
<i>S. aureus</i>	Ehp	C3
<i>S. aureus</i>	SCIN	C3b
<i>S. aureus</i>	SSL7	C5

The recruitment or mimicry of host complement regulators results in reduced complement activation on pathogenic surfaces. *B. burgdorferi* uses both of these methods to evade complement. It expresses a complement inhibitory protein in the membrane which is CD59-like (Pausa et al., 2003). CD59 is a complement regulator that binds to C8 and C9 to inhibit MAC formation (Huang et al., 2006). Therefore, by mimicking the host CD59, *B. burgdorferi* inhibits MAC formation in its membrane (Pausa et al., 2003). Additionally, *B. burgdorferi* expresses five complement regulator acquiring surface proteins (CRASPs) which bind to FH, FH-like protein 1 (FHL-1) or complement FH-related proteins (CFHRs) (Kraiczy and Stevenson, 2013). While FH and FHL-1 are regulators of the alternative pathway (Clark et al., 2014), CFHRs have been shown to have other complement regulatory activity or modulate the activity of FH (Józsi et al., 2015). By expressing CRASPs on their surface, *B. burgdorferi* can recruit these host complement regulators and reduce complement activation via decay of AP C3 convertase and degradation of C3b.

Other bacterial pathogens have also been shown to recruit fluid-phase complement regulators from the host to their surfaces, including FH, C1-INH, C4BP (inhibitor of classical and lectin pathways) and vitronectin (inhibitor of MAC formation) (Blom et al., 2009; Hovingh et al., 2016). Examples are given in **Table 1.4**.

Another strategy of complement evasion by pathogens is the expression of molecules that directly inhibit host complement proteins. Complement inhibitors of both the C3 convertase and C5 convertase have been described in *S. aureus*, and the structural basis for their mechanism of inhibition has been studied. *S. aureus* secretes a 16-kDa protein called extracellular fibrinogen-binding protein (Efb) (Lee et al., 2004). The crystal structure of Efb bound to a fragment of C3 shows that Efb inhibits the conversion of C3 to C3b by binding to the TED domain of C3 (PDB 2GOX, Hammel et al., 2007a). By preventing C3b generation, Efb blocks complement-mediated phagocytosis (Lee et al., 2004). *S. aureus* also secretes the 10-kDa Efb homologous protein (Ehp), which also binds to C3 and inhibits C3 conversion (PDB 2NOJ, Hammel et al., 2007b). Additionally, *S. aureus* produces the 10-kDa staphylococcal complement inhibitor (SCIN), which acts at the level of the C3 convertase (Rooijakkers et al., 2005). SCIN binds directly to C3b and inhibits the activity of the AP C3 convertase; it stabilises the C3 convertase and locks it in a stable but non-functional state (PDB 3NSA, Garcia et al., 2010; PDB 2WIN, Rooijakkers et al., 2009). Furthermore, *S. aureus* secretes staphylococcal superantigen-like protein 7 (SSL7), which acts at the level of the C5 convertase (Langley et al., 2005). SSL7 bind to C5 and inhibits C5 cleavage by the C5 convertase (PDB 3KLS, Laursen et al., 2010).

Studies on the molecular mechanisms of complement evasion and complement inhibition have improved our understanding of host-pathogen interactions, and of regulatory mechanisms of the human complement system. This will in turn provide insights into the role of complement in the pathology of infectious and inflammatory diseases. Structural and functional studies of complement inhibitory molecules have the potential to be translated into the development of complement-targeting therapeutics (Lambris et al., 2008). Additionally, complement evasion molecules from bacterial pathogens could be good vaccine targets (Hovingh et al., 2016). More importantly, structural studies of complement inhibitors have resulted in improved understanding of the human complement system. For example, the ability of SCIN to stabilise the AP C3 convertase,

which is otherwise short-lived, allowed the crystallisation of the C3bBb-SCIN complex (Rooijakkers et al., 2009). The structure provided molecular insights into substrate specificity and enzymatic activity of the C3 convertase.

1.3.2 Complement evasion by ticks

Ticks are obligate blood-feeding parasites and they have developed ways to counterattack the deleterious effects of the host complement. Ticks can remain attached to the host for relatively long periods, compared to other blood-feeding arthropods; the blood meals of *Ixodes* ticks, for example, can last from several days up to weeks depending on the life stage and tick species (Šimo et al., 2017). Ticks inject many proteins into their feeding site, which include immune system and coagulation inhibitors so that they can prolong their feed without being damaged (Schroeder et al., 2009). Therefore, tick saliva represents an interesting target for the discovery of novel immune system inhibitors (Šimo et al., 2017). In particular, inhibitors of the complement system in tick saliva could protect the ticks from complement-mediated inflammation in the gut, as well as from the effects of complement deposition and anaphylatoxins. Tick complement inhibitors have also been identified as crucial virulence factors for tick-borne pathogens, such as *B. burgdorferi* (Schuijt et al., 2011; Wagemakers et al., 2016).

Complement inhibitory proteins have been identified from the salivary glands of different tick species. OmCI is a well-characterised 17-kDa protein from the soft tick *Ornithodoros moubata* belonging to the lipocalin family (Nunn et al., 2005). It inhibits the terminal pathway of complement by binding tightly to C5 and preventing its cleavage (Fredslund et al., 2008; Roversi et al., 2007). Tick complement inhibitors represent opportunities to increase our knowledge of the host-tick interface, as well as our mechanistic understanding of complement. Structures of C5 in complex with C5-binding tick proteins (OmCI and the *Rhipicephalus appendiculatus* complement inhibitor (RaCI) family) in addition to the Fab fragment of Eculizumab have provided insights into substrate recognition and specificity of the C5 convertases (Jore et al., 2016).

Furthermore, tick complement inhibitors are a source of complement therapeutics. Tick salivary proteins have multiple advantages as therapeutic agents. Firstly, they are highly potent due to the significant evolutionary pressures exerted on ticks by the complement

system (Schroeder et al., 2009). Tick complement inhibitors characterised so far bind to their targets with high affinity. Secondly, tick salivary proteins are likely poorly immunogenic, as ticks need to remain attached to the host for weeks without inducing an immune response (Šimo et al., 2017; Wikel and Bergman, 1997). Recombinant OmCI (Coversin, Akari Therapeutics) was shown to inhibit C5 in multiple species and was effective in rodent models of complement-mediated disease (Hepburn et al., 2007). Coversin has completed Phase I and Phase II clinical trials for the treatment of PNH (Hill et al., 2017; Weston-Davies et al., 2014).

1.3.3 Properdin-binding complement inhibitors from ticks

Most tick salivary complement inhibitors cloned to date have been identified in ticks belonging to the *Ixodes* genus (Lawrie et al., 1999). The first characterised tick protein was from the tick *Ixodes scalpularis* and was named *I. scalpularis* anti-complement protein (Isac) (Valenzuela et al., 2000). Interestingly, Isac specifically inhibited the alternative pathway and had no effect on the classical pathway. This was different from previously identified non-specific inhibitors from ticks at the time. Isac was found to prevent C3b and FB deposition on the surface, and displace pre-bound FB from the C3 convertase (Valenzuela et al., 2000). The authors of the study suggested that due to the presence of four cysteines in Isac, it could be a functional homolog of a short consensus repeat (SCR) domain of FH, acquired by convergent evolution (Valenzuela et al., 2000).

Since then, homologous sequences of Isac have been found from cDNAs collected from the salivary glands of the ticks *I. scalpularis*, *I. ricinus* and *I. pacifus* (Daix et al., 2007; Francischetti et al., 2005; Ribeiro et al., 2006). Two different homologs of Isac were isolated from the salivary gland transcriptome of *I. ricinus*; these were named *I. ricinus* anti-complement (IRAC) proteins I and II (Daix et al., 2007). Salp20 was identified from *I. scalpularis* and it shares 83% sequence identity with Isac (Das et al., 2001). IRAC I, IRAC II and Salp20 also inhibit C3b deposition, inhibit FB binding to C3b, and displace pre-bound FB from AP C3 convertase (Couvreur et al., 2008; Tyson et al., 2007). Therefore, these tick proteins inhibit the alternative pathway at the C3 convertase level, resulting in decreased C3a production and C3b deposition (Tyson et al., 2007).

Salp20 and IRACs were shown to bind to properdin (Couvreur et al., 2008; Tyson et al., 2008). They were shown to inhibit the interaction of properdin with C3b, as well as displace pre-bound properdin (Couvreur et al., 2008). The binding affinity of properdin for Salp20 was 100-fold higher than for C3b (Tyson et al., 2008). By binding tightly to properdin, these inhibitors probably reduce the stability of the C3 convertase. Their inhibitory effect is similar to that of monoclonal antibodies that bind to properdin and inhibit the alternative pathway (Gupta-Bansal et al., 2000). Additionally, displacing properdin from the C3 convertase may leave C3bBb susceptible to FH-mediated decay, since Salp20 and FH were shown to have synergistic inhibitory effects in AP haemolysis assays (Hourcade et al., 2016).

IRACs were shown not to interact directly with C3, C3b, FB or C3bBb (Couvreur et al., 2008). Therefore, these tick proteins could function by competing with C3b for properdin binding, or by altering the conformation of properdin to one with lower C3b affinity (Hourcade et al., 2016). These inhibitors have predicted molecular weights of around 20 kDa, but are much larger (50-70 kDa) due to glycosylation (Couvreur et al., 2008; Tyson et al., 2007). It has been hypothesised that the sugar molecules are responsible for binding the TSR domains of properdin (Tyson et al., 2008).

In summary, multiple *Ixodes* species express complement inhibitory proteins that bind properdin. By reducing complement activation via the alternative pathway, these inhibitors probably allow successful feeding of the tick without triggering an inflammatory response. These properdin-binding proteins are useful tools to investigate the role of properdin in the alternative pathway and C3 convertase regulation. They may also be useful to study the involvement of properdin in human diseases affecting the alternative pathway.

1.3.4 Novel complement inhibitors from *Rhipicephalus pulchellus*

Ixodes and *Rhipicephalus* ticks have different hosts as well as different geographical distributions, which made ticks of the *Rhipicephalus* genus interesting to get immune modulators from. The work described in this section of the identification of novel complement inhibitors from the zebra tick *Rhipicephalus pulchellus* was performed by Terence Tang and Matthijs Jore. Previous work in the lab had shown the presence of

complement inhibitory activity in the salivary gland extract (SGE) of *R. pulchellus*. Female ticks which had been feeding for 6 days were used for salivary gland dissection. To identify novel complement inhibitors from tick saliva, salivary glands from *R. pulchellus* were extracted, homogenised and fractionated using a series of chromatographic methods such as anion exchange chromatography, reverse-phase high performance liquid chromatography (HPLC), hydrophobic interaction chromatography (HIC) and size exclusion chromatography (SEC). In each step of fractionation, the elution fractions were tested for their ability to inhibit MAC formation in ELISA-based complement activation assays (WIESLAB) using human serum, and then the active fractions were further fractionated. Fractions were tested for inhibitory activity in AP and CP WIESLAB assays. The proteins in the final enriched fractions were analysed by mass spectrometry following trypsin digest.

The mass spectrometry data was used to update the cDNA library by (Tan et al., 2015) (Accession no.: PRJNA170743, NCBI). Sequence analysis using the novel transcriptome database narrowed down seven AP and twelve CP candidate inhibitors. These were cloned into pExpres2-2 vector and used to transfect *Drosophila melanogaster* S2 cells (Expres2ion Biotechnologies). Growth media of S2 cells expressing the candidate proteins were then tested for complement inhibition. This resulted in the identification of two novel complement inhibitors, comp52_seq0 and comp53_seq2, which inhibit the AP and CP, respectively. Serum pulldown subsequently showed that comp52_seq0 and comp53_seq2 bind properdin and C5, respectively. comp52_seq0 was subsequently named CIRp-A1 (Complement Inhibitor from R. *pulchellus* of the Alternative Pathway) and comp53_seq2, CIRp-T1 (Complement Inhibitor from R. *pulchellus* of the Terminal Pathway). Additionally, homologs of these proteins were identified by searching for similar sequences in other tick species using the expressed sequence tag (EST) database (NCBI), and the group's *R. pulchellus* and *R. appendiculatus* cDNA libraries. This thesis aims to build on the preliminary work on the novel CIRp-A1 family of properdin-binding complement inhibitory tick proteins described here.

1.4 Aims of this thesis

Properdin

At the beginning of this thesis, the atomic structure of properdin was unknown. There was a 23 Å EM structure of the AP C3 convertase bound to properdin, but the atomic model of properdin could not be built into the density due to the low resolution of the data (Alcorlo et al, 2013). Therefore, one of the aims of this thesis was to determine the structure of properdin to gain insights into the molecular mechanisms underlying the function of properdin in the alternative pathway, as an initiator and/or positive regulator. Properdin was purified from serum, or recombinantly expressed in various expression systems.

Properdin-binding tick complement inhibitors

A novel properdin-binding protein was identified in the lab from the salivary glands of the tick *Rhipicephalus pulchellus*, named CIRp-A1 (Jore, unpublished). In addition to CIRp-A1, five homologs were identified by searching for similar sequences in other tick species. In this thesis, I investigated the molecular basis of alternative pathway inhibition by this novel set of properdin-binding tick proteins. Structural characterisation of CIRp-A1 and homologs was performed, and the effects of CIRp-A1 on properdin function and ligand binding were investigated.

CHAPTER 2: MATERIALS AND METHODS

2.1 Cell culture

2.2 Molecular biology

2.2.1 Cloning

For DNA amplification, polymerase chain reactions (PCRs) were carried out with Q5 High-Fidelity DNA Polymerase (NEB) using a T100™ Thermal Cycler (Bio-Rad) (**Table 2.1**). For plasmid assembly, restriction enzyme digestion and ligation were carried out using FastDigest restriction enzymes (Thermo Scientific) and the LigaFast™ Rapid DNA Ligation System (Promega) (**Table 2.2**). Amino acid mutations were introduced using the Q5® Site-Directed Mutagenesis Kit (NEB) or the NEBuilder HiFi DNA Assembly Master Mix (NEB) (**Table 2.3**).

Table 2.1. PCR protocol for Q5 High-Fidelity DNA Polymerase.

Components of 25 µl reaction		Cycling conditions		
Component	Volume	Cycling step	Temperature	Time
5x Q5 Reaction Buffer	5 µl	1) Initial denaturation	98 °C	10 sec
10 mM dNTPs	0.5 µl	2) Denaturation	98 °C	10 sec
10 µM forward primer	1.25 µl	3) Annealing	55 °C	30 sec
10 µM reverse primer	1.25 µl	4) Extension	72 °C	30 sec/kb
Q5 DNA Polymerase	0.25 µl	Repeat 2-4 x 35 times		
Template DNA	1 µl	5) Final extension	72 °C	2 min
dH ₂ O	15.75 µl	6) Hold	10 °C	∞

Table 2.2. Protocols for restriction enzyme digestion and ligation.

Restriction enzyme digestion		Ligation	
Component	Volume	Component	Volume
10x FastDigest Green Buffer	2 µl	2x Rapid Ligation Buffer	5 µl
Enzyme 1	1 µl	T4 DNA Ligase	1 µl
Enzyme 2	1 µl	Vector DNA	1 µl
DNA template	16 µl	Insert DNA	3 µl
Incubate at 37 °C for 1 h		Incubate at room temperature for 15 min	

Table 2.3. Protocols for site-directed mutagenesis and Gibson Assembly.

Q5 Site-Directed Mutagenesis		Gibson Assembly	
Component	Volume	Component	Volume
PCR product	1 μ l	NEBuilder Master Mix	5 μ l
2x KLD Reaction Buffer	5 μ l	Vector DNA	1 μ l
10x KLD Enzyme Mix	1 μ l	Insert DNA	3 μ l
dH ₂ O	3 μ l	dH ₂ O	1 μ l
Incubate at room temperature for 5 min		Incubate at 50 °C for 1 h	

2.2.2 DNA visualisation and purification

DNA samples were visualised in 1% (w/v) agarose gels, prepared as outlined in **Table 2.4**. DNA samples were mixed with GelPilot DNA Loading Dye, 5x (Qiagen) before loading onto the gel. GeneRuler 1 kb DNA Ladder (Thermo Scientific) was used as the molecular weight marker. Gels were visualised with the ChemiDoc XRS+ System (Bio-Rad).

Plasmid DNA was extracted from bacterial overnight cultures using the QIAprep Spin Miniprep Kit (Qiagen), according to the manufacturer's instructions. DNA from agarose gel slices or from PCR reactions were purified using the QIAquick Gel Extraction Kit (Qiagen) or QIAquick PCR Purification Kit (Qiagen), respectively, according to the manufacturer's instructions.

Table 2.4. Buffers and solutions for DNA visualisation.

Solution	Components
50x TAE buffer (per litre)	242 g Tris base 57.1 ml glacial acetic acid 100 ml 0.5 M EDTA pH 8.2
Agarose gel	1% (w/v) agarose in TAE buffer SYBR Safe DNA Gel Stain (Invitrogen), 1:10,000 dilution

2.2.3 DNA sequencing

Purified DNA products were sequenced using Source Bioscience Sequencing or Eurofins Genomics. Sequencing primers are listed in **Table 2.5**.

Table 2.5. Sequencing primers used in this thesis.

Primer	Sequence (5'–3')	Vector
T7 (F)	TAATACGACTCACTATAGGG	pET15b, pETM-14, pETM-44
T7 (R)	GCTAGTTATTGCTCAGCGG	pET15b, pETM-14, pETM-44
pExpreS2-2 (F)	CGTCTACGGAGCGACAATTCAAT	pExpreS2-2
pExpreS2-2 (R)	GGAGTGTGTAAATGGACAATGTTG	pExpreS2-2
pKLAC2 (F)	GAAGAAGCCTTGATTGGA	pKLAC2
pKLAC2 (R)	TTATCGCACAAAGACAAT	pKLAC2
CMV (F)	CAACGGGACTTTCCAAAATG	pcDNA3.1(+)
BGH (R)	TAGAAGGCACAGTCGAGG	pcDNA3.1(+)

2.2.4 Bacterial cell transformation by heat shock

Bacterial cell transformations were carried out by heat shock. 1 µl of plasmid DNA at ~100 ng/µl was added to 50 µl of chemically competent *E. coli* cells in a microcentrifuge tube and incubated on ice for 30 min. Cells were heat shocked at 42 °C for 30 sec and then incubated on ice for 5 min. 500 µl of 2x YT medium was added, and the cells were allowed to recover at 37 °C with shaking for 40 min. Cells were then plated on LB agar plates containing the appropriate antibiotic.

2.2.5 Bacterial culture

Bacterial cultures were grown in Miller's Luria Broth (LB) (10 g tryptone, 5 g yeast extract, 10 g NaCl). LB was supplemented with 1.5% (w/v) agar to make solid LB agar plates. Where appropriate, ampicillin and kanamycin were used at working concentrations of 100 µg/ml and 50 µg/ml, respectively. Antibiotics were filter-sterilised and stored at -20 °C. *E. coli* were grown in liquid and solid media at 37 °C, unless otherwise stated. Bacterial overnight cultures were prepared by inoculating 10 ml of LB with a single isolated colony and incubating in an orbital shaker at 180 rpm. Bacterial growth was monitored by measuring the optical density at 600 nm (OD₆₀₀). Bacterial strain stocks were made by mixing 750 µl of an overnight culture and 250 µl of 60% (v/v) glycerol in a CryoTube (Thermo Scientific) and stored at -80 °C.

2.2.6 Insect culture

For insect expression, *Drosophila melanogaster* S2 suspension cells (Expres2ion Biotechnologies) were grown in EX-CELL 420 medium (Sigma-Aldrich) at 100 rpm and

25 °C. The cells were sub-cultured to a density of 5×10^6 cells/ml in medium supplemented with 10% (v/v) fetal bovine serum (FBS) (Life Technologies), or 8×10^6 cells/ml in serum-free medium. Transfections to obtain stable cell lines were carried out per manufacturer's instructions (Expres2ion Biotechnologies). The cells were frozen in 40% (v/v) EX-CELL 420, 50% (v/v) FBS and 10% (v/v) DMSO.

2.2.7 Yeast culture

For yeast expression, *Kluyveromyces lactis* GG799 cells were transformed using the *K. lactis* Protein Expression Kit (NEB) following the manufacturer's instructions. *K. lactis* were grown in YPGal Media at 250 rpm and 30 °C, or on YCB Agar Medium plates containing 5 mM acetamide at 30 °C. All growth media were made according to the manufacturer's instructions (NEB). *K. lactis* cells were stored at -80 °C suspended in a final concentration of 20% (v/v) glycerol.

2.2.8 Mammalian culture

Chinese hamster ovary (CHO) (Sigma-Aldrich) and human embryonic kidney 293T (HEK293T) (Sigma-Aldrich) cells were maintained in cell culture flasks at 37 °C and 5% CO₂ in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich) supplemented with 10% (v/v) FBS (Life Technologies), 2 mM L-Glutamine (Life Technologies), Minimum Essential Medium Non-Essential Amino Acids (MEM-NEAA) (Life Technologies) and Pen Strep (Life Technologies). For passage, the cells were detached from the culture flasks by washing with Dulbecco's Phosphate-Buffered Saline (PBS) (Sigma-Aldrich) followed by incubation in TrypLE Express (Life Technologies) diluted 1:5 in PBS, and then sub-cultured into fresh medium. The cells were frozen in 90% (v/v) FBS and 10% (v/v) DMSO.

FreeStyle 293-F cells (Thermo Fisher Scientific) were grown in FreeStyle 293 Expression Medium (Life Technologies) at 135 rpm, 37 °C and 5% CO₂. The cells were sub-cultured to a density of 0.2×10^6 cells/ml. The cells were frozen in 45% (v/v) conditioned medium, 45% (v/v) fresh medium and 10% (v/v) DMSO.

2.3 Biochemistry

2.3.1 Standard procedure for purifying soluble recombinant protein

Protein purification in this thesis was largely based on the following protocol, unless otherwise stated. For bacterial expression, a 1 litre culture was inoculated with 10 ml of overnight culture, and cells were then grown at 37 °C with shaking at 180 rpm until the OD₆₀₀ reached 0.6-0.8, at which point 1 mM IPTG was added to induce recombinant protein expression at 21 °C overnight. The lower temperature reduces the risk of overgrowing the culture overnight. Cells were harvested by centrifugation at 4,000 rpm for 20 min at 10 °C. The cell pellet was re-suspended in 40 ml of running buffer containing 50 µg/ml bovine pancreas deoxyribonuclease I (Sigma-Aldrich) and 1 mg/ml lysozyme from chicken egg white (Sigma-Aldrich). Cells were lysed using an Emulsiflex C5 homogeniser (Avestin) and the insoluble fraction was separated by centrifugation at 20,000 rpm for 30 min at 4 °C. The supernatant was then used for purification. For mammalian, insect and yeast expression, cultures were harvested and centrifuged at 1,000 g for 20 min at 10 °C. For eukaryotic expression, the proteins were expected to be secreted into the media due to the inclusion of a signal peptide. Therefore, the supernatant was collected and filtered before purification.

His-tagged proteins were purified by affinity chromatography using a Ni-NTA Superflow Cartridge (Qiagen) following the manufacturer's instructions. The supernatant was loaded onto a pre-equilibrated column, and then the column was washed with twenty column volumes (CVs) of running buffer containing 20 mM imidazole. The protein was eluted with five CVs of running buffer containing 300 mM imidazole, and then the column was washed with five CVs of running buffer containing 1 M imidazole. Proteins were further purified by size exclusion chromatography (SEC) with a HiLoad Superdex column (GE Healthcare) of appropriate pore size and/or ion exchange chromatography with a Mono Q or Mono S column (GE Healthcare), using an AKTA pure chromatography system (GE Healthcare). Detailed purification methods are outlined in the Results section. Following purification, proteins were concentrated using Amicon Ultra Centrifugal Filters (Millipore) with an appropriate molecular weight cut-off.

Table 2.6. Buffers and solutions for SDS-PAGE gel electrophoresis.

Solution	Components	Concentration
3x loading dye	Tris-HCl (pH 6.8)	150 mM
	SDS	6% (w/v)
	Glycerol	30% (v/v)
	Bromophenol blue	0.3% (w/v)
	DTT (for reducing gels)	100 mM
15% SDS-PAGE gel	<i>Running gel:</i>	
	Acrylamide Bis-Acrylamide (19:1)	15% (w/v)
	1.5 M Tris-HCl (pH 8.8)	375 mM
	SDS	0.1% (w/v)
	Ammonium persulfate (APS)	0.1% (w/v)
	Tetramethylethylenediamine (TEMED)	0.01% (v/v)
	<i>Stacking gel:</i>	
	Acrylamide Bis-Acrylamide (19:1)	5% (w/v)
	1 M Tris-HCl (pH 6.8)	125 mM
	SDS	0.1% (w/v)
	APS	0.1% (w/v)
	TEMED	0.01% (v/v)
10x running buffer	Tris base	0.25 M
	Glycine	1.92 M
	SDS	1% (w/v)
Coomassie Blue staining solution	Coomassie blue	0.12% (w/v)
	Ethanol	40% (v/v)
	Glacial acetic acid	10% (v/v)
Destaining solution	Ethanol	30% (v/v)
	Glacial acetic acid	10% (v/v)

2.3.2 SDS-PAGE

Protein samples were separated by SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis). 10 µl of the sample was mixed with 2x loading dye and heated at 95 °C for 5 min. Dilute samples were concentrated with StrataClean resin (Agilent). 10 µl of the resin was mixed with 100-500 µl of the sample, and the mixture was incubated at room temperature for 5 min, before pelleting by centrifugation at 2,000 g. The pellet containing the concentrated protein was re-suspended in 10 µl of 1x loading dye. Samples were loaded onto a 15% SDS-PAGE gel, which was then run in 1x SDS-PAGE running buffer at 200 V until completion. The gels were stained by Coomassie Blue staining. All buffers and solutions used for SDS-PAGE analysis are shown in **Table 2.6**.

2.3.3 Western blotting

After running samples on a SDS-PAGE gel, proteins were transferred onto Amersham Hybond P 0.45 PVDF blotting membrane (GE Healthcare) using the Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad). The transfer buffer was 48 mM Tris, 39 mM glycine, 0.0375% (w/v) SDS and 20% (v/v) methanol. Following transfer, the membrane was incubated in blocking buffer for 1 h; the blocking buffer was 2% (w/v) skimmed milk in PBST (PBS with 0.1% (v/v) Tween 20). The membrane was then incubated with anti-properdin antibody (Complement Technology) for 1 h, diluted 1:2,000 in blocking buffer. It was washed three times for 10 min with blocking buffer, and then incubated with horseradish peroxidase-conjugated anti-goat IgG (Promega) for 1 h, diluted 1:10,000 in blocking buffer. The membrane was washed three times with blocking buffer, and then three times with PBST, before detection with the ECL Western Blotting Substrate (Promega) and imaging. For His-tagged proteins, the Penta-His HRP Conjugate Kit (Qiagen) was used following the manufacturer's instructions.

2.4 Biophysical techniques

2.4.1 SEC with Multi-Angle Light Scattering (SEC-MALS)

Samples were prepared at desired concentrations as detailed in the Results section. Size exclusion chromatography (SEC) was performed prior to mass determination. The scattering of light from each sample was detected by a Dawn Heleos-II light scattering detector and an Optilab-rex refractive index monitor. Data analysis was carried out using the ASTRA 5.3.4.14 software. SEC-MALS experiments were performed by Dr. Steven Johnson.

2.4.2 Mass spectrometry

Native mass spectrometry was performed on a SYNAPT G1 Q-TOF. Proteins were buffer-exchanged into 200 mM ammonium acetate, pH 7.5, by SEC or using a Bio-Spin Size Exclusion Spin Column (Bio-Rad). Native mass spectrometry experiments and data analyses were performed by Lucas Kuhlen.

For protein identification by proteomics analysis, samples were run on a Mini-PROTEAN TGX Precast Gel (Bio-Rad) and stained with InstantBlue stain (Expedeon). Protein bands were cut out for in-gel digestion. Samples were analysed by the Advanced Proteomics Facility, University of Oxford.

2.4.3 Circular Dichroism (CD)

CD spectra were collected using a Jasco J-815 CD Spectrophotometer. Proteins were dialysed against 10 mM sodium phosphate buffer of appropriate pH, and then diluted to 0.1-0.2 mg/ml and 150 μ l. Experiments were performed at 20 °C using a cuvette of 1 mm path length (Starna Scientific). CD spectra were collected in the wavelength range of 190-260 nm, with four accumulations. Protein stability was investigated by performing a temperature interval scan measurement with data pitch of 5 °C and temperature gradient of 2 °C/min.

2.4.4 Surface Plasmon Resonance (SPR)

SPR studies were performed on a Biacore T200 system (GE Healthcare). Proteins were immobilised onto a Series S Sensor Chip CM5 (GE Healthcare) via amine coupling. Coupling was carried out at a flow rate of 10 μ l/min. The chip surface was activated with a 1:1 mixture of 0.4 M 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and 0.1 M N-hydroxysuccinimide (NHS) for 5 min, and then the proteins were covalently immobilised in 10 mM sodium acetate buffer. Remaining sites were blocked by injecting 1 M ethanolamine for 10 min. SPR experiments were carried out at a flow rate of 30 μ l/min. Triplicate measurements were taken for each protein concentration.

2.4.5 Protein preparation for Isothermal Titration Calorimetry (ITC)

ITC was performed with a Malvern PEAQ-ITC microcalorimeter. Proteins were dialysed against PBS, pH 7.4, using Slide-A-Lyzer MINI Dialysis Devices (Thermo Scientific). Both the ligand and the sample were dialysed against the same buffer, as their buffers must be identical. ITC measures the heat absorbed or released following a binding event. If the buffers of the ligand and the sample are different, the chemical potential difference will generate heat upon mixing and mask the desired observation. Samples were diluted

to desired concentrations as detailed in the Results section. ITC data were collected by Dr. David Staunton.

2.4.6 Protein labelling for Micro-Scale Thermophoresis (MST)

Monolith NT Protein Labeling Kit RED-NHS (Amine-Reactive) (NanoTemper Technologies) was used to label proteins for MST via coupling of a fluorescent dye to primary amines of proteins, following the manufacturer's instructions. The protein for labelling was first buffer-exchanged into PBS, pH 7.4, which does not interfere with amine coupling. Buffers that contain primary amines (for example, Tris, glycine, ethanolamine, glutathione) will reduce protein labelling. Labelling reactions were carried out with protein and dye at 20 μ M and 60 μ M, respectively. The labelled protein was purified by SEC to remove excess dye and to buffer-exchange into the running buffer for MST (PBS, 0.05% (v/v) Tween 20, pH 7.4). MST was performed on a Nanotemper Monolith NT.115 Red Blue instrument.

2.4.7 Crystallisation trials

Crystallisation trials were carried out using the vapour-diffusion sitting drop method. The mother liquor and protein stock were mixed at 1:1 or 1:3 ratios in 200 or 300 nl drops. All trials were set up in Swisisci 96-well 2-drop MRC Crystallisation Plates (Molecular Dimensions) and sealed with ClearVue Sheets (Molecular Dimensions), and incubated at 21 °C. Crystallisation screens (Molecular Dimensions) were first set up using the Mosquito LCP (TTP Labtech) to determine initial crystallisation conditions. Optimisation trays were set up using the Dragonfly Crystal (TTP Labtech).

Table 2.7. Crystallisation screens used throughout this thesis. The properties of the broad screens (all Molecular Dimensions) are described.

Broad screen	Properties
Stura FootPrint	Screen of protein precipitant solubility curve with conditions at acidic, neutral and basic pH for polyethylene glycols and salts
JCSG- <i>plus</i>	Sparse matrix screen incorporating PEG, salts, neutralised organic acids or organic precipitants across wide pH range
Structure Screen 1+2	Enhanced buffer selection for greater crystallisation space; enables assessment of protein solubility in a varying range of pH and precipitants
Clear Strategy I&II	Complementary screens developed to aid rational design of subsequent trials; buffers at 4 pH values (5.5, 6.5, 7.5 and 8.5)
PACT premier	Systematic pH/anion/cation screen designed to test pH within pH/ion environment using PEG as the precipitant
Morpheus	Screen covering a range of pH, PEGs and salt additives plus a range of low molecular weight ligands found in more than 33,000 PDB structures
MIDAS <i>plus</i>	Diversified precipitant and polymer screen with narrow range of pH and salt concentrations
LMB	Sparse matrix screen based on published conditions from the LMB, Cambridge, UK
ProPlex	Targeted sparse matrix screen for protein complexes with lower PEG concentrations and fewer organic precipitants

2.5 Complement work

2.5.1 Biotinylation of C3b

The protocol is based on the method by (Berends et al., 2015). 1 mg/ml C3 (Complement Technology) was activated with 10 µg/ml trypsin (Sigma-Aldrich) at 37 °C for 10 min in the presence of 100 µg/ml EZ-Link maleimide-PEG2-biotin (Life Technologies). The reaction was stopped by adding 50 µg/ml soybean trypsin inhibitor (Life Technologies) and 20 mM iodoacetamide (Sigma-Aldrich). The mixture was incubated on ice for 30 min and then diluted 1:1 into 20 mM HEPES, 10 mM NaCl, pH 7.5, and applied onto a Mono Q 5/50 GL column (GE Healthcare). C3b-biotin was eluted by a gradient to 20 mM HEPES, 300 mM NaCl, pH 7.5. C3b-bioton came off the column when the conductivity reached ~24 mS/cm. Fractions containing C3b-biotin were further purified by SEC using a Superdex 200 10/300 GL column (GE Healthcare). C3b-biotin was eluted at ~0.56 CV.

2.5.2 WIESLAB assays

Wieslab® Complement system Screen kits (Euro Diagnostica) were used following the manufacturer's instructions. For CP, MP and AP, serum was diluted 1:101, 1:101 and 1:18 with the provided diluent, respectively. For inhibition assays, 2.5-fold dilution series of inhibitor proteins were prepared; 2 µl of inhibitor or control were added per well.

2.5.3 Haemolysis assays

The protocols are based on the methods by (Morgan, 2000). Haemolysis in the alternative pathway (AP50) was measured using rabbit erythrocytes. 5 ml of rabbit erythrocytes in Alsever's solution (TCS Biosciences) were mixed with 20 ml of AP haemolysis buffer (0.1 M HEPES, 0.15 M NaCl, 8 mM EGTA, 5 mM MgCl₂, 0.1% (w/v) gelatin, pH 7.4), and centrifuged for 5 min at 1,250 g. The supernatant was discarded and the cells were re-suspended in 25 ml of AP buffer. The wash was repeated until the supernatant was clear. The cells were re-suspended in 15 ml of AP haemolysis buffer after washing. The concentration of the cells was calculated by lysing 100 µl of the cells with 2.9 ml of water and measuring the absorbance at 412 nm; absorbance of 0.29 equates to 1×10^8 cells/ml. The cells were diluted to a final concentration of 2×10^8 cells/ml.

Haemolysis in the classical pathway (CP50) was measured using sensitised sheep erythrocytes. 5 ml of sheep erythrocytes in Alsever's solution (TCS Biosciences) were mixed with 45 ml PBS and 0.9 ml of 0.5 M EDTA, pH 7.4. The cells were incubated for 15 min at 37 °C and then centrifuged for 5 min at 2,000 g. The supernatant was discarded and the cells were re-suspended in 50 ml of PBS. The wash was repeated until the supernatant was clear. After washing, the cells were re-suspended in 10 ml of CP haemolysis buffer (0.1 M HEPES, 0.15 M NaCl, 0.15 mM CaCl₂, 0.5 mM MgCl₂, 0.1% (w/v) gelatin, pH 7.4). The concentration of the cells was calculated by lysing 100 µl of the cells with 1.4 ml of water and measuring the absorbance at 541 nm; absorbance of 0.7 equates to 1×10^9 cells/ml. The cells were diluted to a final concentration of 1×10^9 cells/ml. To sensitise the cells, 5 ml of the washed sheep erythrocytes were mixed with 5 ml of CP buffer and 6 µl of haemolysin (Anti-Sheep Red Blood Cell Stroma antibody, S1389, Sigma-Aldrich), and incubated for 10 min at 37 °C. The sensitised cells were subsequently stored at 4 °C.

For performing haemolysis inhibition assays, 2.5-fold dilution series of inhibitor proteins were prepared and 2 μ l of inhibitor or control were added to empty wells of a Clear Flat-Bottom Immuno Nonsterile 96-Well Plate (Thermo Scientific). 50 μ l of diluted serum was added to the wells, followed by 50 μ l of appropriate red blood cells. The plate was incubated for 1 h at 37 °C with gentle shaking. 100 μ l of PBS was added to each well, and then the plate was centrifuged for 2 min at 2,000 g. 100 μ l of the supernatant was transferred to a clean 96-well plate and the absorbance at 405 nm was measured.

CHAPTER 3: EXPRESSION AND PURIFICATION OF PROPERDIN

3.1 Introduction

At the beginning of this thesis, the atomic structure of properdin was unknown. There was an EM structure of the AP C3 convertase bound to properdin (Alcorlo et al, 2013); however, properdin could not be modelled into the density due to the low resolution of the data (23.4 Å). A crystal structure of the AP C3 convertase bound to properdin was later published (Pedersen et al., 2017), but again at a resolution (6 Å) that an atomic model of properdin could not be constructed into the density. Therefore, one of the main aims of this thesis was to determine the structure of properdin to further our understanding of its role in complement. To do this, properdin was purified from serum or by recombinant expression. Properdin had been purified from serum before (Medicus et al., 1980; Pangburn, 1989; Sun et al., 2004); however, due to its low concentration in plasma at 5 µg/ml (Nolan and Reid, 1993), the yield may be too low for structural studies. In addition, serum-derived properdin exists in multiple oligomeric forms, which would be unsuitable for crystallisation trials. Similarly, mammalian expression resulted in multiple oligomeric forms of properdin (Higgins et al., 1995) or highly polymerised properdin (Ali et al., 2014). Bacterial expression of properdin had been done before, where individual domains were expressed as MBP fusion constructs (Kouser et al., 2016; Perdikoulis et al., 2001); however, it is unclear whether all TSR domains of properdin were correctly folded or remained soluble once the MBP tag was cleaved off. Therefore, I set out to establish a method to express and purify sufficient quantities of recombinant properdin and its domains suitable for structural studies.

3.2 Bioinformatics analysis of properdin sequence to define domain boundaries

The human properdin monomer is 442 amino acids long, without the signal peptide, and composed of thrombospondin type 1 repeat (TSR) domains. The TSR domain boundaries used in this thesis are shown in **Figure 3.1A**. Initially, properdin was annotated as containing five and a half TSR domains (Nolan et al., 1992), based on the consensus TSR sequence of six cysteine, three tryptophan and two arginine residues. Properdin was later expanded to six TSR domains, with TSR6 containing a large insertion (Higgins et al, 1995). The N-terminal region was then re-defined as an additional TSR domain, named TSR0 (Sun et al., 2004). However, it contains six cysteine residues with an incorrect spacing and none of the conserved tryptophan or arginine residues (**Figure 3.1C**).

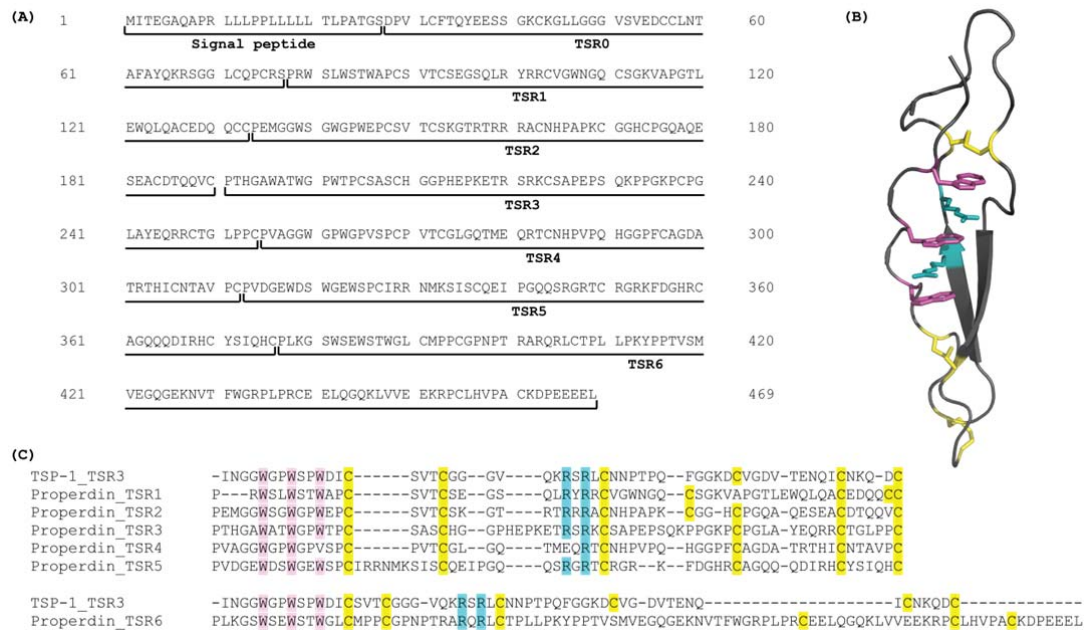


Figure 3.1. TSR domains of properdin. (A) Properdin TSR domain boundaries used in this thesis. (B) The crystal structure of TSR3 domain of thrombospondin-1 (PDB 1LSL) shows the stacking of cysteine (yellow), tryptophan (magenta) and arginine (cyan) residues of the CWR-layered motif. Image was produced with PyMOL. (C) The sequence alignment of TSR3 domain of human thrombospondin-1 and TSR domains of human properdin shows that residues of the CWR-layered motif are highly conserved.

The crystal structure of the TSR3 domain of thrombospondin-1 shows the stacking of cysteine, tryptophan and arginine side chains forming a CWR-layered motif (Tan et al., 2002) (**Figure 3.1B**). The sequence alignment of TSR domains of properdin and TSR3 domain of thrombospondin-1 shows that these motif residues are highly conserved, with the exception of TSR4, which only has two tryptophan and one arginine residues (**Figure 3.1B**). TSR1 and TSR2 both have an extra cysteine residue, which may form an inter-domain disulphide bond.

As mentioned in **Section 1.2.1**, properdin is post-translationally modified by N-linked glycosylation, O-linked glycosylation and C-mannosylation (Hartmann and Hofsteenge, 2000; Higgins et al., 1995; Yang et al., 2016). The glycosylation pattern of properdin may be important for protein folding, and may affect recombinant expression, particularly the unusual C-mannosylation of tryptophan residues.

3.3 Purification of properdin from serum using CIRp-A1

3.3.1 Generation of affinity column with CIRp-A1 via amine coupling to purify properdin from serum

The identification of a properdin-binding complement inhibitor CIRp-A1 from the tick *Rhipicephalus pulchellus* by the lab (Jore, unpublished) provided a tool for purifying properdin from serum. An affinity column was generated with CIRp-A1 using the Pierce NHS-Activated Agarose Slurry (Thermo Scientific) following the manufacturer's instructions. In brief, CIRp-A1 was mixed with the slurry on a rotary wheel overnight at 4 °C. After coupling, the remaining active sites were blocked with 1 M ethanolamine. The slurry was then packed into an empty cartridge to generate a CIRp-A1 'column'. As a trial, 15 ml of serum was used to purify properdin. Human serum was acquired from the John Radcliffe Hospital, Oxford. After loading the serum onto the CIRp-A1 column and washing with PBS, properdin was eluted with 0.2 M glycine-HCl, pH 3. The pH of the elution fraction was adjusted by adding 50 µl of neutralisation buffer (1 M Tris, pH 9) per 1 ml of eluate. SDS-PAGE analysis showed excess of CIRp-A1 in the flow-through of the coupling reaction. There was a band around 56 kDa in the elution, corresponding to the molecular weight of properdin (**Figure 3.2A**). The presence of properdin in the elution

fraction was confirmed by a Western blot with α -properdin antibody (**Figure 3.2B**). The small-scale trial purification indicated that using CIRp-A1 was a viable method to purify properdin from serum.

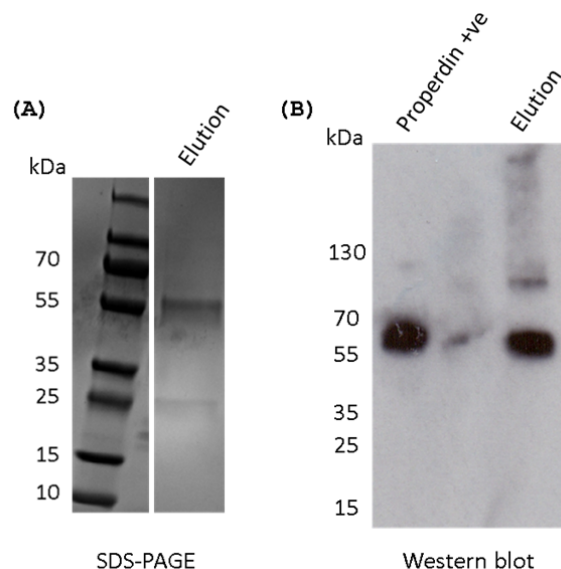


Figure 3.2. Small-scale trial of serum purification of properdin using CIRp-A1. (A) The eluate from CIRp-A1-coupled NHS-Activated Agarose resin was analysed by reducing SDS-PAGE. 4–20% Mini-PROTEAN TGX Precast Protein Gel (Bio-Rad) was used. The elution fraction showed a band at 56 kDa, where properdin is expected to run. (B) Western blot with α -properdin antibody confirmed the presence of properdin in the eluate. Purified properdin (Complement Technology) was used as a positive control for the blot.

3.3.2 Large-scale purification of properdin from serum using CIRp-A1

Following a successful pulldown trial with 15 ml of serum, 50 ml of serum was used for the purification of properdin using CIRp-A1. Human serum was acquired from the John Radcliffe Hospital, Oxford. This time, the elution fractions off the CIRp-A1 column were further purified by SEC; the running buffer was PBS, pH 7.4. The SEC profile had two main peaks (**Figure 3.3A**). The first peak ran as two bands under non-reducing condition, but as a single band under reducing condition (**Figure 3.3B**). The different gel patterns for non-reducing and reducing conditions were observed for properdin before (Nolan and Reid, 1993). Proteomics analysis of the gel bands confirmed the presence of properdin in the first peak. Based on the elution volume of the first peak, it was likely a properdin trimer or tetramer. The second peak from SEC likely contained immunoglobulins, which

dissociate into heavy (50 kDa) and light (25 kDa) chains under reducing condition (**Figure 3.3B**). Using the same method, a larger-scale purification with 500 ml of serum yielded 0.61 mg of properdin (data not shown), which corresponds to a concentration of 1.2 µg/ml. This was lower than the reported serum concentration of properdin (5 µg/ml), but loss of protein is expected at different steps of purification.

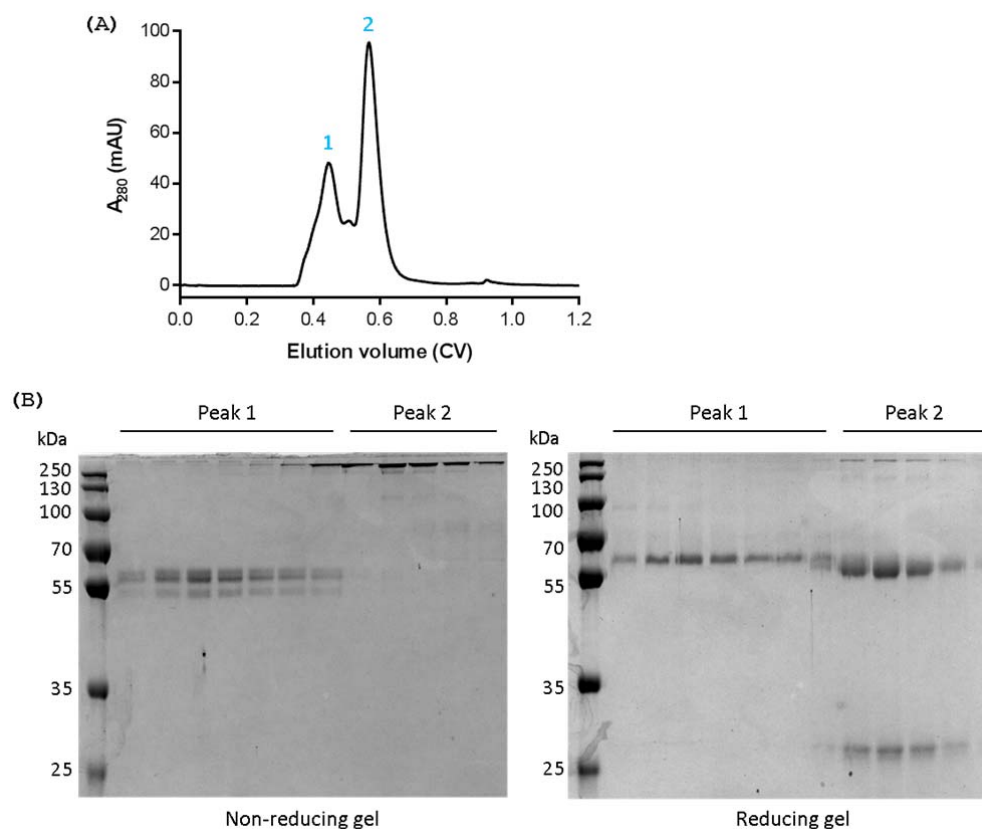


Figure 3.3. Purification of properdin from serum. (A) S200 10/30 size exclusion profile of serum properdin showed two main peaks (Peak 1 and Peak 2). The running buffer for SEC was PBS, pH 7.4. (B) Elution fractions from SEC were analysed by 15% SDS-PAGE under non-reducing (left) and reducing (right) conditions.

3.3.3 SEC-MALS analysis of serum-purified properdin

SEC combined with multi-angle light scattering (SEC-MALS) was performed with properdin purified from serum (**Figure 3.4**). Superdex 200 10/300 GL column was used and the running buffer was PBS, pH 7.4. As expected, serum properdin existed in multiple oligomeric forms. The overlapping nature of the peaks made it difficult to

interpret the masses accurately, but the predominant peak was likely a properdin tetramer or trimer.

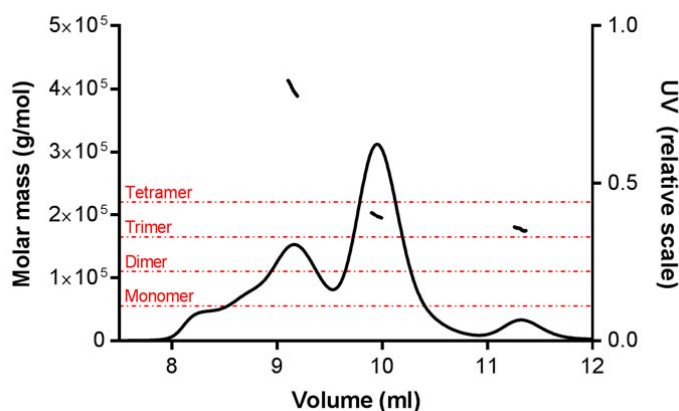


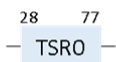
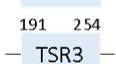
Figure 3.4. SEC-MALS analysis of serum properdin. Serum properdin was analysed by SEC-MALS using a Superdex 200 10/300 GL column. The running buffer for SEC-MALS was PBS, pH 7.4. Multiple SEC peaks suggested multiple oligomeric forms of properdin, with the predominant peak being a tetramer or trimer. Expected masses of different oligomers of properdin are indicated with red dotted lines across the graph.

3.4 Expression of properdin in bacterial cells

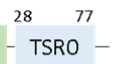
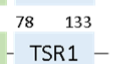
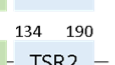
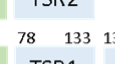
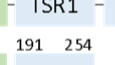
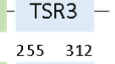
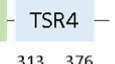
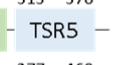
3.4.1 Summary of constructs for bacterial expression

Recombinant expression of properdin was attempted in bacteria, from soluble expression or refolding from inclusion bodies. Soluble expression trials were conducted without an affinity tag (**Section 3.4.2**) or with a His-MBP tag (**Section 3.4.3**). Refolding from inclusion bodies was attempted (**Section 3.4.5** and **Section 3.4.6**). The constructs used for bacterial expression are summarised in **Figure 3.5**, including the plasmid vector and TSR domain(s) used.

Expression trials (Section 3.4.2)

pET15b TSR0	—  —	MW = 5 kDa , pI = 6.0
pET15b TSR3	—  —	MW = 7 kDa , pI = 8.9

Expression and purification (Section 3.4.3)

pETM-44 TSR0	— His — MBP —   —	MW = 48 kDa , pI = 5.5
pETM-44 TSR1	— His — MBP —   —	MW = 49 kDa , pI = 5.7
pETM-44 TSR2	— His — MBP —   —	MW = 49 kDa , pI = 5.7
pETM-44 TSR1-2	— His — MBP —    —	MW = 56 kDa , pI = 6.0
pETM-44 TSR3	— His — MBP —   —	MW = 50 kDa , pI = 6.1
pETM-44 TSR4	— His — MBP —   —	MW = 49 kDa , pI = 5.7
pETM-44 TSR5	— His — MBP —   —	MW = 50 kDa , pI = 6.0
pETM-44 TSR6	— His — MBP —   —	MW = 53 kDa , pI = 5.9

Refolding (Section 3.4.5 and 3.4.6)

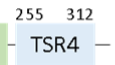
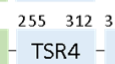
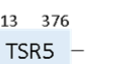
pETM-44 TSR4	— His — MBP —   —	MW = 49 kDa , pI = 5.7
pETM-44 TSR4-5	— His — MBP —    —	MW = 56 kDa , pI = 6.1

Figure 3.5. Constructs for bacterial expression of properdin. Constructs were designed without an affinity tag (pET15b) or with a His-MBP tag (pETM-44). Numbers above TSR domains indicate residue number within properdin. The sequences of the TSR domains are shown in **Figure 3.1**. The molecular weights (MW) and theoretical pI of the constructs are shown.

3.4.2 Expression trials of TSR and MBP-TSR fusion constructs

The original strategy for the purification of individual TSR domains of properdin was to express them without an affinity tag as inclusion bodies and then purify the proteins from solubilised inclusion bodies. This method has been performed before for other modular disulphide-containing complement proteins. Due to the predicted disulphide bonds within each TSR domain, the domains were predicted to form protein aggregates when expressed in bacterial cells.

For expression trials, TSR0 and TSR3 were cloned into pET15b vector and transformed into three different *E. coli* strains – BL21 (DE3), Rosetta 2 (DE3) and Origami B. BL21 (DE3) is the standard expression strain used in the lab. Rosetta 2 (DE3) is designed to enhance the expression of eukaryotic proteins with codons rarely used in *E. coli*; this strain was tried as TSR domains begin with a proline. Origami B is designed to enhance disulphide formation in the cytoplasm. Empty pET15b was transformed and used as the negative control. The transformation plates were re-suspended in 8 ml of LB, and 100 µl of the re-suspension was inoculated into 10 ml of LB with appropriate antibiotics. At OD₆₀₀ of 0.6, 500 µl samples were taken and the cells spun down for SDS-PAGE analysis. The cultures were induced with 1 mM IPTG and incubated for four hours before taking a second set of samples. A third set of samples was taken after overnight induction. As shown in **Figure 3.6**, neither TSR0 (~6 kDa) nor TSR3 (~7 kDa) was expressed by any of the tested strains since there was no difference on the gel between the empty vector and the domain-containing vector after induction. I did not continue expression trials using this method for other TSR domains, as they were assumed to be similar to TSR0 and TSR3.

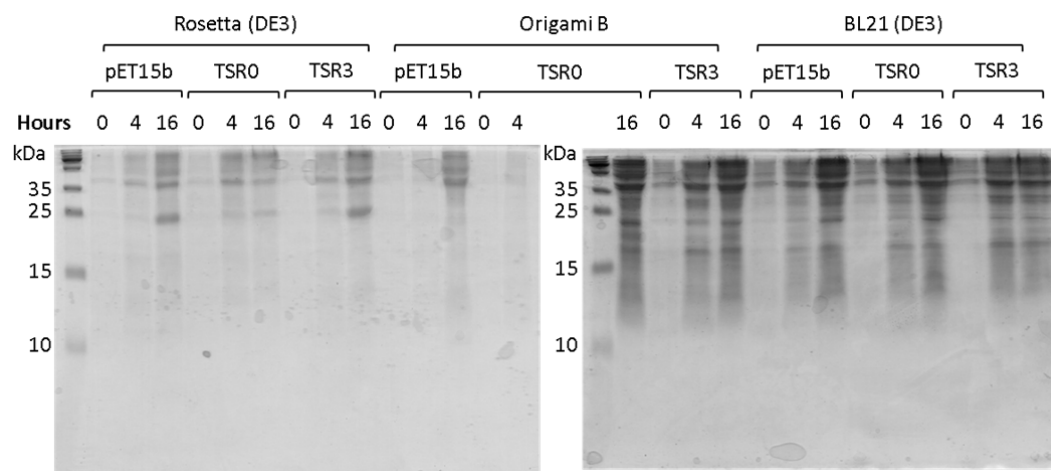


Figure 3.6. Expression trials of pET15b TSR0 and TSR3 in *E. coli*. Three *E. coli* strains were tried: Rosetta 2 (DE3), Origami B and BL21 (DE3). Samples taken throughout the expression trials were analysed by reducing 20% SDS-PAGE. For each construct, the lanes are before induction, four hours after induction, and after overnight induction, from left to right.

When expressed alone, translation of TSR domains might be impacted by the domains beginning with rare codons. Therefore, to aid the expression and solubilisation of the TSR domains, an MBP tag was used. MBP-TSR fusion constructs were previously

successfully purified (Perdikoulis et al., 2001). TSR0, TSR3 and TSR4 were cloned into pETM-44 vector, which contains an N-terminal His-tagged MBP protein. Empty pETM-44 was transformed and used as the negative control. Expression trials were carried out as before using three different *E. coli* strains – BL21 (DE3), Rosetta 2 (DE3) and SHuffle. *E. coli* SHuffle T7 cells (NEB) have been engineered to enhance disulphide bond formation by constitutively expressing a disulphide bond isomerase DsbC. As shown in **Figure 3.7**, the empty vector expressed the MBP protein alone (~42 kDa), whereas there was a shift in the bands for the TSR domain-containing vectors (~50 kDa) indicating successful expression of the MBP-tagged TSR domains in all of the strains tested. Although expression levels were higher in Rosetta 2 (DE3) and BL21 (DE3), I first decided to try purification from SHuffle cells. These cells promote disulphide bond formation and therefore, it might not be necessary to denature and refold the proteins.

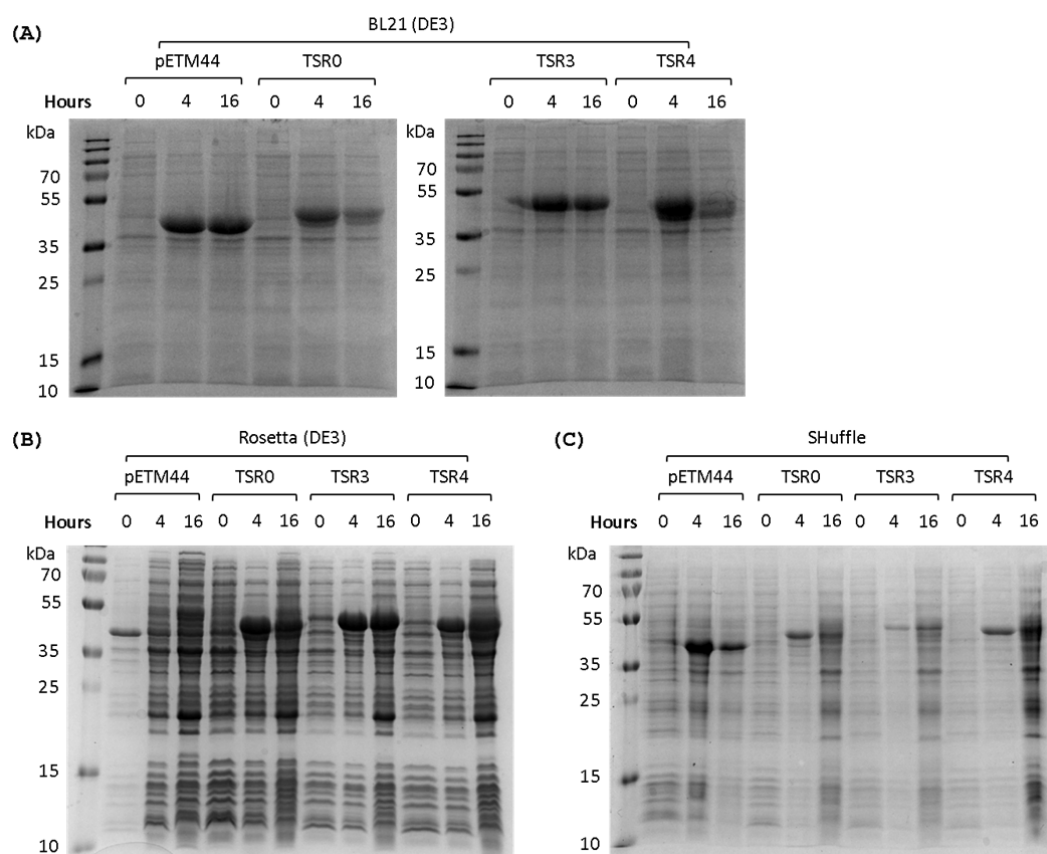


Figure 3.7. Expression trials of pETM-44 TSR0, TSR3 and TSR4 in *E. coli*. Three *E. coli* strains were tried: (A) BL21 (DE3), (B) Rosetta 2 (DE3) and (C) SHuffle. Samples taken throughout the expression trials were analysed by reducing 15% SDS-PAGE. For each construct, the lanes are before induction, four hours after induction, and after overnight induction, from left to right.

3.4.3 Purification of MBP-TSR fusion constructs

E. coli SHuffle T7 cells were grown in LB media in an orbital shaker at 30 °C and induced overnight at 21 °C. Proteins were purified from the supernatant of lysed cells by affinity chromatography using a 5 ml Ni-NTA Superflow column. The running buffer was 50 mM Tris, 300 mM NaCl, pH 8. Following affinity chromatography, the His-MBP tag was cleaved overnight with 3C protease and simultaneously dialysed to remove imidazole. The dialysis buffer was 5% (v/v) glycerol, 50 mM Tris, 300 mM NaCl and the pH was varied depending on the theoretical pI of the cleaved TSR domain (**Table 3.1**). Then, the cleaved MBP tag and uncleaved MBP-TSR fusion protein were removed by reverse nickel purification. The flow-through containing the untagged TSR domain was purified further by SEC; the buffer was 50 mM Tris, 150 mM NaCl at the same pH as for 3C cleavage. The purification of TSR0, TSR3, TSR4, TSR5, and TSR6 are shown in **Figure 3.8**, **Figure 3.9**, **Figure 3.10**, **Figure 3.11**, and **Figure 3.12**, respectively.

Table 3.1. Purification conditions for TSR domains. The table shows the pH of the buffers used for 3C cleavage, reverse nickel purification and SEC for each TSR domain. The pH for purification was chosen to be at least 1 pH unit away from the theoretical pI of the TSR domain.

Domain	TSR0	TSR1	TSR2	TSR1-2	TSR3	TSR4	TSR5	TSR6
pH	8	7	9	7	8	8.5	7	7
pI	6.2	8.3	7.7	8.3	8.9	7.0	8.7	8.6

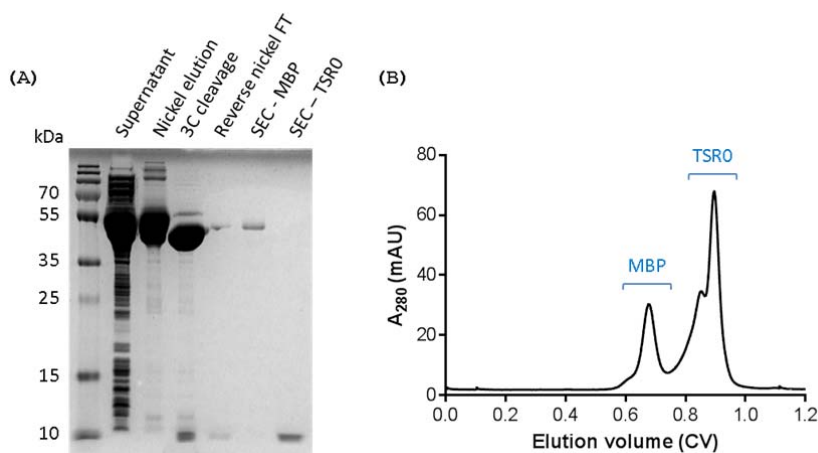


Figure 3.8. Purification of TSR0 domain of properdin. (A) Reducing 15% SDS-PAGE analysis following each purification step. (B) S200 16/60 size exclusion profile of TSR0 domain. The running buffer for SEC was 50 mM Tris, 150 mM NaCl, pH 8.

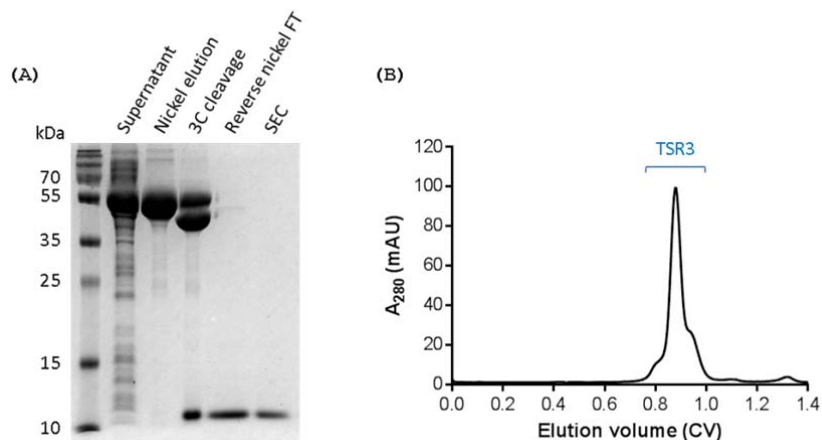


Figure 3.9. Purification of TSR3 domain of properdin. (A) Reducing 15% SDS-PAGE analysis following each purification step. (B) S200 16/60 size exclusion profile of TSR3 domain. The running buffer for SEC was 50 mM Tris, 150 mM NaCl, pH 8.

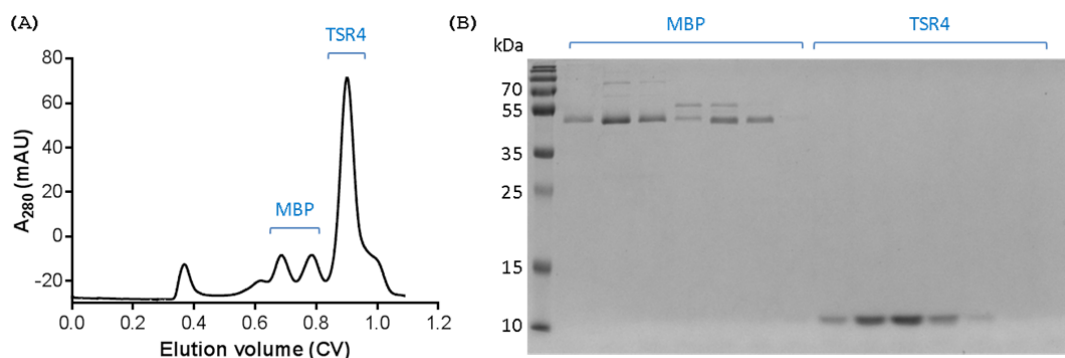


Figure 3.10. Purification of TSR4 domain of properdin. (A) S200 16/60 size exclusion profile of TSR4 domain. The running buffer for SEC was 50 mM Tris, 150 mM NaCl, pH 8.5. (B) Reducing 15% SDS-PAGE analysis of elution fractions from SEC shown in (A).

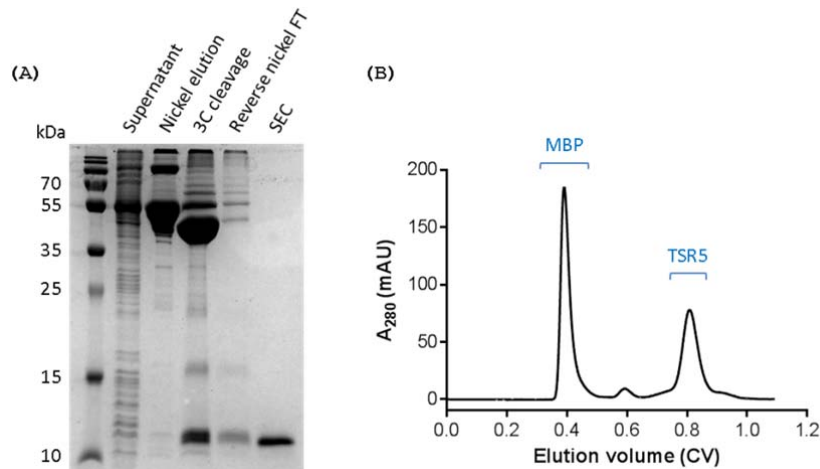


Figure 3.11. Purification of TSR5 domain of properdin. (A) Reducing 15% SDS-PAGE analysis following each purification step. (B) S75 16/60 size exclusion profile of TSR5 domain. The running buffer for SEC was 50 mM Tris, 150 mM NaCl, pH 7.

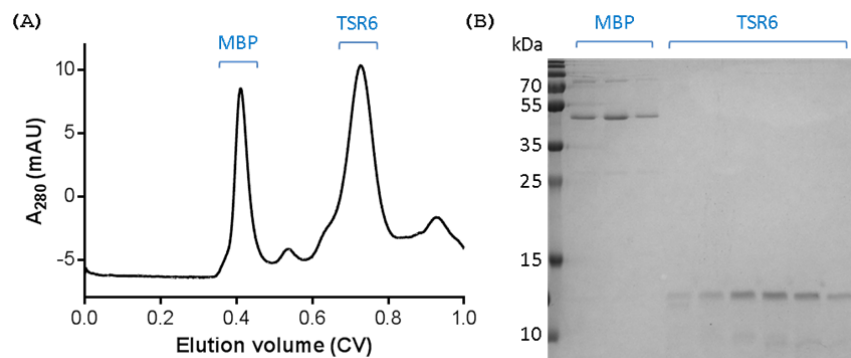


Figure 3.12. Purification of TSR6 domain of properdin. (A) S75 26/60 size exclusion profile of TSR6 domain. The running buffer for SEC was 50 mM Tris, 150 mM NaCl, pH 7. (B) Reducing 15% SDS-PAGE analysis of elution fractions from SEC shown in (A).

Purification of TSR1 and TSR2 was also attempted using the same approach. For both of these domains, once the MBP-TSR fusion protein was cleaved with 3C protease, the purification protocol used (reverse nickel purification followed by SEC) did not separate the TSR domain from the cleaved MBP tag and the uncleaved MBP-TSR fusion protein (TSR1 shown in **Figure 3.13** as a representative). In addition, TSR1-2 was expressed and purified as a duplet, due to the possibility of an inter-domain disulphide linkage, but the same issue occurred as with TSR1 and TSR2 expressed individually. These TSR domains might not have folded properly and could have aggregated.

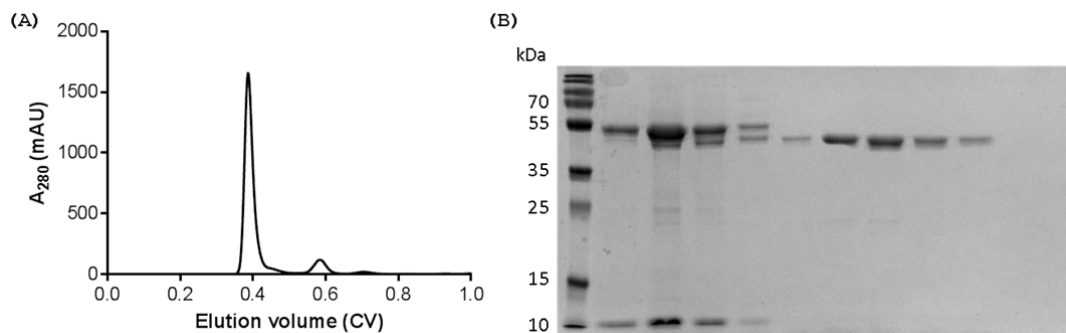


Figure 3.13. Purification of TSR1 domain of properdin. (A) S75 16/60 size exclusion profile of TSR1 domain. The running buffer for SEC was 50 mM Tris, 150 mM NaCl, pH 7. (B) Reducing 15% SDS-PAGE analysis of elution fractions from SEC shown in (A).

3.4.4 Biophysical analyses of purified TSR domains from MBP-TSR fusion constructs

SEC-MALS was carried out for purified TSR0, TSR3, TSR4 and TSR5 using a Superdex 75 10/300 GL column with 50 mM Tris, 150 mM NaCl, pH 8 as the running buffer. SEC-MALS experiments were performed by Dr. Steven Johnson. The calculated masses suggested that TSR0 and TSR3 were monomeric (**Figure 3.14B**). The calculated mass of TSR4 corresponded to a dimer, whereas the calculated mass of TSR5 was between a monomer and a dimer. However, the mass calculations for TSR4 and TSR5 are unreliable due to low signal. The molecular masses for TSR0 and TSR3 could be determined since higher amounts of TSR0 and TSR3 were used for SEC-MALS than TSR4 and TSR5, as indicated by the relative scale (**Figure 3.14A**). Since TSR4 and TSR5 came off the size exclusion column later than TSR0 and TSR3, they were unlikely dimeric.

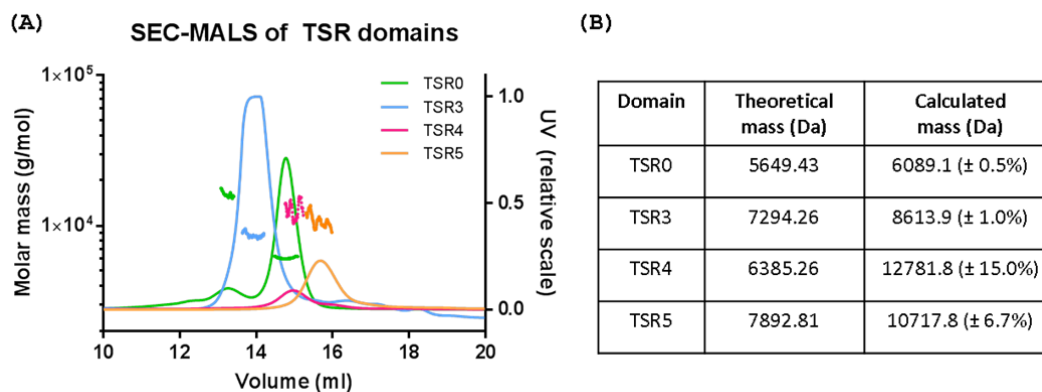


Figure 3.14. SEC-MALS analysis of TSR domains. (A) The molecular masses of TSR0 (green), TSR3 (blue), TSR4 (magenta) and TSR5 (orange) were calculated from the elution profiles examined by SEC-MALS on a Superdex 75 10/300 GL column. The running buffer for SEC-MALS was 50 mM Tris, 150 mM NaCl, pH 8. (B) The table shows theoretical and calculated masses of TSR0, TSR3, TSR4 and TSR5.

For native mass spectrometry analysis of TSR3, the protein was buffer-exchanged into 200 mM ammonium acetate, pH 7.5, using a Superdex 200 Increase 5/150 GL column. Native mass spectrometry experiments and data analysis were performed by Lucas Kuhlen. Analysis was performed on a SYNAPT G1 Q-TOF at low collision energy (cone voltage of 10 V and trap voltage of 10 V). The theoretical mass of TSR3 based on its amino acid sequence is 7294.26 Da; the calculated mass was 7288.69 ± 0.17 Da, thus TSR3 was monomeric (**Figure 3.15**). The calculated mass was 6 Da lower than the theoretical mass, which suggested that the six cysteine residues in TSR3 formed disulphide bonds.

Circular dichroism (CD) analysis was performed with TSR domains to assess their secondary structure. TSR3, TSR4, TSR5 and TSR6 were dialysed against 10 mM sodium phosphate buffer at the pH in **Table 3.1**. The CD spectra of TSR4, TSR5 and TSR6 suggested that these domains were disordered and lacked secondary structure (**Figure 3.16B-D**). The CD spectrum of TSR3 also showed no secondary structure, but had a positive peak at 230 nm (**Figure 3.16A**), which has been shown to correlate with stacking tryptophan residues (Andersson et al., 2001). This positive peak was previously observed for TSR3 (Perdikoulis et al., 2001) and serum properdin (Medicus et al., 1980). A single TSR domain contains three tryptophan residues, which when folded correctly, form a ladder (**Figure 3.1B**). CD analysis indicated that only TSR3 folded correctly to form a

tryptophan ladder, which would provide a strong enough absorbance at 230 nm. The positive peak at 230 nm decreased when TSR3 was heated and eventually became more like the others at temperatures above 40 °C, suggesting that it was indicative of folding (**Figure 3.17**). Temperature interval scan CD analysis suggested the melting temperature (T_M) of TSR3 to be around 37 °C (**Figure 3.17B**).

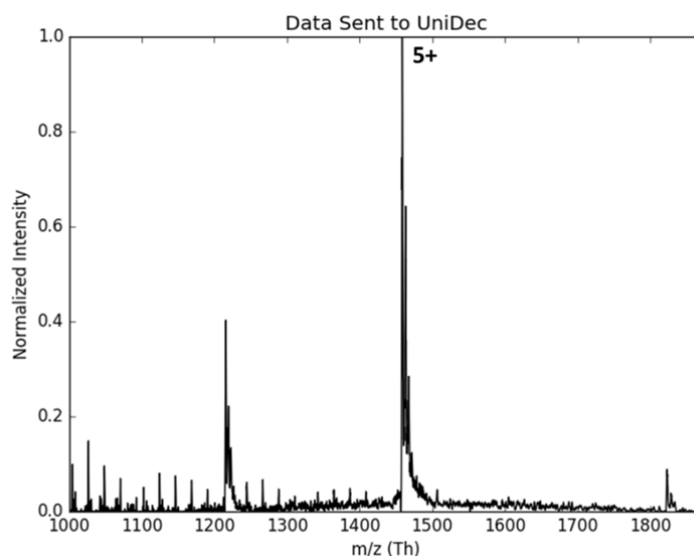


Figure 3.15. Native mass spectrometry analysis of TSR3 domain of properdin. Native mass spectrometry was performed on a SYNAPT G1 Q-TOF at cone voltage of 10 V and trap voltage of 10 V. The sample buffer was 200 mM ammonium acetate, pH 7.5. The calculated molecular mass of 7288.69 ± 0.17 Da corresponds to a monomer of TSR3, which has a theoretical mass of 7288.26 Da with disulphide bonds.

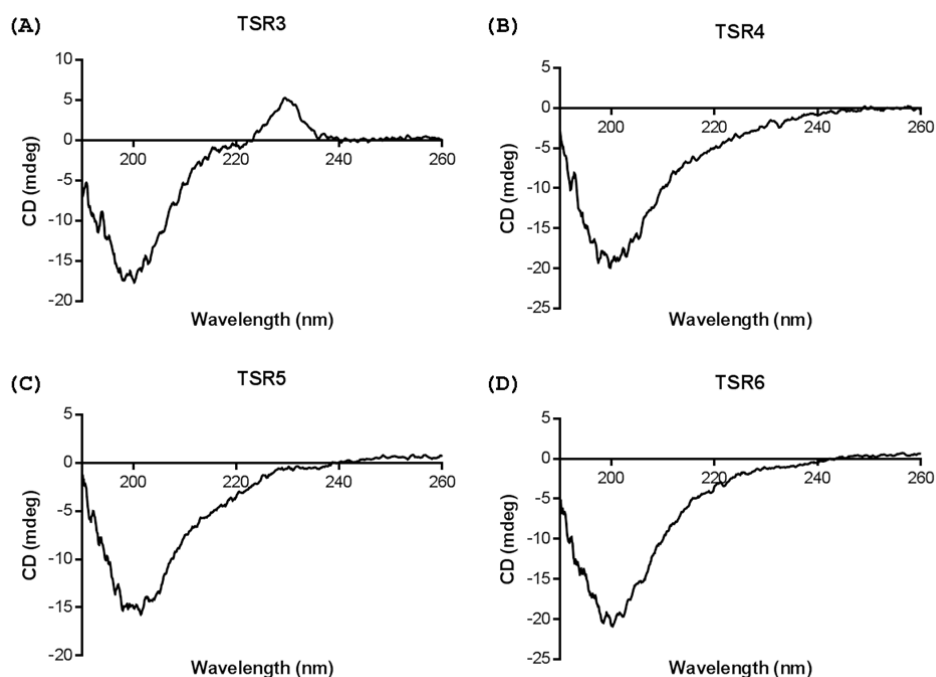


Figure 3.16. CD analysis of TSR domains. (A) TSR3 had a positive peak at 230 nm, suggesting correct folding and formation of a tryptophan ladder. The CD spectra of (B) TSR4, (C) TSR5, and (D) TSR6 suggested a lack of secondary structure. The CD buffers for TSR3, TSR4, TSR5 and TSR6 were 10 mM sodium phosphate buffer at pH 8, 8.5, 7 and 7, respectively.

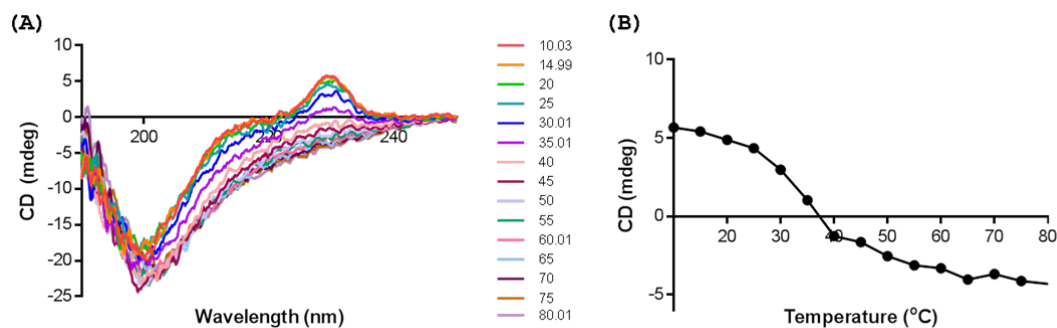


Figure 3.17. Temperature interval scan measurement of CD with TSR3. (A) CD spectra of TSR3 were recorded as the temperature was increased from 10 °C to 80 °C at 5 °C intervals. (B) The CD at 230 nm was positive at lower temperatures, and decreased as the temperature was increased and the protein unfolded. The CD buffer was 10 mM sodium phosphate buffer, pH 8.

3.4.5 Attempts to purify TSR4 by refolding

Since no evidence suggested that TSR4 was correctly folded in the soluble fraction, I attempted refolding. Following nickel and reverse nickel purification, as outlined in **Section 3.4.3**, fractions containing TSR4 were used for refolding trial. The refolding buffers were based on a study where the refolding of TSR3 was demonstrated (Perdikoulis et al., 2001). For refolding, TSR4 was dialysed against 1 l of 20 mM Tris, 100 mM NaCl, pH 9, 0.01% (v/v) Tween 20, 2 M urea, at 4 °C for 1 h. It was then dialysed against the same buffer containing 100 mM β -mercaptoethanol at 4 °C overnight. The protein was then added dropwise into 2 l of 20 mM Tris, 100 mM NaCl, pH 9, 0.01% (v/v) Tween 20, 0.3 M urea, 1.25 mM glutathione-reduced, 0.25 mM glutathione-oxidised, 10 mM lysine, and left at 4 °C overnight. This buffer containing TSR4 was concentrated down to ~40 ml using a 5 kDa Vivaflow 200 filtration device, and then dialysed against 1 l of 50 mM Tris, 150 mM NaCl, pH 8.5, at 4 °C overnight.

Following the refolding protocol, TSR4 was purified by SEC using a Superdex S75 26/60 column; the running buffer was 50 mM Tris, 150 mM NaCl, pH 8.5. The protein came off the column at the expected volume for its molecular weight. However, the yield was very low, with the peak fraction at ~3 mAU (**Figure 3.18A**). The peak fraction was concentrated for CD analysis; the protein was dialysed against 10 mM sodium phosphate buffer, pH 8. The CD spectrum looked similar to TSR4 purified from the soluble fraction (**Figure 3.16B**). The lack of a positive peak at 230 nm suggested that TSR4 did not refold correctly (**Figure 3.18B**). However, the lack of a positive CD signal at 230 nm might not be an appropriate indicator of folding for TSR4. TSR4 only contains two of the three conserved tryptophan residues, and the resulting CD signal could be too weak.

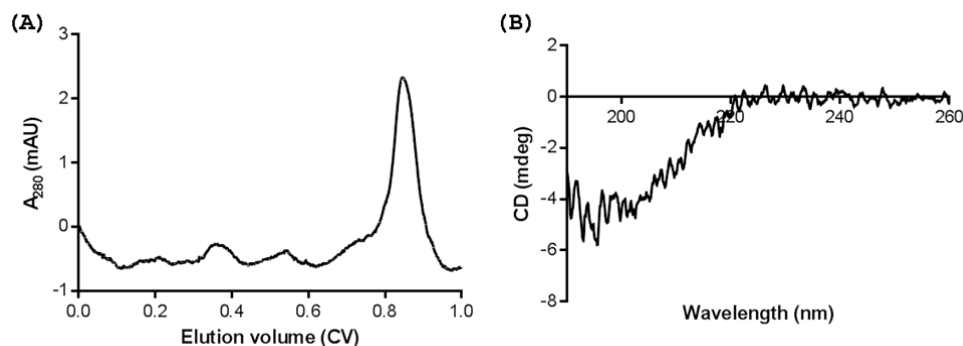


Figure 3.18. SEC and CD analyses of TSR4 following refolding trial. (A) S75 26/60 size exclusion profile of refolded TSR4 domain. The running buffer for SEC was 50 mM Tris, 150 mM NaCl, pH 8.5. (B) CD spectrum of refolded TSR4 suggested a lack of secondary structure. The CD buffer was 10 mM sodium phosphate buffer, pH 8.

3.4.6 Attempts to purify TSR4-5 by refolding

Following the refolding trial of TSR4 in **Section 3.4.5**, a more systematic approach was taken with TSR4-5 using different refolding conditions. TSR4-5 was used as having two TSR domains might contribute to a stronger CD signal at 230 nm. MBP-TSR4-5 was expressed in *E. coli* BL21 (DE3); cells were grown in LB media in an orbital shaker at 37 °C and induced overnight at 21 °C. MBP-TSR4-5 was purified from the supernatant of lysed cells by affinity chromatography using a 5 ml Ni-NTA Superflow column. The running buffer was 50 mM Tris, 300 mM NaCl, pH 8. Following nickel purification, the MBP tag was cleaved overnight with 3C protease and simultaneously dialysed against 50 mM Tris, 300 mM NaCl, pH 7.7. Following cleavage of the tag, precipitation occurred; SDS-PAGE analysis showed that the precipitate mainly consisted of TSR4-5 (**Figure 3.20A**). The precipitate was re-suspended in 30 ml of 8 M urea, 1 mM EDTA, 100 mM Tris, 25 mM DTT, pH 8. For small-scale refolding by rapid dilution, six refolding buffers were tested (**Table 3.2**). Refolding buffer F was based on the purification of amphioxus properdin (Gao et al., 2017). For refolding, 5 ml of the denatured TSR4-5 was added dropwise into 200 ml of refolding buffer and left to stir at 4 °C for five days. 50 ml of each refolding buffer containing TSR4-5 was concentrated down to 1 ml to be analysed by SEC. The running buffer for SEC was 50 mM Tris, 150 mM NaCl, pH 7.

Table 3.2. Refolding buffers tested for the refolding of TSR4-5 expressed in *E. coli*.

Refolding buffer	Buffer components
A	20 mM ethanolamine, 2 mM cystine, 1 mM cysteine, 10 mM NaCl, 1 mM EDTA, pH 11
B	Buffer A containing 500 mM arginine
C	Buffer A containing 5% (v/v) glycerol
D	Buffer A containing 1 mM DTT
E	20 mM ethanolamine, 1 mM glutathione-oxidised, 1 mM glutathione-reduced, 10 mM NaCl, 1 mM EDTA, pH 11
F	25 mM Tris, 1 mM glutathione-oxidised, 1 mM glutathione-reduced, 10 mM NaCl, 1 mM EDTA, 1 mM EDTA, 1% (w/v) glycine, 5% (v/v) glycerol, pH 8

With the exception of Refold C, other refolding buffers resulted in distinct, non-overlapping SEC peaks (**Figure 3.19**). Based on the SDS-PAGE analysis, the peak at 0.65 CV corresponds to TSR4-5, which has a theoretical mass of 13830.57 Da (**Figure 3.20**). This peak was absent in Refold E, and TSR4-5 could not be observed on the gel. The peaks containing TSR4-5 (peak 2 from Refold A; peak 3 from Refold B; peak 3 from Refold D; peak 2 from Refold F) were concentrated down to 100 μ l, and then dialysed against 10 mM sodium phosphate buffer, pH 7, for CD analysis. The CD spectra of the samples indicated that the proteins were disordered; none of the samples had a positive signal at 230 nm, expected from a folded tryptophan ladder, suggesting that TSR4-5 did not refold correctly in any of the conditions tested (**Figure 3.21**).

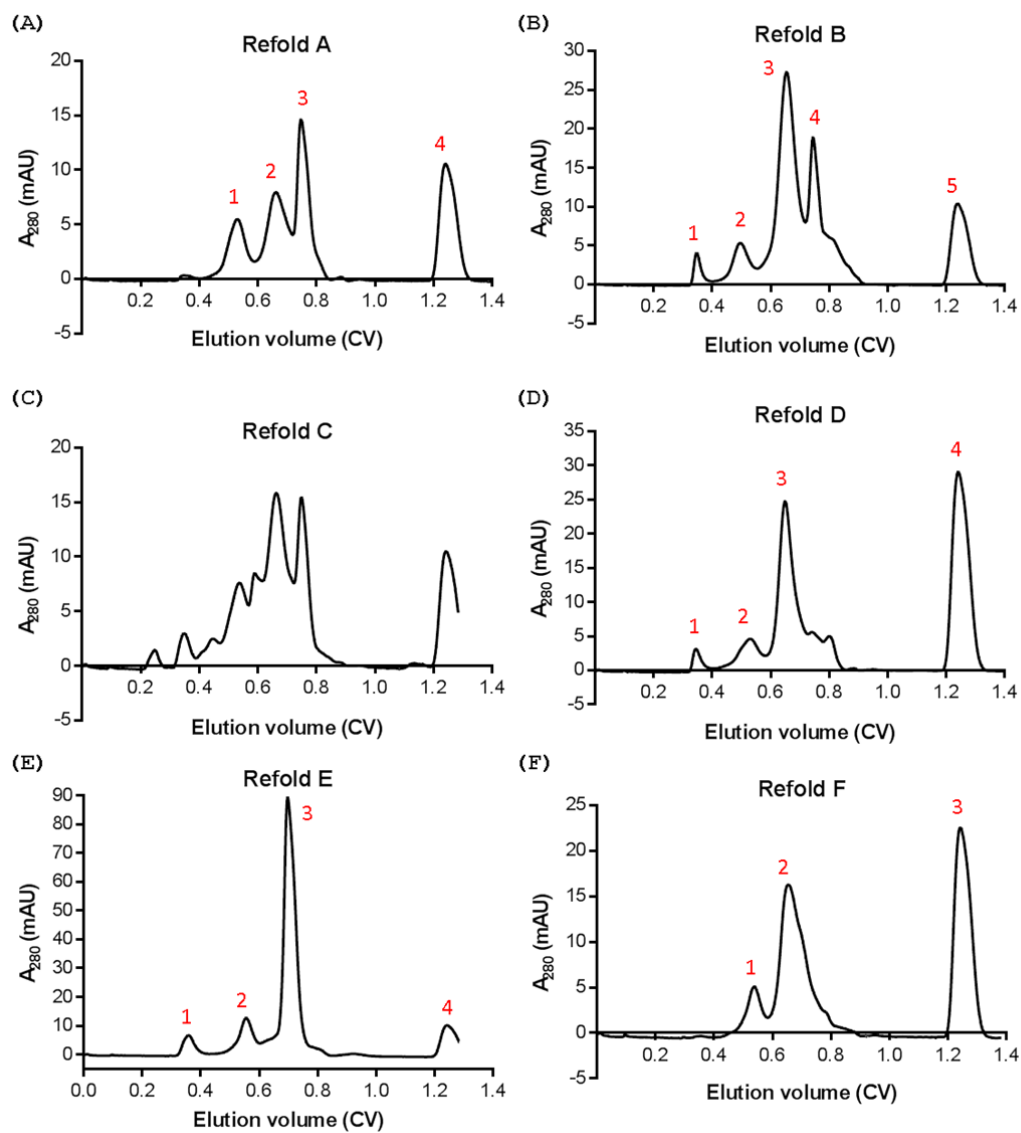


Figure 3.19. SEC analysis of TSR4-5 refolding trials. Following incubation in the refolding buffer, TSR4-5 was concentrated before being analysed by SEC. S75 10/30 size exclusion profiles of Refold A to F are shown. The running buffer for SEC was 50 mM Tris, 150 mM NaCl, pH 7.

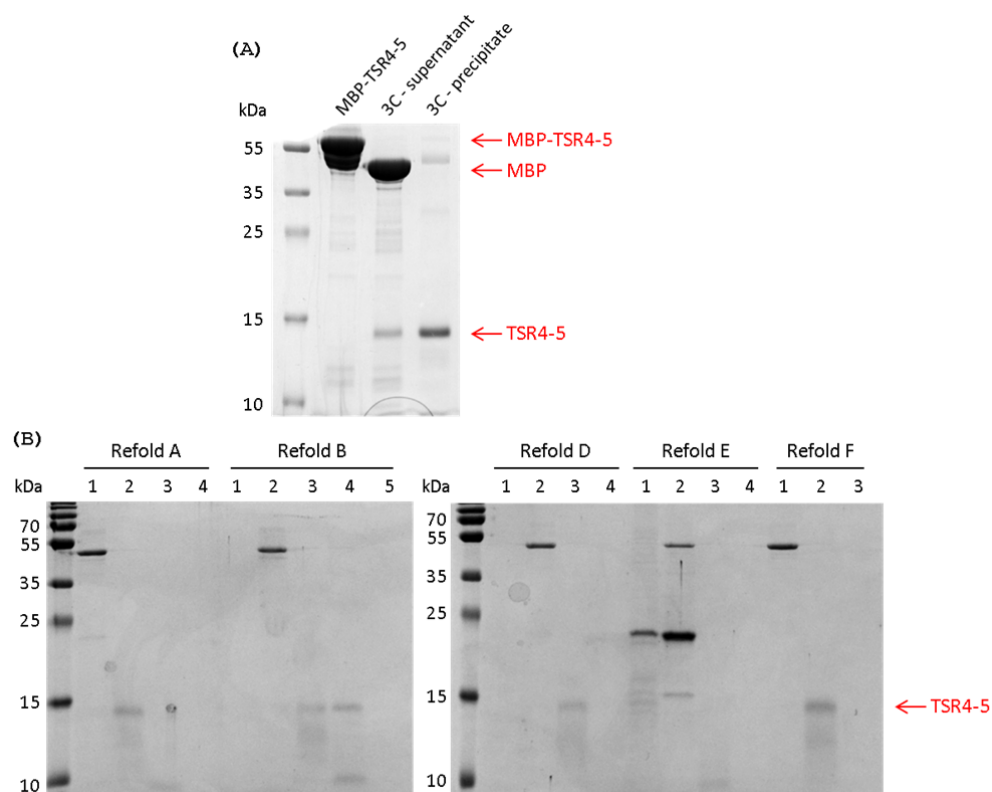


Figure 3.20. SDS-PAGE analysis of TSR4-5 refolding trials. (A) Reducing 15% SDS-PAGE analysis of MBP-TSR4-5 purification and 3C cleavage. MBP-TSR4-5 was expressed in *E. coli* BL21 (DE3). Following purification by affinity chromatography, the MBP tag (43 kDa) was cleaved with 3C protease. The tag cleavage resulted in the precipitation of TSR4-5 (14 kDa). (B) Denatured TSR4-5 was refolded in different buffers, as outlined in **Table 3.2**. Numbered peak fractions from SEC shown in **Figure 3.19** were analysed by reducing 15% SDS-PAGE.

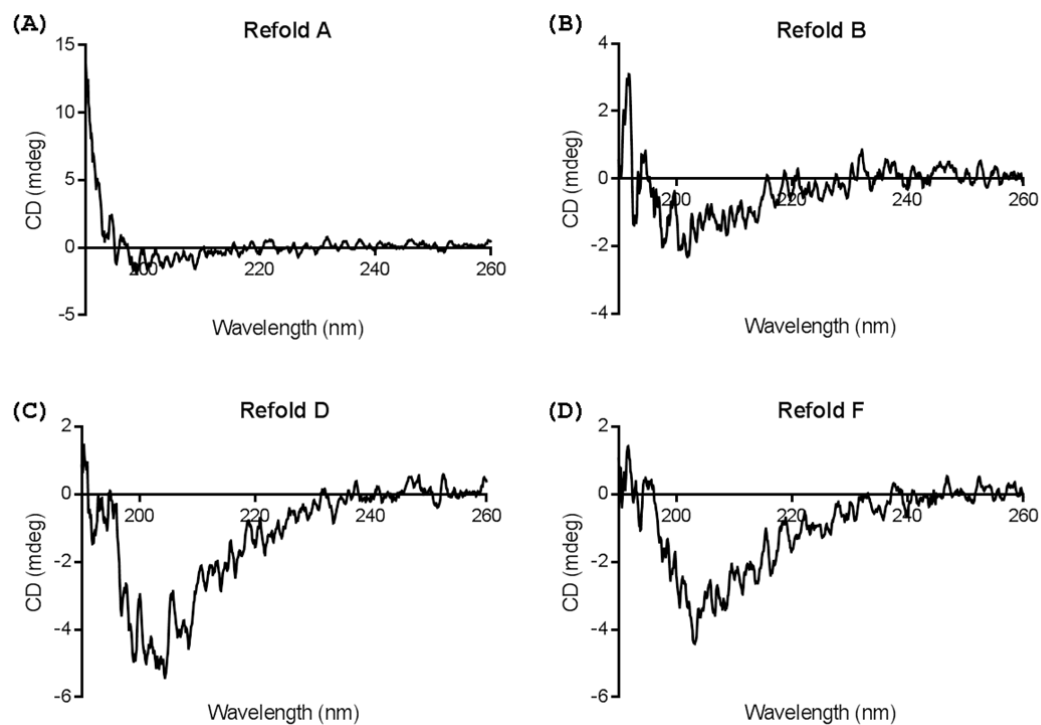


Figure 3.21. CD analysis of TSR4-5 from refolding trials. TSR4-5 from (A) Refold A, (B) Refold B, (C) Refold D, and (D) Refold F were analysed by CD to assess secondary structure. The lack of a positive peak at 230 nm indicated that TSR4-5 did not fold correctly in any of the refolding conditions tested. The CD buffer was 10 mM sodium phosphate buffer, pH 7.

3.5 Expression of properdin in insect cells

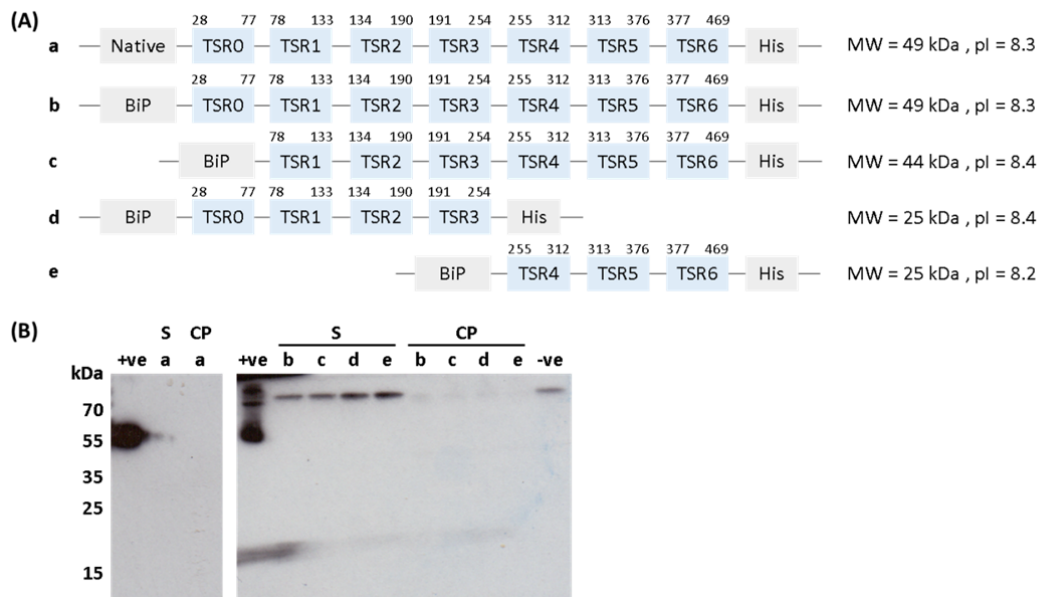


Figure 3.22. Expression of full-length properdin and TSR domains in *Drosophila melanogaster* S2 suspension cells. (A) Constructs were designed with either (a) the native signal peptide or (b)–(e) the insect BiP signal peptide. Numbers above TSR domains indicate residue number within properdin. The sequences of the TSR domains are shown in **Figure 3.1**. The molecular weights (MW) and theoretical pI of the constructs after signal peptide removal are shown. (B) Western blots were performed for both the supernatant (S) and the cell pellet (CP) fractions of the insect cell cultures of the different constructs from (A) with α -properdin antibody. None of the constructs were expressed by insect cells. The positive control was purified properdin (Complement Technology) and the negative control was the supernatant of non-transfected insect cells.

Insect expression of properdin was attempted in *Drosophila melanogaster* S2 suspension cells (Expres2ion Biotechnologies). The properdin gene was codon-optimised for insect expression. Different combinations of properdin TSR domains were cloned into pExpres2-2 vector with the native signal peptide or the insect BiP signal sequence, and a C-terminal 6-His tag. The constructs were full-length properdin, TSR1–6, TSR0–3 and TSR4–6 (**Figure 3.22A**). Transfection to obtain stable cell lines was carried out per manufacturer's instructions (Expres2ion Biotechnologies). Following transfection, Western blots were performed for both the supernatant and the cell pellet fractions of the insect cell cultures using α -properdin antibody. None of the transfected cell lines expressed full-length properdin or TSR domains, as none of the expected bands were present (**Figure 3.22B**). Since TSR domains of human thrombospondin-1 had been

successfully expressed and purified from *Drosophila* S2 cells before (Miao et al., 2001; Tan et al., 2002), it is not that insect cells cannot support expression and folding of TSR domains. Those studies used pMT/BiP/V5-HisA (Invitrogen) as the expression vector, which contains a different promoter from pExpreS2-2 that I used. Therefore, the promoter used here might have resulted in poor expression of properdin. Additionally, no *Drosophila* Kozak sequence was included within pExpreS2-2, which might have affected translation and protein expression (Cavener, 1987).

3.6 Expression of properdin in yeast cells

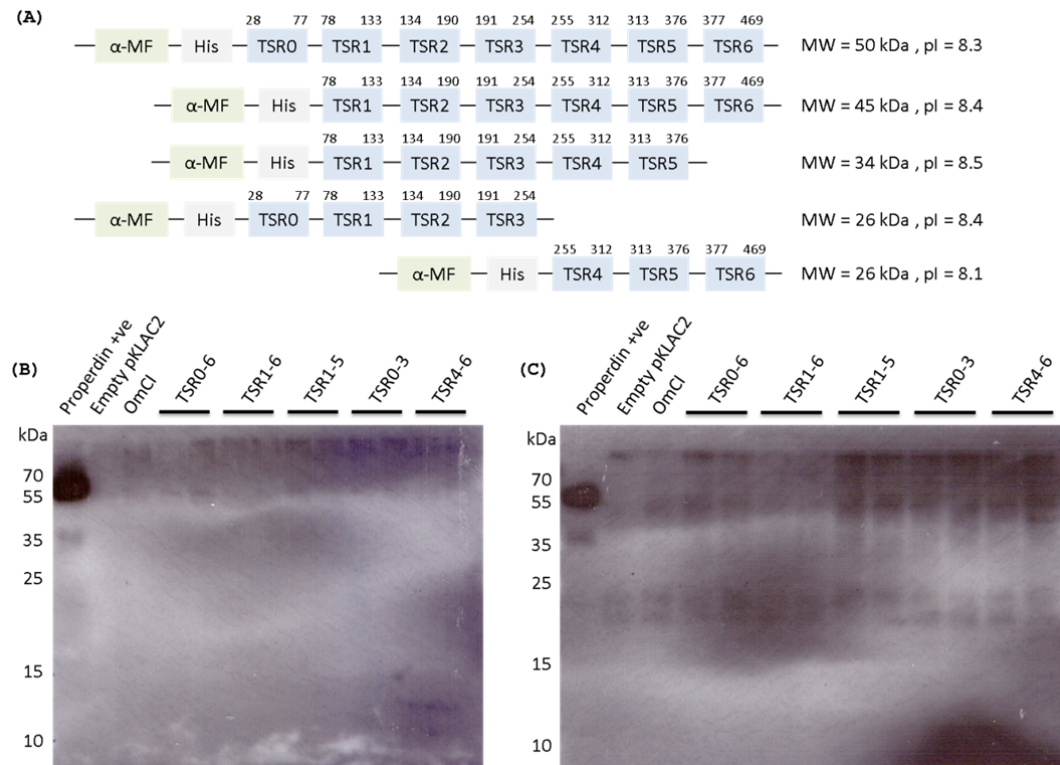


Figure 3.23. Yeast expression of properdin. (A) Expression of full-length properdin and TSR domains was attempted in *K. lactis* GG799 cells. Constructs were designed with the α -MF secretion leader sequence. Numbers above TSR domains indicate residue number within properdin. The sequences of the TSR domains are shown in **Figure 3.1**. The molecular weights (MW) and theoretical pI of the constructs after signal sequence removal are shown. Western blots of (B) the supernatant and (C) the cell pellet fractions of yeast cultures were performed with α -properdin antibody. None of the constructs were expressed by yeast cells. Purified properdin (Complement Technology) was used as a positive control for the blots.

K. lactis GG799 cells were transformed using the *K. lactis* Protein Expression Kit (NEB) following the manufacturer's instructions. Transformants were selected by growth on Yeast Carbon Base (YCB) agar medium containing 5 mM acetamide. YCB medium contains glucose and all nutrients required for growth except a source of nitrogen. Cells can utilise acetamide as a source of nitrogen only after it is broken down to ammonia by acetamidase, the product of the *amdS* gene present in the pKLAC2 vector used for transformation. Five properdin constructs were tried: TSR0-6, TSR1-6, TSR1-5, TSR0-3 and TSR4-6. All constructs used the *K. lactis* α -mating factor (α -MF) domain sequence as the secretion leader sequence, and had a 10-His tag at the N-terminus. Empty pKLAC2 and pKLAC2-OmCI (which had been previously expressed and purified from *K. lactis* in the lab) were used as controls. Following transformation and selection, a 5 ml pre-culture was grown from a single colony, and then, 200 μ l of the pre-culture was inoculated into a 10 ml culture. The final culture was centrifuged to pellet cells, and both the supernatant and cell pellet fractions were analysed by Western blots. Expression of OmCI was confirmed with a Western blot with α -His antibody (data not shown), suggesting that the transformation protocol worked. Western blots with α -properdin antibody of the supernatant and cell pellet fractions of the properdin transformant cultures showed that none of the properdin constructs were expressed by *K. lactis* (**Figure 3.23**). Post-translational modifications of properdin by yeast may not be appropriate for correct protein folding. In addition, the gene used for cloning was optimised for mammalian expression, which could have been sub-optimal for yeast expression.

3.7 Expression and purification of properdin in mammalian cells

3.7.1 Expression trials in HEK293T and CHO

Mammalian expression of properdin and its TSR domains was attempted using pcDNA3.1(+) as the vector. All constructs were designed with the native signal peptide for secretion to the media and a C-terminal 6-His tag to enable purification by affinity chromatography. The native Kozak translation initiation sequence of properdin is CTCAACATGA (the ATG start codon is underlined); however, the Kozak consensus sequence is GCC(G/A)CCATGG (Kozak, 1991). Since I wanted to keep the native signal

peptide which immediately follows the start codon, the Kozak sequence GCCACCATGA was used in the constructs for the mammalian expression of properdin.

HEK293T cells were tested first for properdin expression. The cells were maintained in supplemented DMEM before transfection and transfected when 80% confluent. For the transfection of a 10 ml culture in a T-75 flask, 18 µl of GeneJuice transfection reagent (Novagen) was added to 800 µl of serum-free medium Opti-MEM (Life Technologies) and incubated for 5 min. 6 µg of DNA was then added to the GeneJuice/Opti-MEM mixture and incubated for 15 min. During incubation, the cells were washed with 10 ml of PBS and 9 ml of growth medium was added to the flask. The DNA/GeneJuice/Opti-MEM mixture was added dropwise to the cells. After 6 h, the flask was replaced with 10 ml of fresh growth medium, and then left at 37 °C for four days.

For the expression trial, two different growth media were tested – supplemented DMEM and serum-free Opti-MEM. The negative control was without any DNA, and three constructs were tested: TSR0-6, TSR0-3 and TSR4-6 (**Figure 3.24A**). Following transfection, the supernatants were harvested for a Western blot with α-properdin antibody. As shown in **Figure 3.24B**, TSR0-6 was expressed in DMEM; TSR0-3 was expressed in both DMEM and Opti-MEM; TSR4-6 expression was not observed in DMEM or Opti-MEM. As the expression of full-length properdin was observed in DMEM and the expression of TSR0-3 was higher in DMEM than in Opti-MEM, supplemented DMEM was subsequently used for transfection.

Polyethylenimine (PEI) was used for expression trials, as a cheaper alternative to GeneJuice (Longo et al., 2013). For transfection of a 20 ml culture in a T-175 flask, 30 µl of PEI (1 mg/ml in PBS, filter-sterilised) was added to 500 µl Opti-MEM, while 10 µg of DNA was added to 500 µl Opti-MEM. These mixtures were incubated separately for 5 min, mixed together, and then incubated for 30 min. During incubation, the cells were washed with 20 ml of PBS and 20 ml of supplemented DMEM was added to the flask. The PEI/DNA/Opti-MEM mixture was added dropwise to the cells, and then left at 37 °C for three days. As shown in **Figure 3.24C**, the results with PEI were the same as when GeneJuice was used as the transfection reagent: TSR0-6 and TSR0-3 were expressed, but not TSR4-6. Therefore, PEI was subsequently used for transfection.

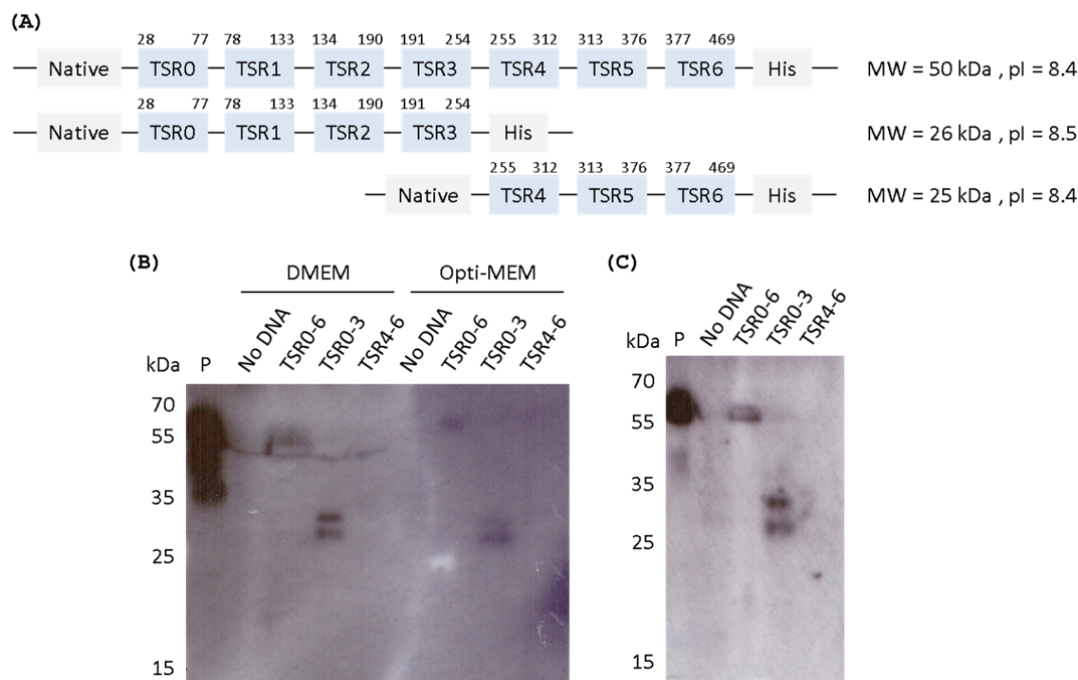


Figure 3.24. Transient expression trials of properdin in HEK293T. (A) Properdin constructs were designed with the native signal peptide. Numbers above TSR domains indicate residue number within properdin. The sequences of the TSR domains are shown in **Figure 3.1**. The molecular weights (MW) and theoretical pI of the constructs after signal peptide removal are shown. (B) GeneJuice was used as the transfection reagent for the transient expression of properdin constructs. Supplemented DMEM and Opti-MEM were compared as the transfection medium. Western blot with α -properdin antibody showed that TSR0-6 was expressed in DMEM, and TSR0-3 was expressed in DMEM and Opti-MEM. (C) PEI was used as the transfection reagent, with supplemented DMEM as the transfection medium. Western blot with α -properdin antibody showed that TSR0-6 and TSR0-3 were expressed. P = purified properdin (Complement Technology) was used as a positive control for the blot.

In addition, CHO cells were also tested for properdin expression. The protocol for transfection of CHO using PEI as the transfection reagent was based on (Li, 2015). For transfection of a 30 ml culture in a T-175 flask, 7.5 μ g of DNA was added to 1.5 ml of Opti-MEM and incubated for 2 min. 60 μ l of PEI (1 mg/ml in PBS, filter-sterilised) was added to the DNA/Opti-MEM mixture and incubated for 10 min. During incubation, the cells were washed with 30 ml of PBS and 30 ml of supplemented DMEM was added to the flask. The PEI/DNA/Opti-MEM mixture was added dropwise to the cells. After 4 h, the flask was replaced with 30 ml of fresh growth medium, and then left at 37 °C for five days. Four constructs were tested: TSR0-6, TSR0-5, TSR0-3 and TSR4-6 (**Figure 3.25A**). As shown in **Figure 3.25B**, TSR0-6 and TSR0-3 were expressed in CHO, consistent with HEK293T.

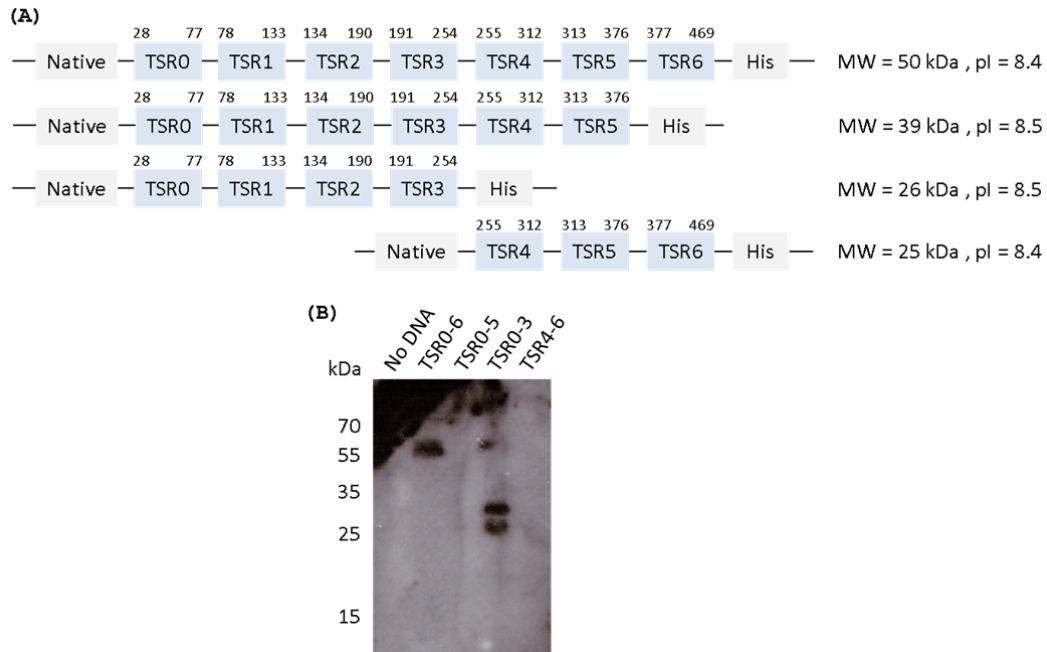


Figure 3.25. Transient expression trials of properdin in CHO. (A) Properdin constructs were designed with the native signal peptide. Numbers above TSR domains indicate residue number within properdin. The sequences of the TSR domains are shown in **Figure 3.1**. The molecular weights (MW) and theoretical pI of the constructs after signal peptide removal are shown. (B) PEI was used as the transfection reagent for the transient expression of properdin constructs in CHO, with supplemented DMEM as the transfection medium. Western blot with α -His antibody showed that TSR0-6 and TSR0-3 were expressed.

3.7.2 Transient expression and purification of TSR0-3 in HEK293T

Following the expression trial, transient expression of TSR0-3 in HEK293T was scaled up to around 1 litre of culture. Four days after transfection, the supernatant was harvested and filtered, before loading onto a 1 ml cComplete His-Tag Purification column (Roche), pre-equilibrated with Buffer A (PBS, pH 7.4). After loading the supernatant, the column was washed with 60 CV of 1% Buffer B (Buffer A containing 250 mM imidazole), and then the protein was eluted by gradient up to 80% Buffer B over 40 CV. SDS-PAGE analysis (**Figure 3.26A**) and Western blot with α -properdin antibody (not shown) confirmed the presence of TSR0-3 in the elution. TSR0-3 appeared as a double band around 30 kDa; the reason for this is unknown. As there were still many contaminants, SEC was performed on a S200 16/60 column; the running buffer was 50 mM Tris, 150 mM NaCl, pH 7.5. SEC managed to get rid of some of the contaminants, but judging by the gel, a trace of albumin (~ 70 kDa) still remained (**Figure 3.26B**). Further purification

by ion exchange chromatography was performed. For this, the sample was first dialysed against Buffer A (50 mM Tris, pH 7.5). The sample was loaded onto a Mono S 5/50 column, and then the column was washed with 10 CV of Buffer A. The protein was eluted by gradient up to 50% Buffer B (Buffer A containing 1 M NaCl) over 40 CV. Ion exchange chromatography managed to separate albumin and TSR0-3, as albumin did not bind to the column and came off in the flow-through (**Figure 3.26C**). Elution fractions containing TSR0-3 were pooled together, and the final yield was 45 μ g from 1 litre.

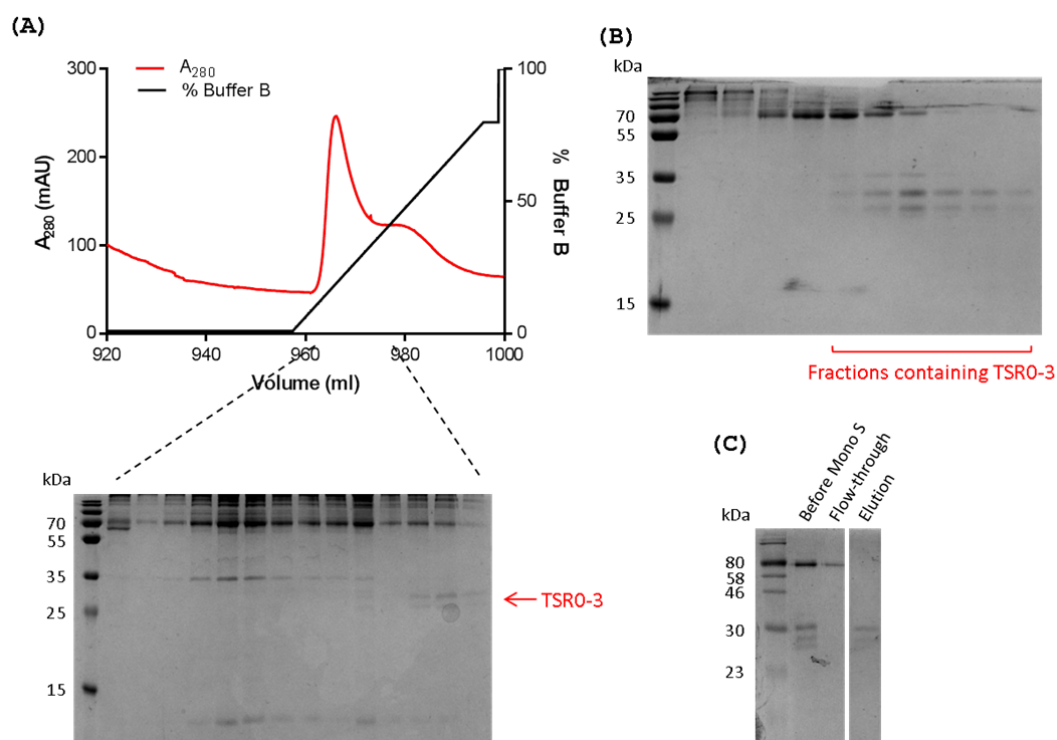


Figure 3.26. Purification of TSR0-3 from transient expression of HEK293T. (A) Affinity chromatography profile of TSR0-3 expressed in HEK293T and reducing 15% SDS-PAGE analysis of elution fractions. TSR0-3 showed as a double band around 30 kDa. (B) S200 16/60 size exclusion profile of TSR0-3 and reducing 15% SDS-PAGE analysis of elution fractions. The running buffer for SEC was 50 mM Tris, 150 mM NaCl, pH 7.5. SEC separated some of the contaminants from TSR0-3. (C) Reducing 15% SDS-PAGE analysis shows the separation of albumin and TSR0-3 following ion exchange chromatography using a Mono S column. Albumin and TSR0-3 were present in the flow-through and eluate, respectively.

3.7.3 Transient expression and purification of full-length properdin in HEK293T

Following the expression trial, transient expression of TSR0-6 in HEK293T was scaled up to around 1 litre of culture. Three days after transfection, the supernatant was harvested and filtered. Purification of TSR0-6 was carried out using the same method as TSR0-3 (**Section 3.7.2**). Following affinity chromatography, non-reducing SDS-PAGE gel (**Figure 3.27**) and Western blot with α -properdin antibody (data not shown) showed TSR0-6 as a double band around 60 kDa. TSR0-6 could not be separated from the higher molecular weight contaminants from nickel column purification, even after SEC and ion exchange chromatography (data not shown). Looking at the size exclusion profile and the mAU readout, the yield of TSR0-6 from this purification method was deemed too low for structural studies and the expression and purification of TSR0-6 from HEK293T was not pursued any further.

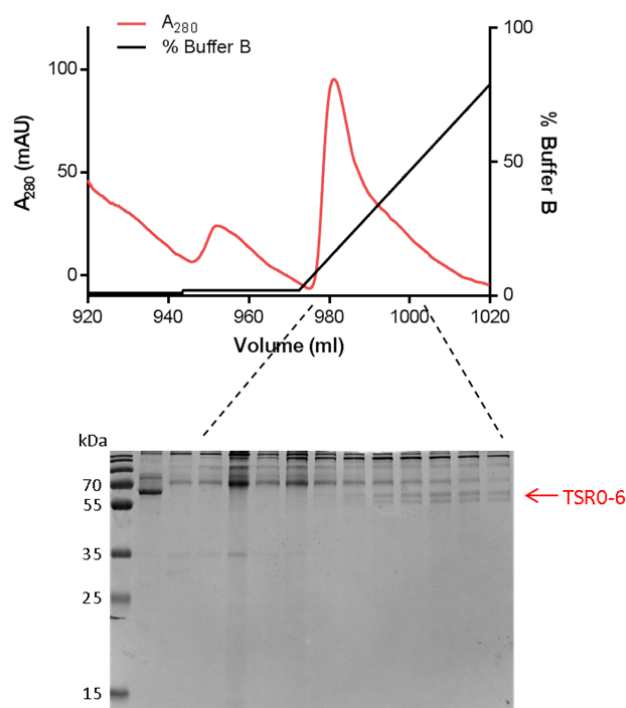


Figure 3.27. Purification of TSR0-6 from transient expression of HEK293T. Affinity chromatography profile of TSR0-6 expressed in HEK293T and non-reducing 15% SDS-PAGE analysis of elution fractions. TSR0-6 showed as a double band around 55 kDa.

3.7.4 Transient expression and purification of full-length properdin in CHO

Following the expression trial, transient expression of TSR0-6 in CHO was scaled up to around 1 litre of culture. Four days after transfection, the supernatant was harvested and filtered. Affinity chromatography purification of the supernatant was carried out using the protocol outlined in **Section 3.7.2**. Fractions containing TSR0-6 were further purified by SEC with a S200 10/30 column, with PBS, pH 7.4, as the running buffer. Non-reducing SDS-PAGE gel and Western blot with α -properdin antibody confirmed the presence of properdin in the elution fractions (data not shown). As there were still contaminants present, ion exchange chromatography with a Mono S 5/50 column was performed. The sample was dialysed against 50 mM Tris, 20 mM NaCl, pH 7, before being injected onto the column. TSR0-6 was eluted by gradient up to 50 mM Tris, 800 mM NaCl, pH 7, over 80 CV. The elution peak from Mono S contained TSR0-6, judging by the non-reducing SDS-PAGE gel and Western blot with α -properdin antibody (**Figure 3.28**). Even after ion exchange chromatography, contaminants around 130 kDa still remained. As TSR0-6 could only be observed as very faint bands on the gel, the expression of TSR0-6 from CHO was considered low and was not optimised further.

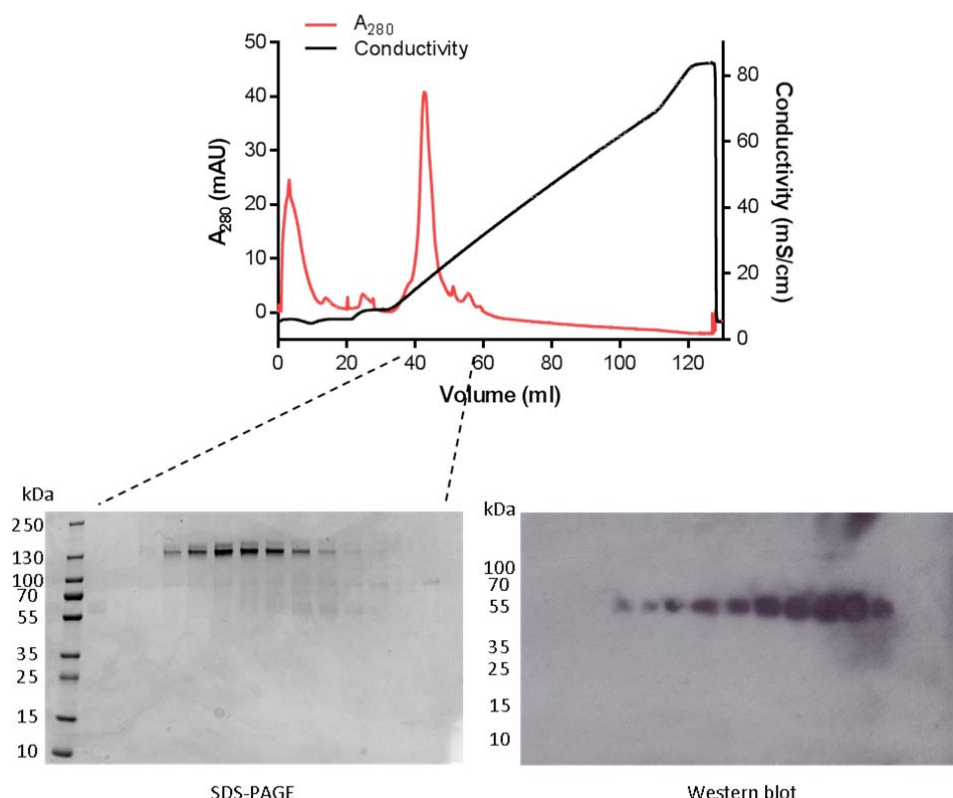


Figure 3.28. Purification of TSR0-6 from transient expression of CHO. Ion exchange chromatography profile of TSR0-6 expressed in CHO. Non-reducing SDS-PAGE analysis (left) and Western blot with α -properdin antibody (right) of elution fractions confirmed the presence of properdin. 4–20% Mini-PROTEAN TGX Precast Protein Gels (Bio-Rad) were used.

3.7.5 Transient expression and purification of full-length properdin in HEK293F

The FreeStyle 293-F cell line is derived from the 293 cell line, so it is similar to HEK293T, but has been adapted for suspension growth. The 293-F cells can reach a density of 3×10^6 cells/ml in a shaking flask; this is higher than the density of HEK293T at 0.78 – 1.17×10^6 cells/ml in an adherent flask, depending on the flask and culture volume used. As the higher cell density of 293-F might increase the yield, the 293-F cell line was used for the transient expression and purification of TSR0-6. For transfection, the cells were diluted to 1×10^6 cells/ml. For direct comparison, the same amount of DNA and PEI were used as with HEK293T transfection (**Section 3.7.1**): for a 10 ml culture, 5 μ g of DNA and 15 μ g of PEI were used. Large-scale expression was performed with 900 ml of HEK293F culture. 450 μ g of DNA was diluted into 18 ml of OptiPro SFM

and 1350 μ l of PEI (1 mg/ml in PBS, filter-sterilised) was diluted into 18 ml of OptiPro SFM. The diluted PEI was added to the diluted DNA, and the mixture was incubated for 30 min, before being added to the flask while swirling.

Three days after transfection, the supernatant was harvested and filtered for purification. The supernatant was adjusted to pH 7 before loading onto a 5 ml Ni-NTA Superflow column; the running buffer was 50 mM HEPES, 300 mM NaCl, pH 7. Following affinity chromatography, the elution fractions containing TSR0-6 were further purified by SEC with a S200 16/60 column; the running buffer was 10 mM HEPES, 150 mM NaCl, pH 7. SDS-PAGE analysis of peak fractions showed that TSR0-6 was quite clean after SEC (**Figure 3.29A,B**). Nonetheless, looking at the size exclusion profile and the mAU readout, the yield of TSR0-6 from this protocol was low and the expression of TSR0-6 from HEK293F was not continued further.

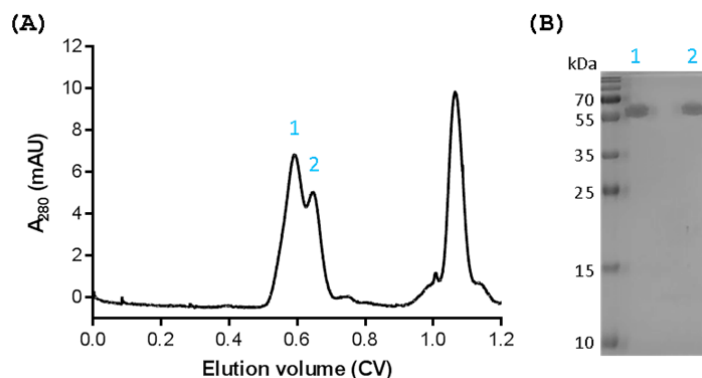


Figure 3.29. Purification of TSR0-6 from transient expression of HEK293F. (A) S200 16/60 size exclusion profile of TSR0-6 expressed in HEK293F. The running buffer for SEC was 10 mM HEPES, 150 mM NaCl, pH 7. (B) Reducing 15% SDS-PAGE analysis of peak fractions from SEC, as highlighted in (A).

3.7.6 Stable expression and purification of properdin using the FPc and FPc Δ 3 constructs

During this thesis, a paper was published which described the purification of properdin using a stable HEK293F cell line (Pedersen et al., 2017). The FPc construct was designed with an internal TEV cleavage site inserted between TSR3 and TSR4 domains (**Figure 3.30A**). The cleavage of recombinant properdin results in the formation of pseudo-

monomeric properdin, which consists of TSR0-3 of one properdin monomer and TSR4-6 of another. The FPc Δ 3 construct contains an additional TEV cleavage site between TSR2 and TSR3 (**Figure 3.30A**); this results in the formation of pseudo-monomeric properdin lacking TSR3 domain (Pedersen et al., 2019). I acquired stable HEK293F cell lines of both of these constructs from the authors of the study.

For the purification of FPc, the supernatant of the HEK293F culture was harvested and filtered. After adjusting to pH 6.4, the supernatant was loaded onto a CIRp-A1 column (as generated in **Section 3.3.1**). Following elution from CIRp-A1, the protein concentrations of the elution fractions were measured, and the fractions containing properdin were pooled. TEV protease was added at TEV:properdin ratio of 1:4, and left for three days at 4 °C. FPc was further purified by SEC with a S200 16/60 column; the running buffer was PBS, pH 7.4. FPc Δ 3 was purified using the same protocol. **Figure 3.30B** shows the SEC profiles of FPc purified from a 2 l culture and FPc Δ 3 purified from a 0.6 l culture. The yield was ~2 mg per litre for both constructs.

When analysed by SDS-PAGE, FPc ran as a single band, with the bands for TSR0-3 and TSR4-6 likely overlapping. FPc Δ 3 ran as two bands; the upper band was likely TSR4-6 and the lower band TSR0-2 (**Figure 3.30C**). The masses of FPc and FPc Δ 3 were analysed by SEC-MALS using purified proteins at 1 mg/ml. Superdex 200 10/300 GL column was used and the running buffer was PBS, pH 7.4. The calculated masses were 58.7 kDa ($\pm$ 0.3%) for FPc and 51.8 kDa ($\pm$ 0.3%) for FPc Δ 3, corresponding to monomeric proteins (**Figure 3.30D**). FPc Δ 3 had another peak which gave a mass of 59.3 kDa ($\pm$ 0.4%); as this peak was at the same position as FPc, this was likely the full-length construct containing TSR3. For both FPc and FPc Δ 3, the calculated masses were higher than the predicted masses from their amino acid sequences, likely due to post-translational glycosylation. These monomeric properdin constructs are suitable for crystallisation trials; as the yields were higher than other mammalian expression systems that I tried, the FPc and FPc Δ 3 cell lines were subsequently used to purify monomeric properdin for structural and interaction studies.

The full-length construct FPc was analysed by CD; the protein was dialysed against 10 mM sodium phosphate buffer, pH 7.4. The CD spectrum had a positive peak at 230 nm

(Figure 3.31), which has been associated with stacking tryptophan residues (Andersson et al., 2001) and observed in serum properdin (Medicus et al., 1980).

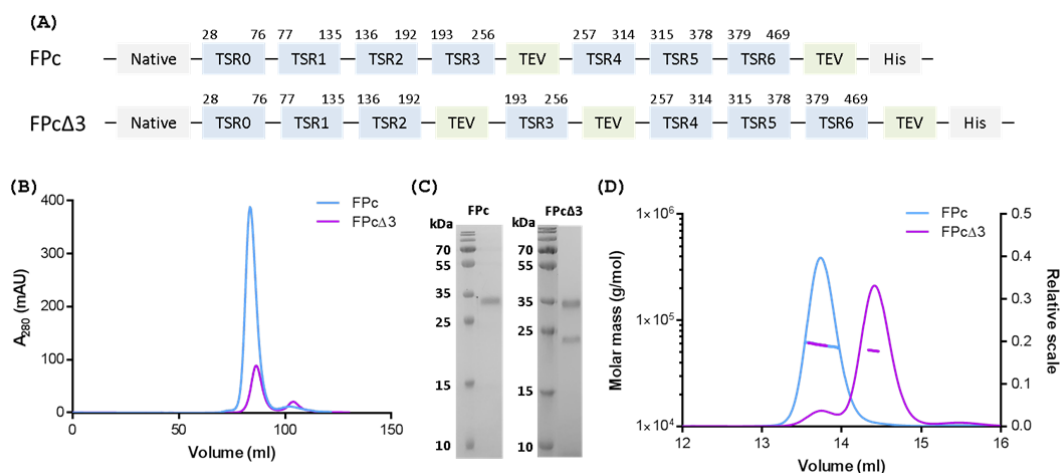


Figure 3.30. Purification of FpC and FpCΔ3. (A) FpC and FpCΔ3 (Pedersen et al., 2019) contain TEV cleavage sites between TSR domains, resulting in pseudo-monomeric properdin. The native signal peptide was used in these constructs. Numbers above TSR domains indicate residue number within properdin. The domain boundaries are from the aforementioned study. (B) S200 16/60 size exclusion profile of FpC and FpCΔ3 purified from stable HEK293F cell lines. The running buffer for SEC was PBS, pH 7.4. (C) FpC ran as a single band on a reducing 15% SDS-PAGE gel (the bands for TSR0-3 and TSR4-6 likely overlap) whereas FpCΔ3 ran as two bands (the upper band is likely TSR4-6 and the lower band is likely TSR0-2). (D) FpC and FpCΔ3 were analysed by SEC-MALS on a Superdex 200 10/300 GL column, with PBS, pH 7.4, as the running buffer. The calculated masses from SEC-MALS were 58.7 kDa ($\pm 0.3\%$) for FpC and 51.8 kDa ($\pm 0.3\%$) for FpCΔ3, corresponding to monomeric proteins.

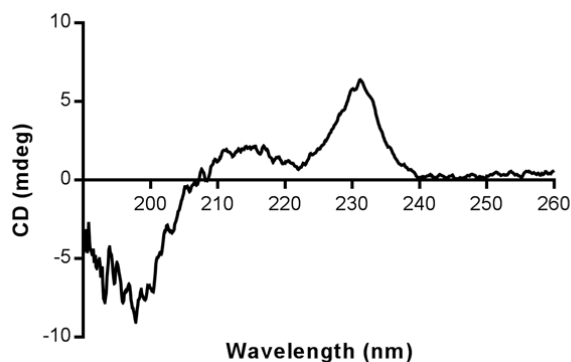


Figure 3.31. CD analysis of FpC. The full-length properdin construct FpC had a positive peak at 230 nm, which has been observed for serum properdin (Medicus et al., 1980) and has been shown to correlate with stacking tryptophan residues (Andersson et al., 2001). The CD buffer was 10 mM sodium phosphate buffer, pH 7.4.

3.8 Discussion

Various methods of properdin expression and purification were tried. The yield of properdin purified from serum using the properdin-binding tick protein CIRp-A1 was 1.2 mg of properdin per litre of serum (**Section 3.3.2**). Although the yield was good, there were multiple oligomeric forms of properdin (**Figure 3.4**), which might be unsuitable for crystallisation. Additionally, EM studies of properdin suggested flexibility in the formation of oligomers (Alcorlo et al., 2013), which is also unsuitable for crystallisation. I did not try separating the different oligomers by further purification, for example, ion exchange chromatography, as purification of properdin from serum was more a proof-of-principle of using CIRp-A1 to pull down properdin. I wanted to establish a method for recombinant expression and purification of properdin. This way, I could try expressing shorter properdin constructs, as well as generate properdin mutants if desired further down the line.

Recombinant expression of full-length properdin and TSR domains was attempted in various expression systems. Purification of TSR domains in bacteria, as soluble proteins or by refolding, resulted in unfolded proteins, as judged by their CD spectra. Although the analysis of protein folding by CD was complicated by a lack of predicted secondary structure in the TSR fold, only TSR3 showed any sign of folding, as indicated by a positive peak in the CD spectrum at 230 nm (**Figure 3.16A**). This peak is consistent with tryptophan stacking interactions (Andersson et al., 2001) and was also observed in full-length properdin (**Figure 3.31**). Thermal denaturation of TSR3 led to the disappearance of this peak and suggested a T_M of 37 °C (**Figure 3.17**). The relatively low T_M was unsurprising since TSR domains lack regular secondary structure and are only held together by disulphide bonds (**Figure 3.1B**).

Eukaryotic expression of properdin was tried in insect, yeast and mammalian systems; expression was only observed in mammalian cells. The vector used for insect expression might have resulted in poor expression, due to the promoter used or the lack of Kozak sequence (**Section 3.5**). Yeast cells might be unable to support the expression and folding of properdin, perhaps due to the inability to provide the appropriate chaperones or enable the unusual post-translational modifications of properdin. Although expression of properdin was observed in all mammalian cells tested – HEK293T, CHO and HEK293F –

expression levels and purification yields were low. The yield of TSR0-3 from HEK293T was 45 µg from a 1 litre culture. The yield of TSR0-6 from HEK293T, CHO and HEK293F was similar or even lower. For any of these transient expression experiments, I did not perform a time-course analysis of expression, as I always harvested the supernatant three or four days post-transfection. Therefore, it is possible that protein expression could be higher at other time points. In addition, I also did not compare expression levels when different amounts of DNA are used during transfection, or when different ratios of DNA/transfection reagent are used. Nonetheless, the ability to recombinantly express properdin, even at low levels, would be useful for pulldown assays, if not for structural studies.

(Pedersen et al., 2017) reported a yield of 1 mg of properdin per litre from transient expression in HEK293F using their FPc construct. This is much higher than the yield from the transient expression in HEK293F in this thesis (**Section 3.7.5**). The constructs used in this thesis and the FPc construct both contained the native properdin signal peptide and a C-terminal His tag. However, there are a few notable differences in the constructs and transfection methods. Their expression vector was pCEP4 with the endogenous Kozak sequence, whereas I used pcDNA3.1(+) with an optimised Kozak sequence (**Section 3.7.1**). For transfection, different amounts of DNA were used, as well as different DNA/PEI ratios. These factors could have contributed to low yields of properdin in this thesis. The stable HEK293F cell lines expressing FPc or FPcΔ3 gave yields of 2 mg per litre (**Section 3.7.6**), which is twice the reported yield from the transient expression of FPc and much higher than other mammalian expression systems that I tried in this chapter. In addition, the stable HEK293F cell lines of FPc and FPcΔ3 enable purification of monomeric properdin, which is suitable for crystallisation trials.

CHAPTER 4: FUNCTIONAL AND STRUCTURAL CHARACTERISATION OF CIRP-A1 AND HOMOLOGS

4.1 Introduction

Ticks inject many proteins into their feeding site, including immune system and coagulation inhibitors, so that they can prolong their feed without being damaged. Complement-modulating tick proteins represent opportunities to increase our knowledge of the host-tick interface; they can also be used as tools to study the molecular mechanisms of complement regulation. A novel complement inhibitor protein, named CIRp-A1 (Complement Inhibitor from Rhipicephalus pulchellus of the Alternative Pathway), was identified in the lab prior to the commencement of this thesis. CIRp-A1 was purified from the salivary glands of the tick *Rhipicephalus pulchellus*, by serial fractionation and mass spectrometry analysis of the enriched fraction (Jore, unpublished). Serum pulldowns subsequently showed that CIRp-A1 binds to properdin. In addition to CIRp-A1, five homologs were identified by searching for similar sequences in other tick species (**Table 4.1**). CIRp-A1 and the homologs share 42-82% amino acid sequence identity with each other, with the four cysteine residues conserved in all six proteins (**Figure 4.1**). In this chapter, I discuss initial structural and functional characterisation of CIRp-A1 and homologs, towards understanding the molecular basis of complement inhibition by these tick proteins.

Table 4.1. Tick species from which CIRp-A1 and homologs were identified. Homologs of CIRp-A1 were identified by searching for similar sequences in the expressed sequence tag (EST) nucleotide sequence database (NCBI) (marked with *), or in the lab's *R. appendiculatus* and *R. pulchellus* cDNA libraries (Jore, unpublished).

Protein	Identifier/Accession number	Tick species
CIRp-A1	comp52_seq0	<i>Rhipicephalus pulchellus</i> female
CIRp-A2	CD794868.1*	<i>Rhipicephalus appendiculatus</i> female
CIRp-A3	CK182034.1*	<i>Rhipicephalus microplus</i>
CIRp-A4	comp194_seq2	<i>Rhipicephalus pulchellus</i> female
CIRp-A5	comp308_seq0	<i>Rhipicephalus pulchellus</i> male
CIRp-A6	comp46007_c0_seq1	<i>Rhipicephalus appendiculatus</i> female

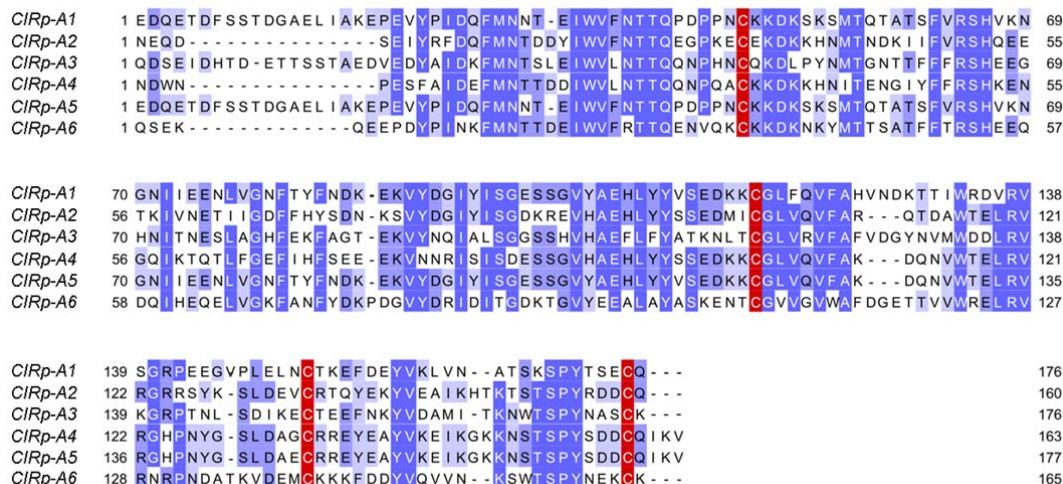


Figure 4.1. Multiple sequence alignment of CIRp-A1 and homologs. Amino acid sequence alignment of CIRp-A1 and homologs shows that the cysteine residues (red) are conserved across the six proteins. The multiple sequence alignment was generated by MUSCLE (EMBL-EBI) and viewed with Jalview. Residues were coloured based on sequence conservation scores by the Blosum62 matrix.

4.2 Expression and purification of CIRp-A1 and homologs in insect cells

4.2.1 Insect expression and purification of CIRp-A1 and homologs

Initial structural and functional characterisations of CIRp-A1 and homologs were carried out using proteins expressed in insect cells (**Figure 4.3A**). This expression system was chosen, as insect cells can support disulphide bond formation and post-translational modifications, which might be necessary for protein expression and folding. The genes of CIRp-A1 and homologs were codon-optimised for insect expression, and cloned into pExpreS2-2 vector with the insect BiP signal sequence followed by an N-terminal 6-His tag. Transfections into *Drosophila melanogaster* S2 suspension cells were carried out following the manufacturer's instructions (Expres2ion Biotechnologies).

For purification, the supernatant was harvested from a 1 l culture, filtered, and then loaded onto a 1 ml cOmplete His-Tag Purification column, pre-equilibrated with Buffer A (PBS, pH 7.4). After loading the supernatant, the column was washed with 60 CV of 1% Buffer B (Buffer A containing 250 mM imidazole), and then the protein was eluted by

gradient up to 80% Buffer B over 40 CV. The protein was further purified by SEC using a S200 16/60 column, with PBS, pH 7.4, as the running buffer. Purification of CIRp-A1 is shown in **Figure 4.2**.

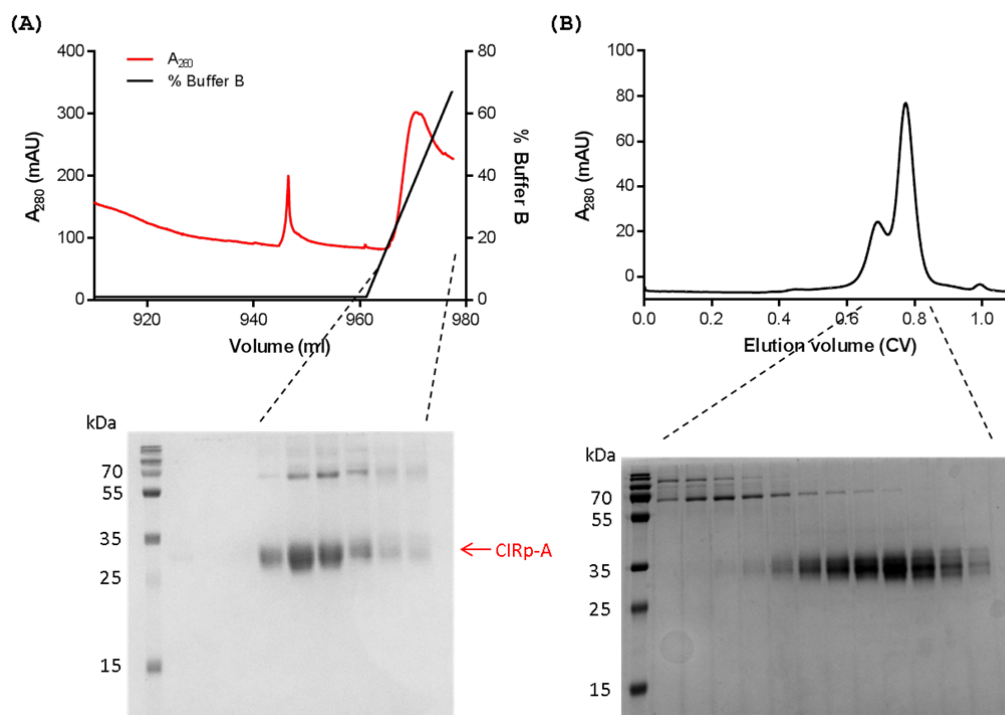


Figure 4.2. Insect expression and purification of CIRp-A1. (A) Nickel purification from insect culture supernatant using a cOmplete His-Tag Purification column and reducing 15% SDS-PAGE analysis of elution fractions. (B) S200 16/60 size exclusion profile of CIRp-A1 and reducing 15% SDS-PAGE analysis of elution fractions. The running buffer for SEC was PBS, pH 7.4.

Homologs of CIRp-A1 were purified using the same protocol. SDS-PAGE analysis of insect-purified CIRp-A1 and homologs showed two families of proteins that correlate with the presence or absence of the added N-terminal sequence (**Figure 4.3B**). CIRp-A1, CIRp-A3 and CIRp-A5, which have the added N-terminal sequence (**Figure 4.1**), ran higher on the gel than CIRp-A2, CIRp-A4 and CIRp-A6. All the proteins ran higher on the gel than the molecular masses calculated from their amino acid sequences (**Figure 4.3**). The gel indicates masses of 25-35 kDa whereas the calculated masses are around 20 kDa. This suggests that these proteins are heavily glycosylated when expressed in insect cells. The proteins also ran as multiple bands or smears, suggesting that they are heterogeneously glycosylated. They have between two and nine predicted N-linked

glycosylation sites (**Figure 4.4**), as determined using the NetNGlyc 1.0 Server (DTU Bioinformatics); they have predicted O-linked glycosylation as well, as determined using the NetOGlyc 4.0 Server (DTU Bioinformatics).

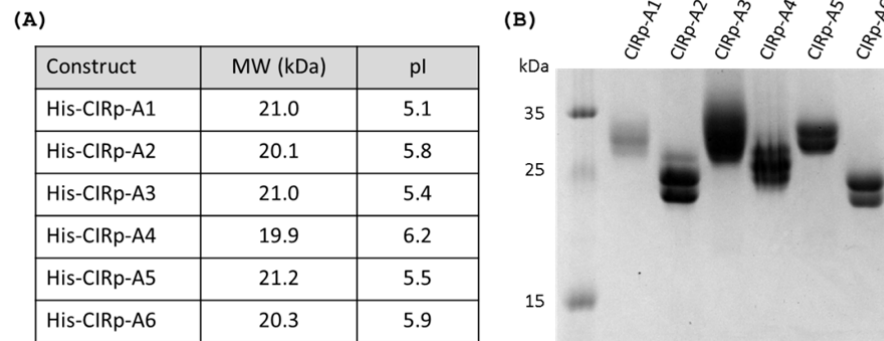


Figure 4.3. Purified CIRp-A1 and homologs. (A) The constructs shown in the table were purified. The molecular weights (MW) and theoretical pI of the constructs after signal peptide removal are shown. (B) CIRp-A1 and homologs were purified from stable insect cell lines and analysed by reducing 15% SDS-PAGE. All six proteins ran higher on the gel than their calculated molecular masses of around 20 kDa. The multiple bands or smears were likely due to glycosylation.

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CIRp-A1  1 EDQETDFSSTDGAELIAKEPEVYPIDQFMNNT-EIWVFNTTQPDPPNCKKDKSKSMTQTATSFVRSHVKN 69
CIRp-A2  1 NEQD-----SEIYRFDQFMNTDDYIWVFNNTTQEGPKCEKDKKHNTNDKIIFVRSHQEE 55
CIRp-A3  1 QDSEIDHTD-ETTSSTAEDVEDYAIKFMNTSLEIWVLNTTQQNPHNCQKDLPYNMTGNTTFFFRSHEEG 69
CIRp-A4  1 NDWN-----PESFAIDEFMNTTDDIWVLNTTQQNPQACKKDKKHNTITENG IYFFRSHKEN 55
CIRp-A5  1 EDQETDFSSTDGAELIAKEPEVYPIDQFMNNT-EIWVFNTTQPDPPNCKKDKSKSMTQTATSFVRSHVKN 69
CIRp-A6  1 QSEK-----QEEPDPYINKFMNTTDEIWVFRTTQENVQCKKDKKNKYMNTSATFFTRSHHEEQ 57

CIRp-A1  70 GNII EENLVGNFTYFNDK-EKVYDGIYISGESSGVYAEHLYYVSEDKKCGLFQVFAHVNDKTTIWRDVRV 138
CIRp-A2  56 TKIVNETIIGDFFHYSDN-KSVYDGIYISGDKREVHAEHLYYSSEDMICGLVQVFAR--QTDAWTEL RV 121
CIRp-A3  70 HNTNESLAGHFKEKAGT-EKVYNQIALSGGSSHVHAEFLFYATKNLTCGLVRVFAFVDGYNVMWDDL RV 138
CIRp-A4  56 GQIKTQTLFGEFIHFSEE-EKVNNRISISDESSGVHAEHLYYSSEDKKCGLVQVFAR--DQNVWTEL RV 121
CIRp-A5  70 GNII EENLVGNFTYFNDK-EKVYDGIYISGESSGVYAEHLYYVSEDKKCGLVQVFAR--DQNVWTEL RV 135
CIRp-A6  58 DQIHEQELVGKFANFYDKPDGVYDRIDITGDKTGVEEALAYASKENTCGVVGWVAFDGETTVVWREL RV 127

CIRp-A1  139 SGRPEEGVPLELCTKEFDEYVKLVN--ATSKSPYTSECQ--- 176
CIRp-A2  122 RGRRSYK-SLDEVCRQYEKYVEAIKHTKTSTSPYRDDCQ--- 160
CIRp-A3  139 KGRPTNL-SDIKECTEEFNKYVDAMI-TKNWTSYPYNASCK--- 176
CIRp-A4  122 RGHPNYG-SLDAGCRREYEAYVKEIKGKKNSTSPYSDDCQIKV 163
CIRp-A5  136 RGHPNYG-SLDAECRREYEAYVKEIKGKKNSTSPYSDDCQIKV 177
CIRp-A6  128 RNRPNDA TKVDEMCKKKFDDYVQVVN--KSWTSPYNEKCK--- 165

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Figure 4.4. Predicted N-linked glycosylation sites of CIRp-A1 and homologs. N-linked glycosylation sites (green) were predicted using the NetNGlyc 1.0 Server, which analyses Asn-Xaa-Ser/Thr sequences. CIRp-A1 and homologs have between two and nine glycosylation sites.

4.2.2 Circular dichroism analysis of CIRp-A1 and homologs to assess secondary structure

Circular dichroism (CD) analysis was performed with CIRp-A1 and homologs to assess their secondary structure. All proteins were dialysed against 10 mM sodium phosphate buffer, pH 7.4, before analysis. Samples were diluted to 0.1-0.2 mg/ml. The CD spectra of CIRp-A1 and homologs suggest the prevalence of β -sheets (**Figure 4.5A** to **Figure 4.10A**). This agrees with the prediction of structural homology by HHPred which suggests that CIRp-A1 and homologs are lipocalins. Lipocalins are characterised by an eight-stranded anti-parallel β -barrel. Temperature interval scan measurements of CD were collected to assess the thermal stability of CIRp-A1 and homologs. The proteins were heated from 10 °C to 80 °C at 5 °C intervals, and then cooled back down to 20 °C. When cooled back down to 20 °C, all proteins could refold back, and the pre-melt (before temperature interval scan) and post-melt (after temperature interval scan and cooling down) CD spectra overlay nicely (**Figure 4.5A** to **Figure 4.10A**). The CD at 195 nm was plotted against temperature, as a positive signal at 195 nm is characteristic of β -sheets (Greenfield, 2007). This showed that CIRp-A1 and homologs are relatively thermally stable, with melting temperatures (T_M) between 58-79 °C (**Figure 4.5B** to **Figure 4.10B**). For all proteins, the signal at 195 nm became negative at temperatures above the T_M as the protein unfolded.

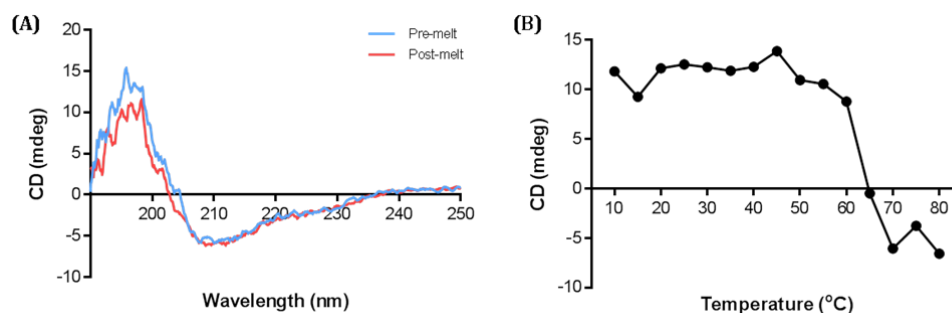


Figure 4.5. Temperature interval scan measurements of CD with CIRp-A1. CD spectra of CIRp-A1 were recorded as the temperature was increased from 10 °C to 80 °C at 5 °C intervals. Following temperature interval scan measurement, the temperature was decreased back down to 20 °C before collecting CD measurements. The CD buffer was 10 mM sodium phosphate buffer, pH 7.4. (A) The pre-melt (blue) and post-melt (red) CD spectra are shown. (B) The CD at 195 nm suggested a T_M of 65 °C.

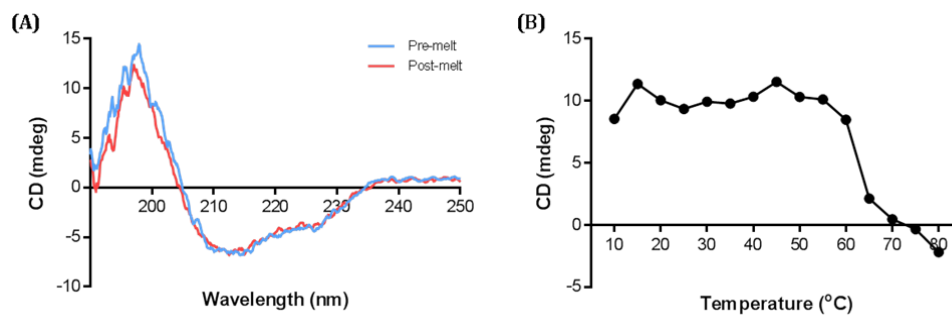


Figure 4.6. Temperature interval scan measurements of CD with CIRp-A2. CD spectra of CIRp-A2 were recorded as in **Figure 4.5**. (A) The pre-melt (blue) and post-melt (red) CD spectra are shown. (B) The CD at 195 nm suggested a T_M of 73 °C.

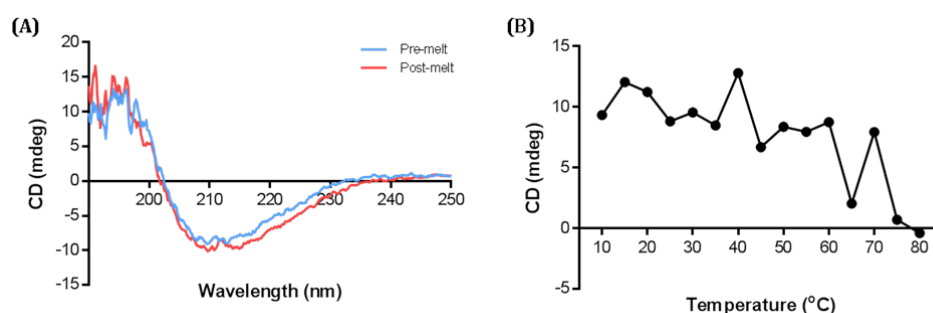


Figure 4.7. Temperature interval scan measurements of CD with CIRp-A3. CD spectra of CIRp-A3 were recorded as in **Figure 4.5**. (A) The pre-melt (blue) and post-melt (red) CD spectra are shown. (B) The CD at 195 nm suggested a T_M of 79 °C.

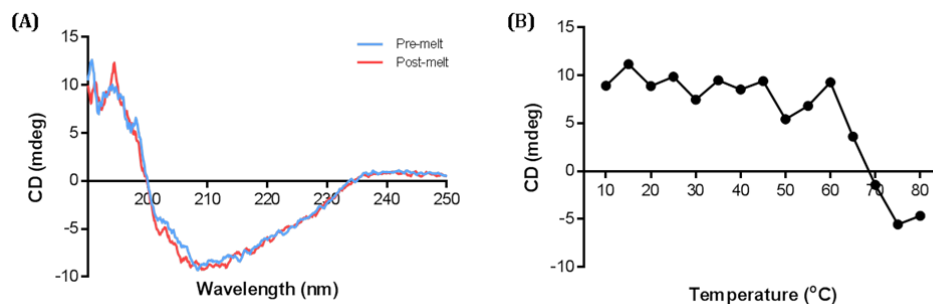


Figure 4.8. Temperature interval scan measurements of CD with CIRp-A4. CD spectra of CIRp-A4 were recorded as in **Figure 4.5**. (A) The pre-melt (blue) and post-melt (red) CD spectra are shown. (B) The CD at 195 nm suggested a T_M of 69 °C.

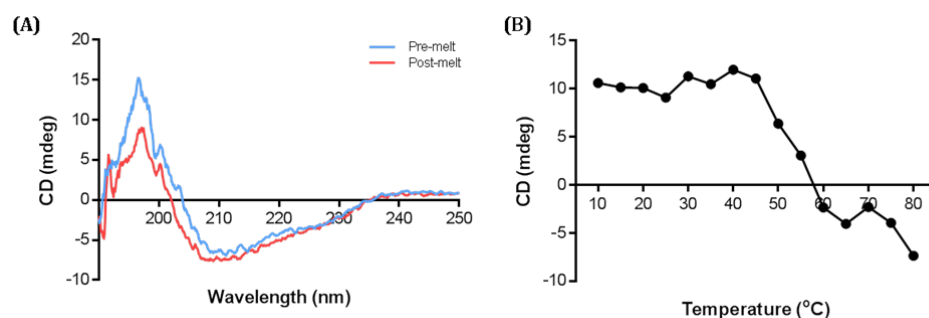


Figure 4.9. Temperature interval scan measurements of CD with CIRp-A5. CD spectra of CIRp-A5 were recorded as in **Figure 4.5**. (A) The pre-melt (blue) and post-melt (red) CD spectra are shown. (B) The CD at 195 nm suggested a T_M of 58 °C.

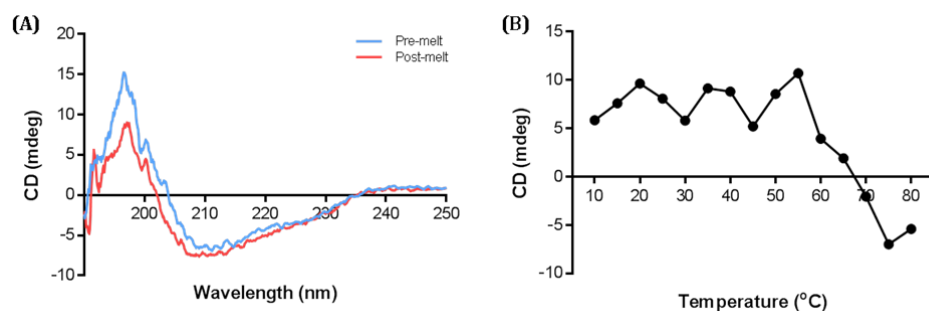


Figure 4.10. Temperature interval scan measurements of CD with CIRp-A6. CD spectra of CIRp-A6 were recorded as in **Figure 4.5**. (A) The pre-melt (blue) and post-melt (red) CD spectra are shown. (B) The CD at 195 nm suggested a T_M of 67 °C.

4.2.3 Native mass spectrometry analysis to investigate glycosylation state before and after deglycosylation

When expressed in insect cells, CIRp-A1 and homologs appeared to be extensively glycosylated (**Figure 4.3**). The heterogeneity and flexibility introduced by glycosylation might hinder crystallisation. Enzymatic deglycosylation of CIRp-A1 and homologs was attempted with Endo H_f and PNGase F (both from NEB). PNGase F removes almost all types of N-linked glycosylation whereas Endo H_f removes only high mannose and some hybrid types of N-linked carbohydrates. PNGase F cleaves between the innermost GlcNAc and asparagine residues, whereas Endo H_f leaves behind one GlcNAc unit. All deglycosylation reactions had a total reaction volume of 20 µl with 5 µg of the protein to be deglycosylated. The reactions were set up following the manufacturer's instructions (NEB). For denaturing conditions, 2 µl of Endo H_f or 1 µl of PNGase F were used; for

native conditions, 5 µl of the enzyme was used. Reactions were incubated at 37 °C overnight.

Figure 4.11 shows SDS-PAGE analysis of CIRp-A1 and homologs before and after deglycosylation with Endo H_f or PNGase F, under denaturing or native condition. The proteins showed differences in their susceptibility to deglycosylation. For most proteins, PNGase F worked better than Endo H_f, as the gel bands were lower following treatment with PNGase F than Endo H_f. Even after enzymatic deglycosylation, most proteins remained heterogeneously glycosylated, and were still seen as multiple bands or smears. The homologs CIRp-A6 and CIRp-A2 showed relatively clean bands following treatment with PNGase F under both denaturing and native conditions. The gel bands were lower and cleaner following treatment in denaturing conditions than in native conditions, likely because PNGase F had better access to the glycans when the proteins were denatured and unfolded, leading to complete deglycosylation. However, the proteins treated in native conditions were chosen for further analysis by native mass spectrometry, as the proteins were to be subsequently used for crystallisation trials.

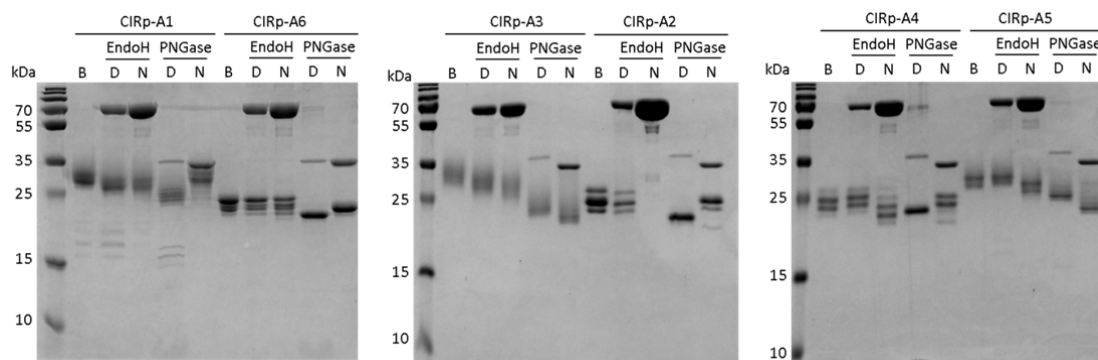


Figure 4.11. Enzymatic deglycosylation of CIRp-A1 and homologs. For each protein, deglycosylation was attempted with Endo H_f or PNGase F under denaturing or native condition. 15% SDS-PAGE gels show the proteins before and after deglycosylation. (B = before deglycosylation, D = deglycosylation under denaturing condition, N = deglycosylation under native condition). Endo H_f is 70 kDa and PNGase F is 36 kDa.

Native mass spectrometry analysis was performed with CIRp-A1 and homologs to assess the level of glycosylation and heterogeneity in the proteins. Native mass spectrometry experiments and data analyses were performed by Lucas Kuhlen. All samples were exchanged into 200 mM ammonium acetate, pH 7.5. The spectra are shown from **Figure**

4.12 to **Figure 4.17**. When analysing the calculated masses of the different species of a protein, a mass difference of ~1040 Da between species was often observed. This mass corresponds to paucimannose (3GlcNAc, 2Man, 1Fuc), which is consistent with the fact that the insect cell line used for the expression of these proteins has been modified to limit N-linked glycosylation at paucimannose. Therefore, species with mass differences corresponding to paucimannose were considered to be species with different numbers of N-linked glycosylation sites occupied. CIRp-A1 has five predicted N-linked glycosylation sites, and species with three, four or five occupied sites were observed (**Figure 4.12**). CIRp-A2 has four predicted sites, and species with two, three or four occupied sites were observed (**Figure 4.13**). When treated with PNGase F, the protein remained heterogeneously glycosylated, with two or three sites occupied, consistent with two bands being observed on the gel. CIRp-A3 has nine predicted sites, which is perhaps why the sample was very heterogeneous. The resolution was too low to distinguish different series, but the most abundant species had seven occupied sites (**Figure 4.14**), with a possibility of species with eight or nine occupied sites. CIRp-A4 has four predicted sites, and species with one, two, three or four occupied sites were observed (**Figure 4.15**). CIRp-A5 has four predicted sites, but a species with five occupied sites was observed, as well as three or four sites (**Figure 4.16**). There was also a species with a mass between four and five occupied sites; this was likely due to O-linked glycosylation. CIRp-A6 has two predicted sites, and species with one or two occupied sites were observed (**Figure 4.17**). When treated with PNGase F, the protein mostly had one N-linked glycosylation site occupied, consistent with a single band being observed on the gel.

The results are summarised in **Table 4.2**. In summary, native mass spectrometry analyses showed that all proteins were heterogeneously glycosylated, and that PNGase F-treated CIRp-A2 and PNGase F-treated CIRp-A6 remained glycosylated. To purify proteins better suited for crystallisation, these N-linked glycosylation sites could be mutated to glutamine residues. The N-glycosylation processing inhibitor kifunensine has also been used in S2 cells; the use of kifunensine in cell cultures could lead to a much more homogeneous protein sample. Alternatively, CIRp-A1 and homologs could be expressed in bacteria.

Table 4.2. Native mass spectrometry analysis of CIRp-A1 and homologs. CIRp-A1 and homologs were analysed by native mass spectrometry to investigate the extent of glycosylation. CIRp-A2 and CIRp-A6 treated with PNGase F were also analysed to investigate the effect of deglycosylation.

Protein	Number of predicted N-linked glycosylation sites	Native mass spectrometry analysis
CIRp-A1	5	Glycosylation on 3/4/5 sites
CIRp-A2	4	Glycosylation on 2/3/4 sites
CIRp-A2 treated with PNGase F		Glycosylation on 2/3 sites
CIRp-A3	9	Species with glycosylation on 7 sites abundant, possibly 8/9 sites
CIRp-A4	4	Glycosylation on 1/2/3/4 sites
CIRp-A5	4	Glycosylation on 3/4 sites, possibly 5 sites
CIRp-A6	2	Glycosylation on 1/2 sites
CIRp-A6 treated with PNGase F		Glycosylation on 1 site

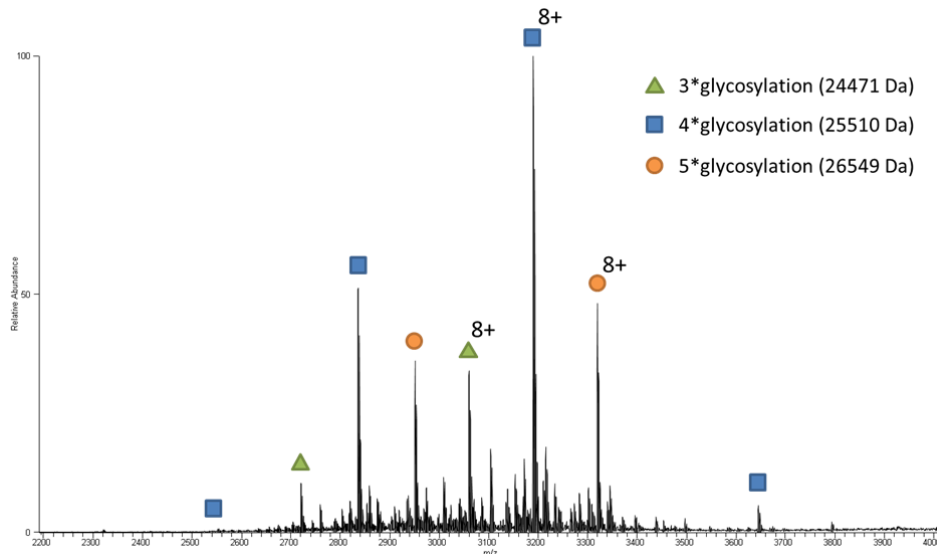


Figure 4.12. Native mass spectrometry analysis of insect-purified CIRp-A1. Native mass spectrometry was performed on a Q Exactive. CIRp-A1 has a theoretical mass of 21015.14 Da with disulphides. The different species of CIRp-A1 showed mass differences of around 1040 Da. As this mass corresponds to the N-linked glycan paucimannose, these species were considered to be species with different numbers of N-linked glycosylation sites occupied. CIRp-A1 has five predicted N-linked glycosylation sites, and species with three, four or five occupied sites were observed.

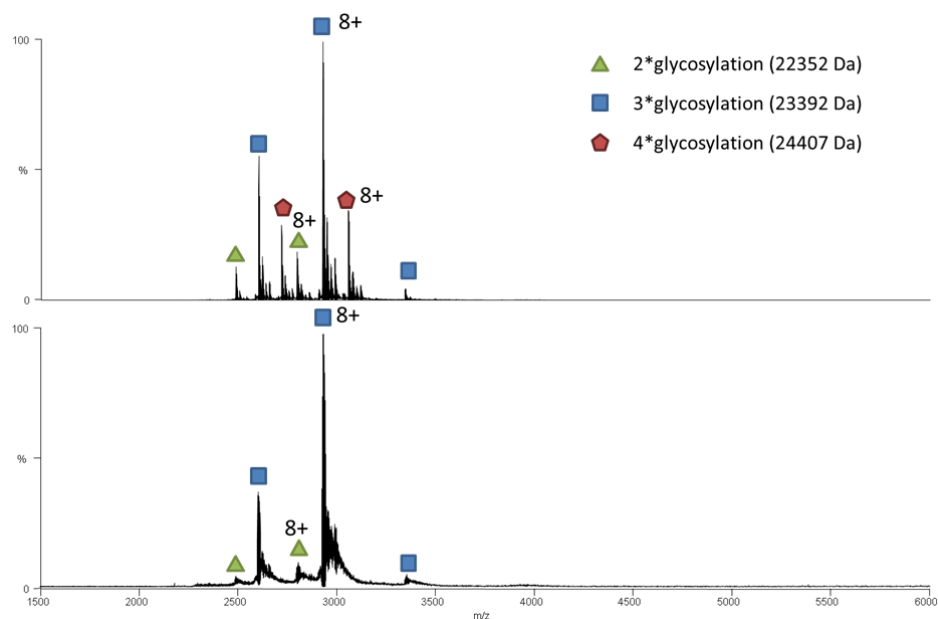


Figure 4.13. Native mass spectrometry analysis of insect-purified CIRp-A2. Native mass spectrometry was performed on a Q Exactive. The spectra of CIRp-A2 (above) and PNGase F-treated CIRp-A2 (below) are shown. CIRp-A2 has a theoretical mass of 20059.07 Da with disulphides. Native mass spectrometry data were analysed as in **Figure 4.12**. CIRp-A2 has four predicted N-linked glycosylation sites, and species with two, three or four occupied sites were observed (above). PNGase F-treated CIRp-A2 had two or three sites occupied (below).

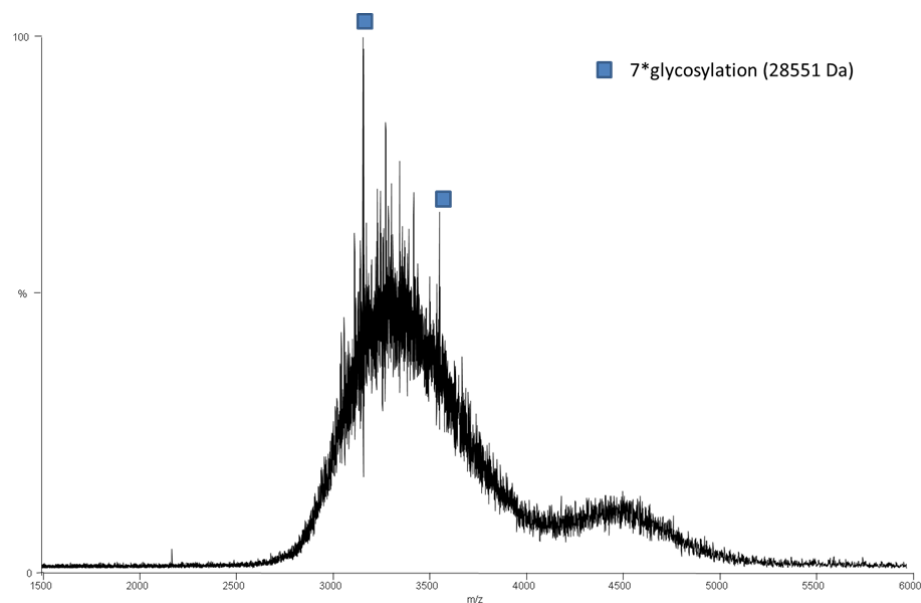


Figure 4.14. Native mass spectrometry analysis of insect-purified CIRp-A3. Native mass spectrometry was performed on a Q Exactive. CIRp-A3 has a theoretical mass of 21022.09 Da with disulphides. The resolution of the native mass spectrometry data for CIRp-A3 was low, but the mass of the most abundant species was 28551 Da, corresponding to seven occupied N-linked glycosylation sites.

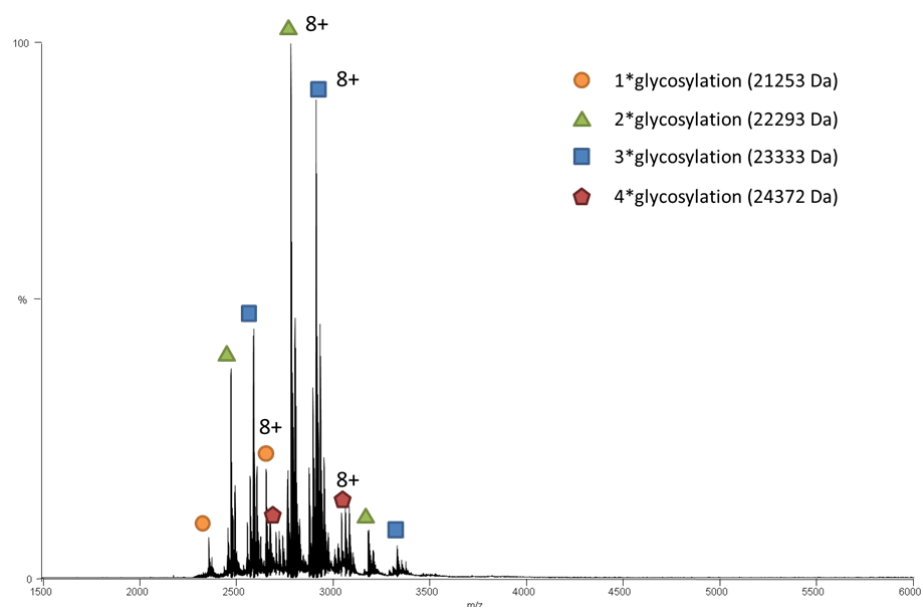


Figure 4.15. Native mass spectrometry analysis of insect-purified CIRp-A4. Native mass spectrometry was performed on a Q Exactive. CIRp-A4 has a theoretical mass of 19853.82 Da with disulphides. Native mass spectrometry data were analysed as in **Figure 4.12**. CIRp-A4 has four predicted N-linked glycosylation sites, and species with one, two, three or four occupied sites were observed.

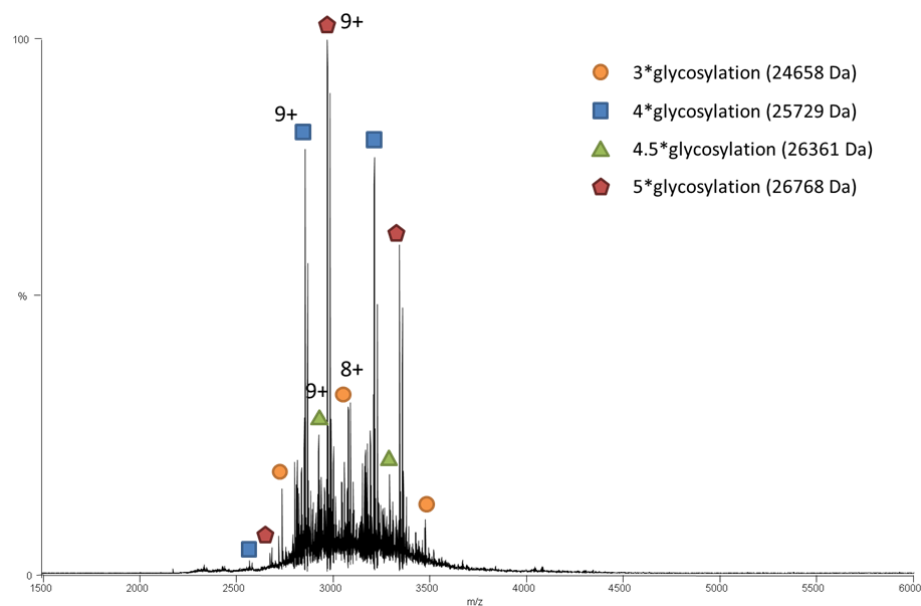


Figure 4.16. Native mass spectrometry analysis of insect-purified CIRp-A5. Native mass spectrometry was performed on a Q Exactive. CIRp-A5 has a theoretical mass of 21202.34 Da with disulphides. Native mass spectrometry data were analysed as in **Figure 4.12**. CIRp-A5 has four predicted N-linked glycosylation sites, and species with three, four or five occupied sites were observed. The species with a mass between four and five occupied sites was likely due to O-linked glycosylation.

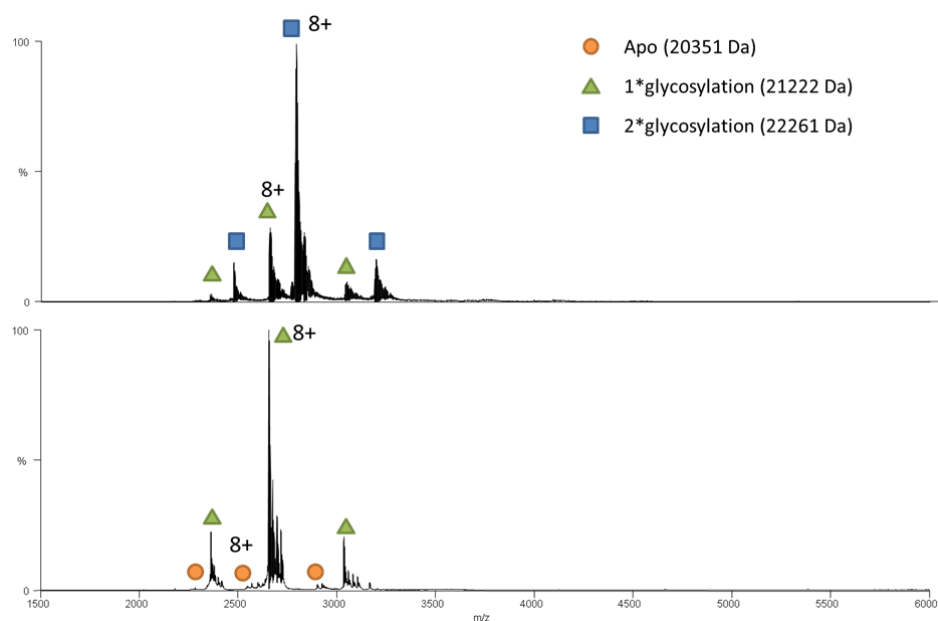


Figure 4.17. Native mass spectrometry analysis of insect-purified CIRp-A6. Native mass spectrometry was performed on a Q Exactive. The spectra of CIRp-A6 (above) and PNGase-F-treated CIRp-A6 (below) are shown. CIRp-A6 has a theoretical mass of 20330.51 Da with disulphides. Native mass spectrometry data were analysed as in **Figure 4.12**. CIRp-A6 has two predicted N-linked glycosylation sites, and species with one or two occupied sites were observed. PNGase F-treated CIRp-A6 had one site occupied.

4.3 Complement inhibition by CIRp-A1 and homologs

4.3.1 WIESLAB assays and C3a/C5a measurements

To investigate the complement inhibitory function of CIRp-A1, commercially available WIESLAB complement assays (Euro Diagnostica) were performed (Seelen et al., 2005). These are enzyme-linked immunosorbent assay (ELISA)-based complement activation assays, where specific activators of each pathway are used to coat the plates. Lipopolysaccharide (LPS), IgM and mannan are used for the AP, CP and LP, respectively. The buffers used in the assays contain different ions that are required by the different pathways. The CP and LP require both calcium and magnesium, whereas the AP only requires magnesium. Therefore, calcium chelator EGTA is used to selectively obtain AP-specific assay. Anti-C1q antibody which inhibits the CP is used to distinguish between the LP and CP (Seelen et al., 2005). Complement activation is measured with a phosphatase-labelled antibody to the MAC. According to the manufacturer's instructions, serum dilution of 1:18, 1:101 and 1:101 were used for the AP, CP and LP, respectively.

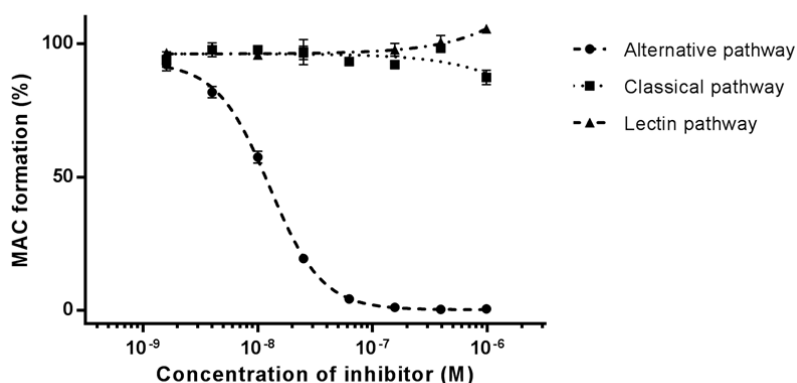


Figure 4.18. Inhibition of complement pathways by CIRp-A1 measured in WIESLAB assays using human serum. CIRp-A1 specifically inhibited the alternative pathway. Values were normalised for no-serum samples (0% MAC formation) and no-additive samples (100% MAC formation). Error bars, s.e.m. (n = 3). Curve fitting was carried out in GraphPad Prism using a dose response inhibition (variable slope) model. The obtained IC₅₀ value for CIRp-A1 in the alternative pathway was 1.3×10^{-8} M (95% CI = 1.15 - 1.37×10^{-8} M; R square = 0.999).

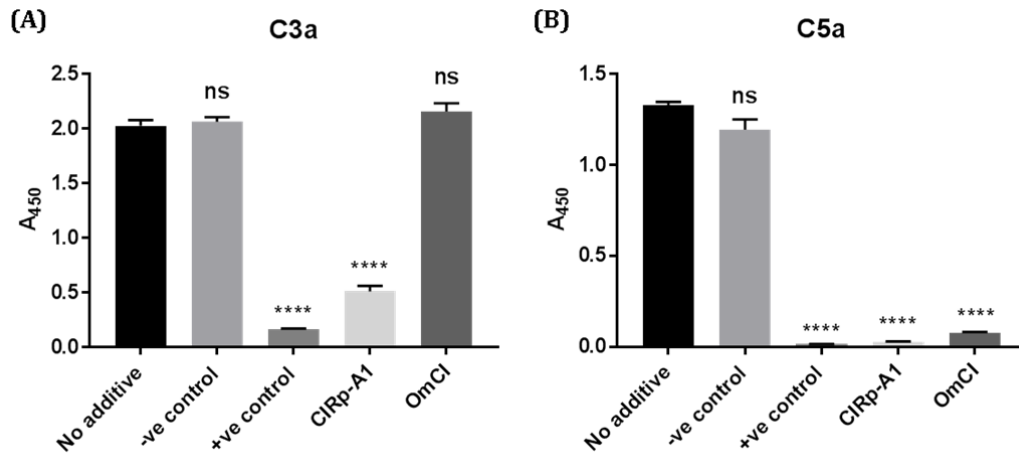


Figure 4.19. C3a and C5a levels in the supernatants of the alternative pathway WIESLAB assay. The levels of (A) C3a and (B) C5a in the supernatants of the alternative pathway WIESLAB assay were measured. CIRp-A1 inhibited C3a and C5a formation through the alternative pathway. The negative control was the functionally unrelated Ra-HBP2 and the positive control was EDTA. All proteins were added at a final concentration of 1 μ M. Values were baseline-corrected for buffer-only samples. Error bars, s.d. (n = 3 WIESLAB samples). ns (not significant); ****P < 0.001 by unpaired two-tailed t-test, with no-additive sample as the reference.

CIRp-A1 specifically inhibited the alternative pathway, but had no effect on the classical and lectin pathways under the assay conditions (**Figure 4.18**). When CIRp-A1 was identified in the lab, it was shown to pull down properdin from serum (Jore, unpublished). It has been shown before that properdin is essential for LPS-induced AP activation (Kimura et al., 2008). CIRp-A1 had no effect on the classical and lectin pathways, even at higher concentrations, which has also been observed with other properdin-binding tick complement inhibitors (Couvreur et al., 2008; Valenzuela et al., 2000), or when using properdin-deficient serum (Kimura et al., 2008).

To determine whether CIRp-A1 acts upstream or downstream of C3 cleavage, the supernatants from the alternative pathway WIESLAB assay were used to assess the formation of C3a and C5a. OmCI, a well-studied C5-binding tick complement inhibitor, was used as a control in the assays. For CIRp-A1 and OmCI, the supernatants from the wells containing the inhibitors at the highest concentration (final concentration of 1 μ M) were used. CIRp-A1 significantly inhibited the formation of both C3a and C5a via the alternative pathway (**Figure 4.19**). In comparison, the C5 inhibitor OmCI had no effect on C3a formation, but inhibited C5a formation. Therefore, CIRp-A1 acts at the point of

C3 cleavage. C3a formation was significantly reduced, but not completely abolished (**Figure 4.19A**), consistent with the notion that properdin is only stabilising the C3 convertase, but is not essential. Compared to the no-additive sample, C3a formation was reduced by 75% when CIRp-A1 was added. Reduced C3 cleavage would affect C3b deposition and the formation of new C3 convertase complexes on the surface, thereby limiting the amplification loop. C5 convertase activity is known to be very sensitive to the density of deposited C3b (Berends et al., 2015), hence without sufficient C3b deposition, the formation of C5 convertase complexes and therefore C5 cleavage would be affected. This could explain why C5a formation was completely abolished when CIRp-A1 was added (**Figure 4.19B**). The final readout of the WIESLAB assay is MAC formation. Although some C3 cleavage occurred, the complete inhibition of C5 cleavage would lead to the inhibition of MAC formation. This could be why CIRp-A1 was observed to completely inhibit the alternative pathway in the WIESLAB assay.

4.3.2 Haemolysis assays using different animal sera

Haemolysis assays measure complement-mediated lysis of red blood cells. CP activity is assayed using antibody-coated sheep red blood cells in buffer containing calcium and magnesium. AP activity is assayed using rabbit or guinea pig red blood cells, which activate the AP, in buffer containing magnesium. The CP and LP are blocked in AP haemolysis assays by adding EGTA to chelate calcium (Harboe et al., 2011). Testing for the alternative pathway in the presence of CIRp-A1 and homologs was performed using rabbit red blood cells. CIRp-A1 and homologs showed different complement inhibitory activity profiles in AP haemolysis assays using different animal sera – human, monkey, rat and guinea pig (**Figure 4.20**). Properdin from the animals that I tested share above 70% amino acid sequence identity with each other (**Table 4.4**). CIRp-A1 and homologs share above 40% amino acid sequence identity with each other (**Table 4.5**).

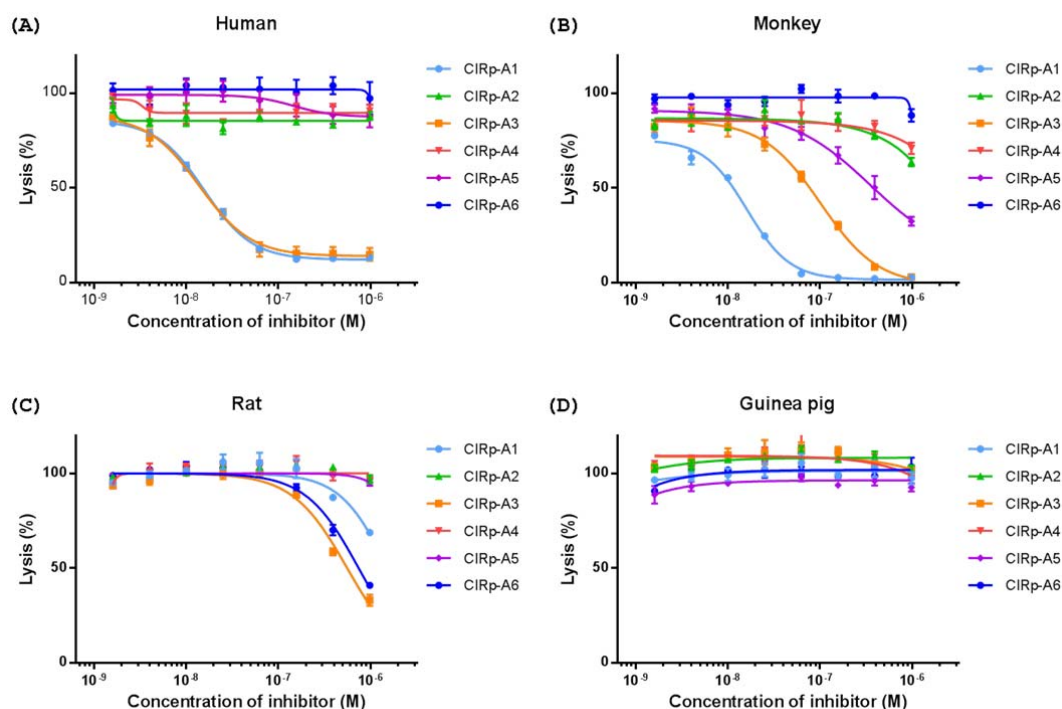


Figure 4.20. Haemolysis assays with CIRp-A1 and homologs using different animal sera. Serum from (A) human, (B) monkey, (C) rat, and (D) guinea pig were tested. Human, monkey, rat and guinea pig sera were diluted 1:5, 1:6, 1:3 and 1:5, respectively. Values were normalised for no-serum samples (0% lysis) and no-additive samples (100% lysis). Error bars, s.e.m. (n = 3). Curve fitting was carried out in GraphPad Prism using a dose response inhibition (variable slope) model.

Table 4.3. Inhibition parameters of CIRp-A1 and homologs in AP haemolysis assays. Curve fitting using a dose response inhibition (variable slope) model and statistical analyses were performed using GraphPad Prism to obtain IC₅₀ values. 95% CI represents the 95% confidence intervals for the obtained IC₅₀ values. R square is a statistical measure of how close the data are to the fitted curve.

Animal	Animal	IC ₅₀ (M)	95% CI (M)	R square
Human	CIRp-A1	1.6 x 10 ⁻⁸	1.39 x 10 ⁻⁸ to 1.77 x 10 ⁻⁸	0.999
Human	CIRp-A3	1.4 x 10 ⁻⁸	9.82 x 10 ⁻⁹ to 1.80 x 10 ⁻⁸	0.997
Monkey	CIRp-A1	1.6 x 10 ⁻⁸	1.12 x 10 ⁻⁸ to 2.14 x 10 ⁻⁸	0.995
Monkey	CIRp-A3	1.0 x 10 ⁻⁷	7.74 x 10 ⁻⁸ to 1.40 x 10 ⁻⁷	0.997
Monkey	CIRp-A5	3.5 x 10 ⁻⁷	2.13 x 10 ⁻⁷ to 6.19 x 10 ⁻⁷	0.993
Rat	CIRp-A3	5.6 x 10 ⁻⁷	4.55 x 10 ⁻⁷ to 6.95 x 10 ⁻⁷	0.977
Rat	CIRp-A6	7.4 x 10 ⁻⁷	6.35 x 10 ⁻⁷ to 8.84 x 10 ⁻⁷	0.984

Table 4.4. Percentage identity matrix of amino acid sequences of properdin from different animals.

	Human	Monkey	Rat	Guinea pig
Human	100	93	78	76
Monkey	93	100	80	75
Rat	78	80	100	73
Guinea pig	76	75	73	100

Table 4.5. Percentage identity matrix of amino acid sequences of CIRp-A1 and homologs.

	CIRp-A1	CIRp-A2	CIRp-A3	CIRp-A4	CIRp-A5	CIRp-A6
CIRp-A1	100	43	48	51	82	45
CIRp-A2	43	100	43	47	45	44
CIRp-A3	48	43	100	56	56	42
CIRp-A4	51	47	56	100	71	42
CIRp-A5	82	45	56	71	100	47
CIRp-A6	45	44	42	42	47	100

CIRp-A1 was active in human and monkey, which was unsurprising since human and monkey properdin share 93% amino acid sequence identity. CIRp-A3 was the only homolog which was active in human; CIRp-A1 and CIRp-A3 share 43% sequence identity. CIRp-A3 was also active in monkey and rat. It is interesting that CIRp-A5 was active in monkey, but inactive in human, despite CIRp-A1 and CIRp-A5 sharing 82% sequence identity. Since CIRp-A1 and CIRp-A5 are identical from the N-terminus up to residue 119, a proportion of the amino acids required for the inhibitory activity of CIRp-A1 likely lie in the region 120-176. The two homologs which were active in rat – CIRp-A3 and CIRp-A6 – share 44% sequence identity. The homologs which did not show complement inhibitory activity in any of the animals tested in this thesis – CIRp-A2 and CIRp-A4 – could be active in some other animals. In summary, there does not seem to be a simple correlation between sequence identity and activity. The regions which are not conserved among the homologs include the loop regions between secondary structural elements (as predicted by HHPred). These regions could confer host specificity. Structural studies of CIRp-A1 and homologs would be needed to address these hypotheses.

4.3.3 The effect of CIRp-A1 on the alternative pathway in the presence of a gain-of-function factor B mutant

Since properdin is known to stabilise the AP C3 convertase (Fearon and Austen, 1975), the ability of CIRp-A1 to inhibit a pre-stabilised C3 convertase complex was investigated. To do this, a “gain-of-function” factor B mutant was used which would boost AP-driven amplification. FB (D279G) mutant was provided by the lab of Santiago Rodriguez de Cordoba. This mutant has a 5-fold increase in C3b binding affinity, and forms a C3

convertase with a longer half-life and increased resistance to decay by complement regulators (Goicoechea de Jorge et al., 2007; Hourcade et al., 1999).

To test the effect of CIRp-A1 on AP activity in the setting of the FB (D279G) mutant, AP WIESLAB assays were performed using serum dilution of 1:18. CIRp-A1 was added to serum first and incubated for 5 min to allow it to bind to properdin, and then the mixture was added to the wells containing FB (D279G) mutant or control (PBS). Taking serum properdin concentration to be 5 µg/ml, the final properdin concentration in the wells was 5 nM. CIRp-A1 was added at a final concentration of 1 µM to ensure sufficient inhibition of properdin. FB (D279G) mutant was added at four different concentrations: 4.5 nM, 9 nM, 22.5 nM and 45 nM. If taking serum FB concentration to be 200 µg/ml, the final wild-type FB concentration in the wells was 118 nM.

As expected, CIRp-A1 completely inhibited MAC formation in normal serum. At lower concentrations of the FB mutant (4.5 nM and 9 nM), CIRp-A1 completely inhibited MAC formation. At higher concentrations of the FB mutant (22.5 nM and 45 nM), CIRp-A1 reduced but did not completely inhibit MAC formation (**Figure 4.21**). The latter results are similar to the initial study describing the FB (D279G) mutant: FB (D279G) mutant had 30–40% maximal haemolytic activity in the absence of properdin, whereas the wild-type FB was dependent on properdin for haemolytic activity (Hourcade et al., 1999).

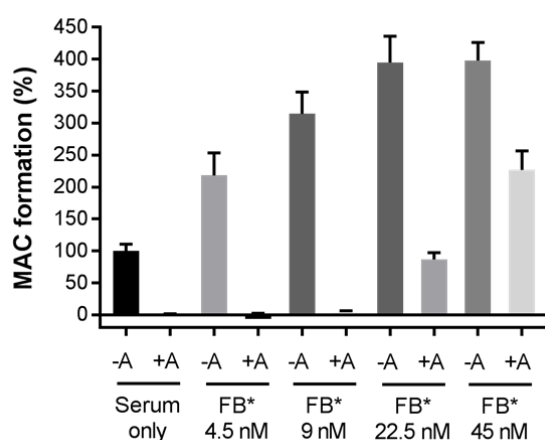


Figure 4.21. The effect of CIRp-A1 on AP activity in the presence of a gain-of-function FB mutant (D279G) as measured in AP WIESLAB assay. FB* denotes FB (D279G) mutant and A denotes CIRp-A1. AP activity was measured when the FB mutant was added to serum at different concentrations, in the absence (-A) or presence (+A) of CIRp-A1. CIRp-A1 was added at a final

concentration of 1 μ M. Values were normalised for no-serum samples (0% MAC formation) and no-additive samples (100% MAC formation). Error bars, s.d. (n = 3).

It is unlikely that properdin is essential for AP initiation as a pattern recognition molecule as this would have resulted in the complete inhibition of MAC formation at all concentrations of the FB mutant. In the absence of CIRp-A1, MAC formation increased with increasing amounts of the FB (D279G) mutant, compared to normal serum. This seems to suggest that the C3 convertase-stabilising ability of the FB (D279G) mutant increased the rate of amplification, leading to increased MAC formation. In this sense, the FB (D279G) mutant could be mimicking the function of properdin as a positive regulator. Perhaps there is some threshold of C3 convertase activity that is required to “activate” the alternative pathway. At lower concentrations of the FB mutant, both the FB mutant and properdin were required to reach this threshold of C3 convertase activity which will ramp up amplification and drive MAC formation. FB mutant alone was not sufficient, as CIRp-A1 inhibited this process. On the other hand, at higher concentrations of the FB mutant, the FB mutant alone could reach this threshold of C3 convertase activity, without properdin.

4.4 Discussion

In this chapter, I discussed initial structural and functional characterisations of the properdin-binding complement inhibitor CIRp-A1 and its homologs. These proteins were purified from insect cells for CD and native mass spectrometry analyses, which revealed that they consist of β -sheets (**Section 4.2.2**) and that they are heterogeneously glycosylated (**Table 4.2**). It might be helpful to mutate the N-linked glycosylation sites on the inhibitors or express the inhibitors in bacteria, to purify proteins better suited for crystallisation trials.

CIRp-A1 specifically inhibited the alternative pathway in WIESLAB assays (**Figure 4.18**), and the analyses of C3a and C5a levels in the supernatants from the alternative pathway WIESLAB assay suggested that CIRp-A1 significantly reduced C3a formation via the AP (**Figure 4.19**). I also looked at the effect of CIRp-A1 on AP activity in the presence of a gain-of-function factor B (D279G) mutant in AP WIESLAB assays.

Properdin was required for maximal MAC formation at all concentrations of the FB mutant, since the addition of CIRp-A1 reduced or completely abolished MAC formation (**Figure 4.21**). Below a certain concentration of the FB mutant, properdin was absolutely necessary for AP activity. Above this concentration, the FB mutant alone was sufficient to induce MAC formation without properdin. It seems that at a certain threshold of C3 convertase activity, when stabilised by properdin and/or the FB mutant, amplification occurs at a sufficient rate to “activate” the AP and induce MAC formation. It could be that at a high enough C3 convertase activity, and therefore C3b deposition, amplification occurs faster than C3b breakdown. This idea of the AP being a rate-governed reaction has been discussed in the field (Harrison, 2017; Lachmann, 2018).

The results of the WIESLAB assays are consistent with the well-established role of properdin as a positive regulator of the AP C3 convertase. AP C3 convertase-driven amplification loop is thought to be at the core of all three complement pathways. However, the inhibition or removal of properdin only specifically affects the AP, as I have shown with CIRp-A1 and others with properdin-binding tick proteins and properdin-deficient serum (Couvreur et al., 2008; Kimura et al., 2008; Valenzuela et al., 2000). Therefore, it is unclear how important AP-driven amplification is for the other pathways.

Quantification of AP-driven amplification in a CP-induced system had been attempted by others using ELISA-based CP activation assays. Anti-factor D antibody abolished MAC formation induced by IgM on the surface (Harboe et al., 2004). Authors of the study concluded that amplification by the alternative pathway accounts for more than 80% of CP-induced MAC formation. However, the study did not show that IgM itself does not induce CP-independent AP activity, for example, by using buffer containing EGTA to chelate calcium and block the CP. In another study, the absence of factor B (either by using factor B-depleted serum or using anti-factor B antibody) led to reduced complement activity induced by ovalbumin (OVA)/anti-OVA immune complexes on the surface (Kimura et al., 2008). The authors of the study concluded that AP-driven amplification was responsible for the reduction. However, similar to the study mentioned above, the anti-FB antibody could just be affecting AP activation rather than amplification. Additionally, the highest serum concentration used by (Kimura et al., 2008) was 10%, which might be insufficient for AP activity. In comparison, (Harboe et al., 2004) used serum concentration of 50%. Finally, anti-FD that inhibited ELISA-based CP activation

had no effect on CP haemolysis (Harboe et al., 2004), suggesting that the requirement of AP amplification could depend on the assay used. In conclusion, these two studies highlight the importance of the experimental set up when measuring CP versus AP complement activation, with regards to types of assays and surface activators used, since different surfaces would affect the relative recruitment and binding of complement regulators in serum (e.g. FH). Preliminary experiments were carried out in an attempt to establish assay conditions under which AP-driven amplification can be observed in a CP or LP-induced system (data not shown), with the intention of using CIRp-A1 to investigate the role of properdin in amplification. However, due to time constraints and the complications mentioned above, these experiments were not pursued any further.

Multiple tick species produce anti-complement proteins that bind properdin indicating the importance of properdin in the alternative pathway. Apart from CIRp-A1 and homologs from this thesis, there are the members of the Isac-like protein (ILP) family, which also target properdin. Salp20 is a member of this family from the tick *Ixodes scapularis*. The addition of Salp20 in a haemolysis assay also diminished C3a formation (Tyson et al., 2007), similar to what I have shown with CIRp-A1 (**Figure 4.19**). There is little sequence homology between CIRp-A1 and Salp20. It is possible that CIRp-A1 and Salp20 bind to different regions of properdin via different mechanisms. Another possibility is that, despite the sequence divergence, the proteins adopt a similar fold in the region responsible for inhibitory activity. Structural studies of CIRp-A1 and Salp20 would be required to address these possibilities. The availability of CIRp-A1 and homologs, and other tick proteins which target properdin, provide tools to improve our understanding of properdin biology. In **Chapter 5**, I discuss structural studies of CIRp-A1 and homologs, towards understanding their molecular mechanisms of inhibition and host specificity, as they had different complement inhibitory profiles in different animals (**Figure 4.20**). In **Chapter 6**, I discuss characterisation of these inhibitors' binding sites on properdin, and their effects on properdin ligand binding.

CHAPTER 5: STRUCTURE DETERMINATION OF CIRP-A1 AND HOMOLOGS

5.1 Introduction

Initial structural and functional characterisation of the properdin-binding complement inhibitor CIRp-A1 and its homologs were discussed in **Chapter 4**. CIRp-A1 and homologs displayed different complement inhibitory activities in different animals (**Section 4.3.2**). CIRp-A1 inhibited AP haemolysis by human and monkey sera, which was perhaps not surprising as human and monkey properdin share 93% sequence identity. The differences in activity were not seen to simply correlate with sequence identity: CIRp-A5 which shares 82% sequence identity with CIRp-A1 was not active in human; in comparison, CIRp-A3 which shares 43% sequence identity with CIRp-A1 was active in human. Towards understanding how these proteins bind to and inhibit properdin, and how host specificity arises, I set out to determine their structures. For the initial structural characterisation, CIRp-A1 and homologs had been expressed and purified from insect cells, which resulted in heterogeneously glycosylated proteins (**Section 4.2.3**). As heterogeneity and flexibility introduced by the sugar moieties might hinder crystallisation, CIRp-A1 and homologs were expressed in bacteria and purified by refolding. The structures of CIRp-A1 and three homologs – CIRp-A2, CIRp-A4 and CIRp-A6 – are described in this chapter.

5.2 Expression and purification of CIRp-A1 and homologs in bacterial cells

5.2.1 Purification of CIRp-A1 by refolding

When expressed in insect cells, CIRp-A1 and homologs were heterogeneously glycosylated (Section 4.2.3). To produce material better suited for crystallisation trials, I attempted to purify CIRp-A1 from bacterial cells. Preliminary experiments in the lab showed that no soluble protein was expressed in *E. coli* (Tang, unpublished). Therefore, I attempted to purify it by refolding from inclusion bodies. The gene for CIRp-A1 was codon-optimised for bacterial expression and cloned into pETM-14 using the restriction enzymes NcoI and NotI, to include the cleavable N-terminal 6-His tag of the vector. The cloned plasmid was transformed into *E. coli* BL21 (DE3), the standard expression strain used in the lab. To test whether CIRp-A1 ended up in inclusion bodies, a 1 l culture was grown in LB media at 37 °C. At OD₆₀₀ of 0.6, the culture was induced with 1 mM IPTG and left overnight at 20 °C. After the cells were lysed by homogenisation and centrifuged at 20,000 rpm at 4 °C for 30 min, SDS-PAGE analysis of the supernatant and the cell pellet fractions indicated that there was a lot more CIRp-A1 in the cell pellet than the supernatant (**Figure 5.1A**).

The purification of CIRp-A1 from inclusion bodies by refolding is shown in **Figure 5.1B**. The cell pellet fraction was re-suspended in 40 ml of Buffer A (50 mM Tris, 150 mM NaCl, pH 8, 0.05% (v/v) Tween 20) using a hand-held homogeniser. After incubation on a rotary wheel at 4 °C for 1 h, the re-suspended pellet was centrifuged at 20,000 rpm at 4 °C for 30 min. This wash step was repeated once more. The cell pellet was then re-suspended in 40 ml of Buffer B (8 M urea, 1 mM EDTA, 100 mM Tris, 25 mM DTT, pH 8) for solubilisation of inclusion bodies, and incubated on a rotary wheel at 4 °C for 2 h. Following centrifugation, the supernatant fraction was retained and filtered. For refolding, the supernatant was added dropwise to Buffer C (1 mM cysteine, 2 mM cystine, 20 mM ethanolamine, 1 mM EDTA, pH 11) with stirring. The refolding buffer containing CIRp-A1 was left overnight at 4 °C, and then concentrated down to ~40 ml using a 10 kDa Vivaflow 200 filtration device. Following concentration, CIRp-A1 was cleaved with 3C protease to remove the His tag whilst dialysing against 2 l of Buffer D (50 mM Tris, 150

mM NaCl, pH 8) overnight at 4 °C. Following reverse nickel purification, CIRp-A1 was further purified by SEC in Buffer D (**Figure 5.1C**).

CD analysis was performed with refolded CIRp-A1 to investigate protein folding. CIRp-A1 was dialysed against 10 mM sodium phosphate buffer, pH 8. The CD spectrum of refolded CIRp-A1 overlaid nicely with the CD spectrum of insect-purified CIRp-A1, suggesting the correct formation of protein secondary structure elements (**Figure 5.2A**). Alternative pathway WIESLAB assay confirmed that the refolded CIRp-A1 was functionally active, suggesting that it was refolded correctly (**Figure 5.2B**).

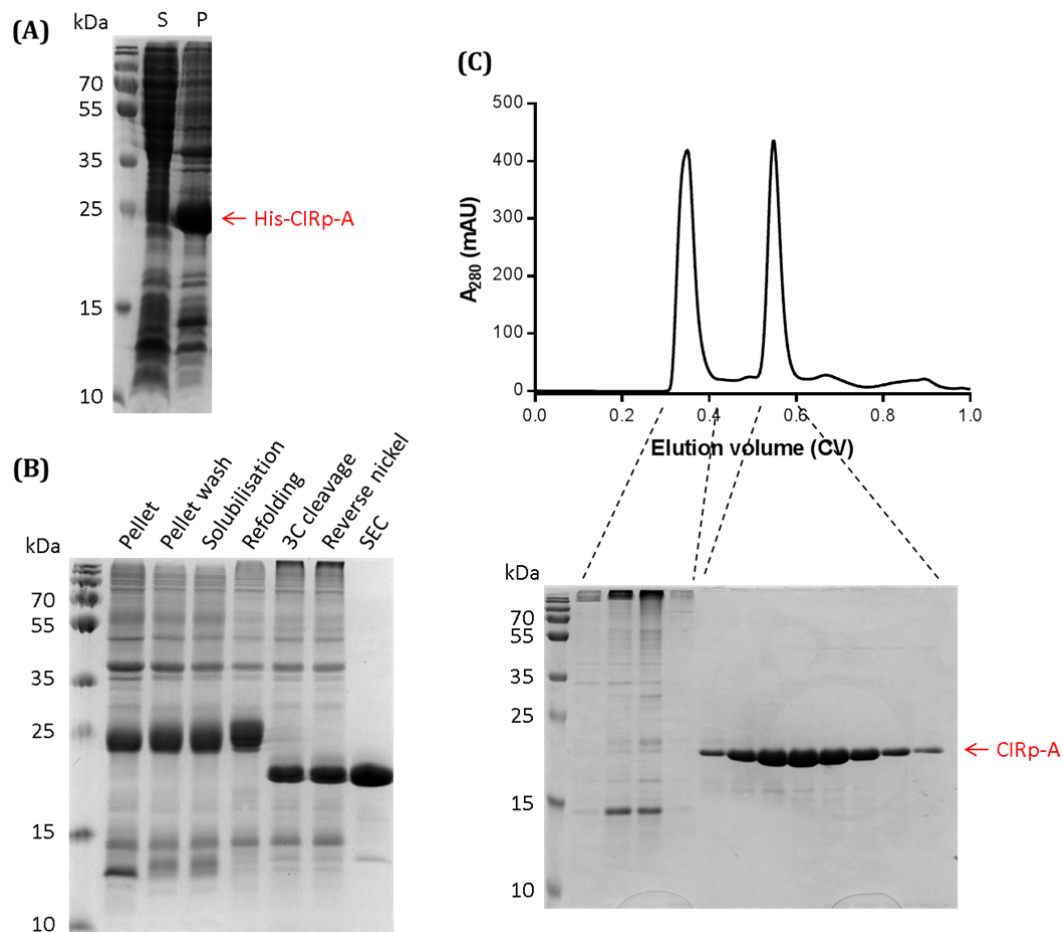


Figure 5.1. Purification of CIRp-A1 by refolding. (A) Reducing 15% SDS-PAGE analysis of the supernatant (S) and pellet (P) fractions after homogenisation of bacterial cells. (B) Reducing 15% SDS-PAGE analysis of CIRp-A1 after each purification step. (C) S75 26/60 size exclusion profile of refolded CIRp-A1 and 15% SDS-PAGE analysis of elution fractions. The running buffer for SEC was 50 mM Tris, 150 mM NaCl, pH 8.

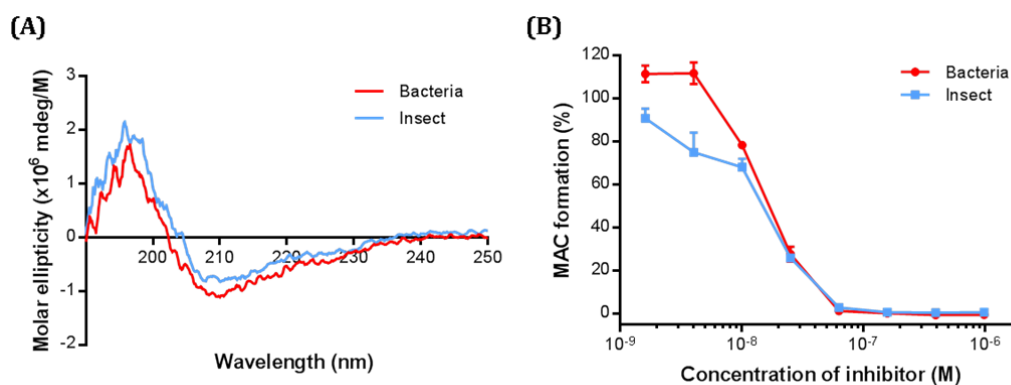


Figure 5.2. Structural and functional analysis of refolded CIRp-A1. (A) CIRp-A1 refolded from bacteria (red) was analysed by CD spectroscopy to assess protein folding and compared with CIRp-A1 expressed in insect cells (blue). The CD buffer was 10 mM sodium phosphate buffer, pH 8. (B) The complement inhibitory activity of CIRp-A1 refolded from bacteria (red) was confirmed by alternative pathway WIESLAB assay using human serum. Insect-purified CIRpA (blue) showed similar activity. Values were normalised for no-serum samples (0% MAC formation) and no-additive samples (100% MAC formation). Error bars, s.e.m. (n = 3).

5.2.2 Purification of CIRp-A1 homologs by refolding

The genes for the homologs were codon-optimised for bacterial expression and cloned into pET15b vector using the restriction enzymes NcoI and XhoI. For CIRp-A1, pETM-14 vector was used for its cleavable His tag. However, the affinity tag was deemed not necessary, as nickel purification was not used for the refolding and purification of CIRp-A1 (**Section 5.2.1**). Therefore, CIRp-A1 homologs were cloned into pET15b without a His tag, which is a standard expression vector used in the lab. The homologs were also present in the cell pellet following homogenisation (data not shown). The homologs were purified using the refolding protocol for CIRp-A1, as outlined in **Section 5.2.1**.

For the homologs CIRp-A2, CIRp-A4 and CIRp-A5, the proteins were still impure following SEC. They were further purified by ion exchange chromatography using a 1 ml Mono Q 5/50 column. The proteins were first dialysed against 50 mM Tris, 20 mM NaCl, pH 8.5, overnight at 4 °C. After loading the sample onto the column and washing, the protein was eluted by gradient elution: 50 mM NaCl to 250 mM NaCl over 50 CV, and then 250 mM NaCl to 1 M NaCl over 10 CV. Purification of CIRp-A4 is shown in **Figure 5.3** as an example; CIRp-A2 and CIRp-A5 were purified in the same way (data not shown).

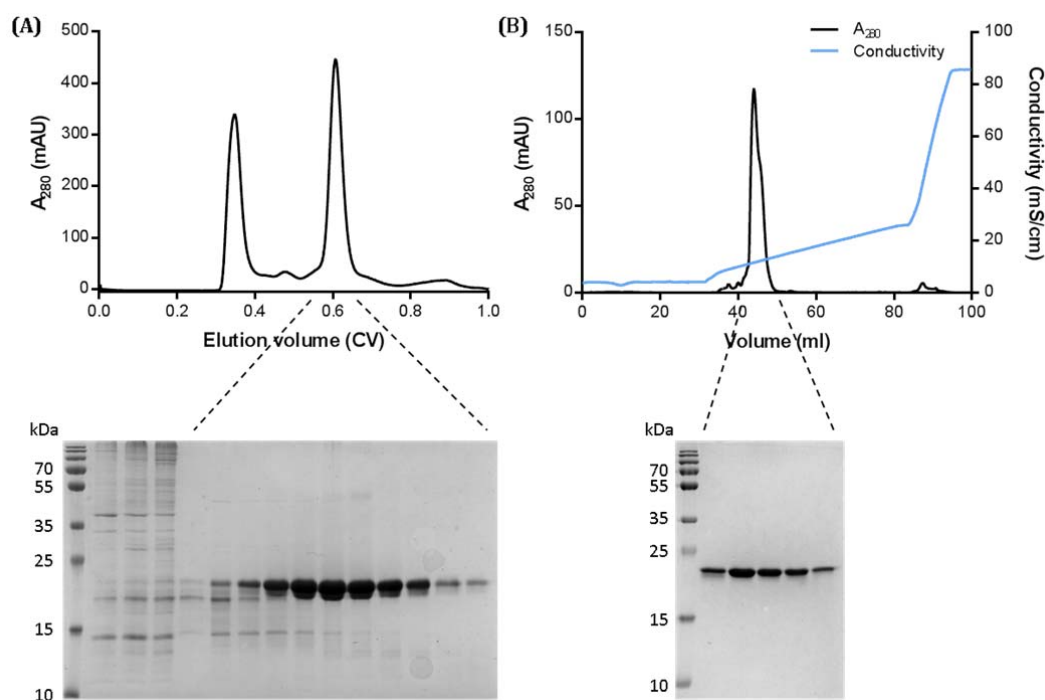


Figure 5.3. Purification of CIRp-A4 by refolding. (A) S75 26/60 size exclusion profile of refolded CIRp-A4 and reducing 15% SDS-PAGE analysis of elution fractions. The running buffer for SEC was 50 mM Tris, 150 mM NaCl, pH 8. (B) Following SEC, CIRp-A4 was further purified by ion exchange chromatography using a Mono Q 5/50 column. Elution fractions were analysed by reducing 15% SDS-PAGE.

When the homolog CIRp-A6 was purified by refolding and SEC, it resulted in multiple species, when analysed by SDS-PAGE (**Figure 5.4A**). Ion exchange chromatography was performed using a 1 ml Mono Q 5/50 column to separate these species. The sample was dialysed against 50 mM Tris, 20 mM NaCl, pH 8.5, overnight at 4 °C. After loading the sample onto the column and washing, the protein was eluted by gradient elution from 20 mM NaCl to 1 M NaCl over 20 CV. As shown in **Figure 5.4B**, the sample consisted of two bands before loading onto the Mono Q column. Some of the lower band could be separated by ion exchange chromatography, as the flow-through consisted of a single band. However, the elution fractions were still a mixture of the two species. Multiple rounds of purification by ion exchange chromatography or elution using a lower gradient might be needed to completely separate the two species. Since the flow-through fraction off the Mono Q column consisted of a single band, this sample was used for CD analysis and subsequent crystallisation trials.

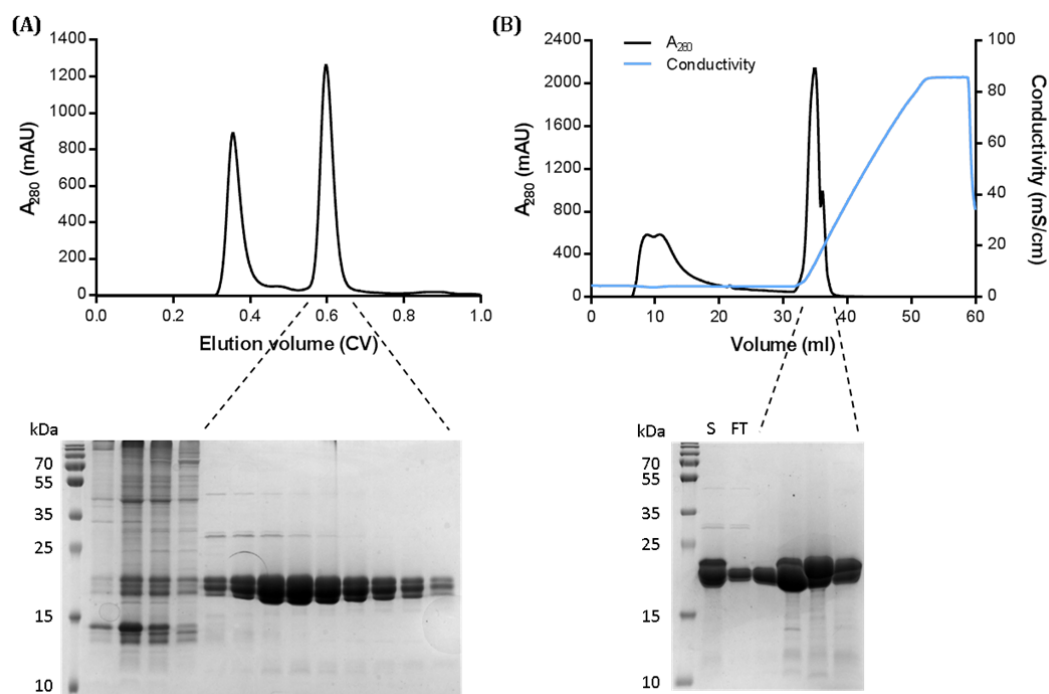


Figure 5.4. Purification of CIRp-A6 by refolding. (A) S75 26/60 size exclusion profile of refolded CIRp-A6 and reducing 15% SDS-PAGE analysis of elution fractions. The running buffer for SEC was 50 mM Tris, 150 mM NaCl, pH 8. (B) CIRp-A6 was further purified by ion exchange chromatography using a Mono Q 5/50 column. The sample loaded onto the column (S), flow-through (FT) and elution fractions were analysed by reducing 15% SDS-PAGE.

CIRp-A3 was the only homolog that could not be refolded using the same protocol as CIRp-A1. As shown in **Figure 5.5**, all proteins eluted near the void volume, and there was no peak at ~0.6 CV where the other refolded homologs were eluted. Since it eluted in the void volume, it was likely refolded incorrectly and aggregated. I also tried refolding with the addition of 500 mM arginine in Buffer C (refolding buffer), but the SEC profile looked the same as in **Figure 5.5** (data not shown). CIRp-A3 has nine predicted N-linked glycosylation sites, at least seven of which were confirmed to be glycosylated by native mass spectrometry (**Section 4.2.3**). Perhaps the glycans are important for protein folding of CIRp-A3 or mask hydrophobic patches on the surface to enable solubilisation.

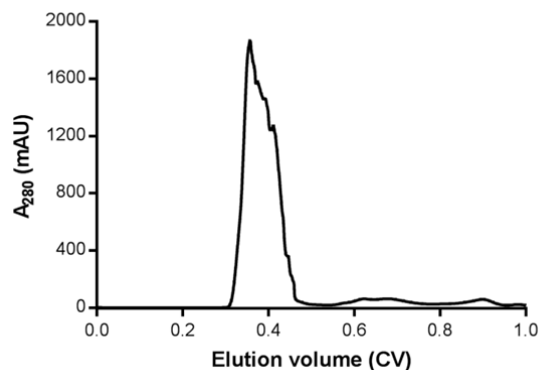


Figure 5.5. SEC analysis of CIRp-A3 purified by refolding. S75 26/60 size exclusion profile of refolded CIRp-A3 indicates that CIRp-A3 was not refolded correctly. The running buffer for SEC was 50 mM Tris, 150 mM NaCl, pH 8.

5.2.3 CD analysis of refolded homologs to assess secondary structure

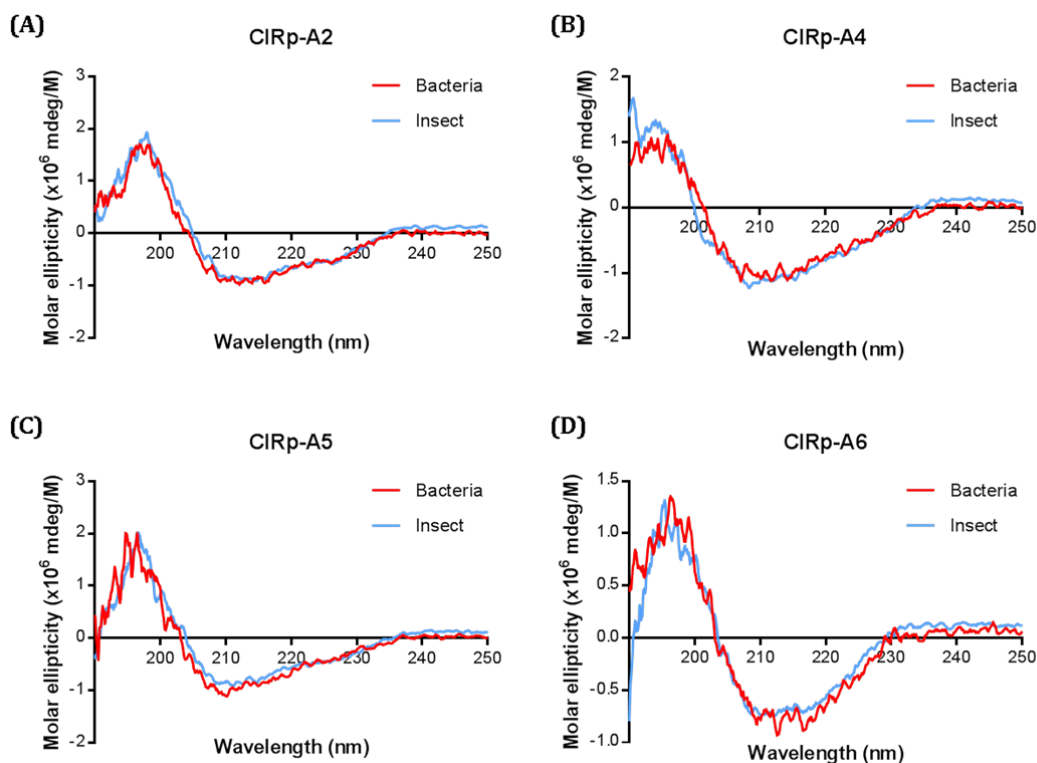


Figure 5.6. CD analysis of refolded homologs. (A) CIRp-A2 (B) CIRp-A4, (C) CIRp-A5 and (D) CIRp-A6, expressed and refolded from bacterial cells, were analysed by CD spectroscopy to assess protein folding. The CD buffer was 10 mM sodium phosphate buffer, pH 7.4. For each homolog, the CD of refolded protein (red) was compared with the CD of insect-purified protein (blue).

The refolded homologs were analysed by CD to assess folding. The proteins were dialysed against 10 mM sodium phosphate buffer, pH 7.4. For plotting the CD spectra, the CD of the refolded protein was corrected for the molar concentration of the corresponding insect-purified protein. For all homologs, the CD spectra of refolded and insect-purified proteins overlaid nicely, indicating the correct formation of protein secondary structure elements (**Figure 5.6**).

5.3 Structure determination of CIRp-A1

5.3.1 Crystallisation trials with CIRp-A1

Crystallisation screens were set up with refolded CIRp-A1, with or without the N-terminal His tag. Crystallisation was carried out using the sitting drop vapour diffusion method. Broad screens were set up with a drop size of 0.3 μ l at 75% (v/v) and 50% (v/v) protein:mother liquor ratios. The drops were left to equilibrate at 21 °C over a well containing 75 μ l of mother liquor. Crystals were observed with cleaved CIRp-A1 (without His tag). Needle clusters grew overnight in 50% (v/v) protein:mother liquor ratio in the condition 0.2 M imidazole malate, pH 6, 30% (w/v) PEG4000 (**Figure 5.7A**). The concentration of CIRp-A1 was 22.5 mg/ml.

Although needles were very promising, they were not of suitable quality for data collection. Trays were set up where the initial crystallisation conditions were optimised with respect to pH and precipitant (PEG4000) concentration. These optimisation trays were set up as shown in **Figure 5.7B**, using CIRp-A1 at 17.8 mg/ml. The stock solutions were 2 M imidazole malate at pH 5.7 and pH 6.4, and 40% (w/v) PEG4000. Trays were incubated at two different temperatures – 21 °C and 10 °C. Plate-like crystals were observed at 21 °C (**Figure 5.7C**), whereas long and thin needles were observed at 10 °C (**Figure 5.7D**).

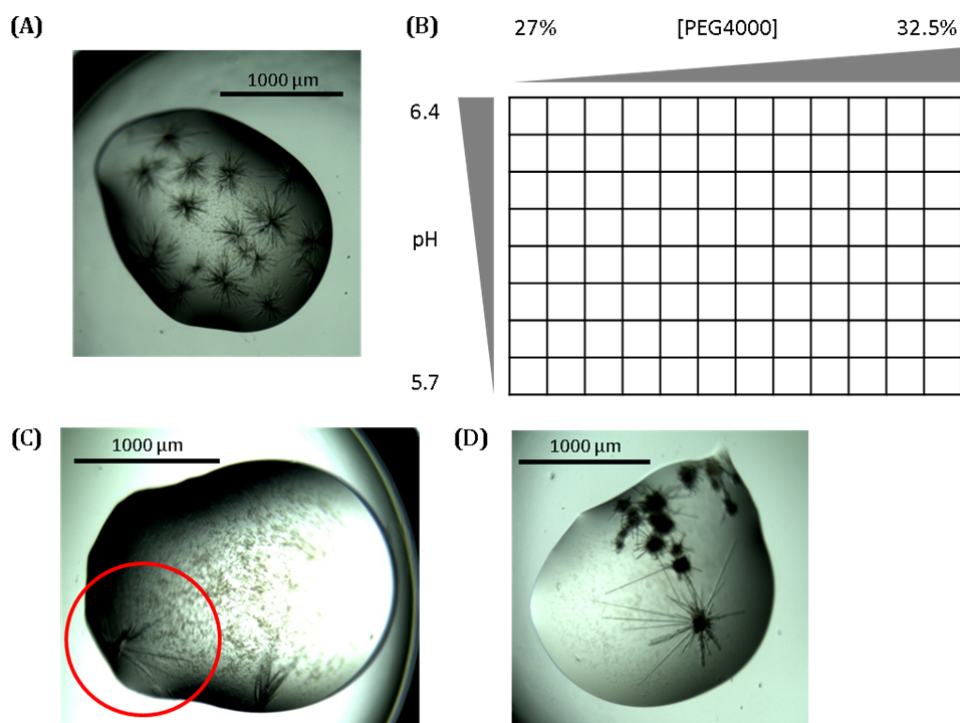


Figure 5.7. Crystallisation trials of CIRp-A1. (A) Initial crystal hit in Stura screen. The drop contained 0.15 μl of CIRp-A1 at 22.5 mg/ml and 0.15 μl of mother liquor (0.2 M imidazole malate, pH 6, 30% (w/v) PEG4000). (B) Optimisation trays were set up based on the initial hit conditions, with respect to pH (vertical) and precipitant concentration (horizontal). (C) Plate-like crystals were obtained in the optimisation tray incubated at 21 $^{\circ}\text{C}$ (highlighted in red circle). (D) Longer needles were obtained in the optimisation tray incubated at 10 $^{\circ}\text{C}$.

5.3.2 CIRp-A1 data collection and processing

The crystals from optimisation trays – both the plate-like crystals (**Figure 5.7C**) and the long needles (**Figure 5.7D**) – were harvested by Prof. Susan Lea for data collection. All crystals were cryoprotected in mother liquor (0.2 M imidazole malate, pH 6, 30% (w/v) PEG4000) supplemented with 20% (v/v) PEG400 and were flash frozen in liquid nitrogen. Data collection was performed by Prof. Simon Newstead at the I04 beamline of Diamond Light Source. Crystals were exposed to an X-ray beam of 0.9795 $\text{\AA}$ wavelength. Three datasets were collected from the plate-like crystals (**Figure 5.7C**). Data were processed by the Diamond automated pipeline using XIA2 in the 3dii or DIALS mode (Winter, 2010). Indexing and integration were performed first, from which the unit cell dimensions and point group were predicted and the intensity for each reflection was determined. This was followed by identification of space group possibilities from the data by POINTLESS (Evans, 2006, 2011). Then, scaling and merging were performed, during which the

intensities of symmetry-related reflections were scaled and averaged to give an I_{hkl} value for each unique reflection. XIA2 3dii uses XDS for indexing and integration (Kabsch, 2010), and XSCALE for scaling and merging (Kabsch, 2010). XIA2 DIALS uses DIALS for indexing and integration (Winter et al., 2018), and AIMLESS for scaling and merging (Evans and Murshudov, 2013). All three datasets were assigned to the orthorhombic $P222_1$ space group with the same unit cell dimensions. The processing statistics are summarised in **Table 5.1**.

Table 5.1. Processing statistics for CIRp-A1 datasets.

	CIRp-A1_1	CIRp-A1_2	CIRp-A1_3
Auto processing	XIA2 3dii	XIA2 3dii	XIA2 DIALS
Space group	$P222_1$	$P222_1$	$P222_1$
Cell dimensions a, b, c (Å) α , β , γ (°)	72.63, 45.74, 57.38 90, 90, 90	72.6, 45.41, 57.96 90, 90, 90	72.6, 45.85, 57.48 90, 90, 90
Resolution	45.02-2.05	45.30-2.18	45.06-1.96
R_{merge} (overall/inner/outer)	0.098/0.030/1.197	0.102/0.043/1.071	0.122/0.044/1.317
R_{pim} (overall/inner/outer)	0.042/0.015/0.496	0.047/0.023/0.476	0.037/0.015/0.515
Unique reflections (overall/inner/outer)	12526/170/909	10469/145/766	14374/205/1047
Multiplicity (overall/inner/outer)	6.4/5.1/6.7	5.6/4.3/5.9	11.4/9.5/7.1
Completeness (overall/inner/outer)	99.9/98.4/99.9	99.7/96.5/99.8	100.0/99.5/100.0
$I/\sigma I$ (overall/inner/outer)	12.0/39.3/1.5	10.7/27.6/1.6	11.5/31.8/1.4
$CC_{1/2}$ (overall/inner/outer)	0.998/0.998/0.704	0.997/0.997/0.632	0.998/0.998/0.571

5.3.3 Structure determination of CIRp-A1 by molecular replacement

In order to determine the likely number of copies of CIRp-A1 in the asymmetric unit, the Matthews Coefficient (Matthews, 1968) and solvent content were calculated in CCP4. Matthews Coefficient of 2.33 and solvent content of 47.35% suggested one copy of CIRp-A1 per asymmetric unit.

The collected dataset only contains the intensity and amplitude for each reflection, and therefore the ‘phase problem’ needs to be solved to generate an electron density map. I obtained the structure solution for CIRp-A1 via molecular replacement (MR), for which a structurally similar model is required as a search probe. The search for structural homologs on the PDB database using the FFAS03 server (Jaroszewski et al., 2005), which performs the search based on fold recognition, returned the protein OmCI as one of the top hits. OmCI is a C5-binding complement inhibitor from the tick *Ornithodoros moubata* (PDB 3ZUI, Roversi et al., 2007) and has a lipocalin-like fold. Additionally, crystal structures of moubatin (Roversi et al., unpublished) and TSGP4 (lab data, unpublished) were used to generate search models. These proteins are also tick lipocalins: moubatin is an inhibitor of platelet aggregation from the tick *O. moubata* (Keller et al., 1993); TSGP4 is an inflammatory modulator from the tick *Ornithodoros savignyi* which scavenges leukotriene C₄, D₄ and E₄ (Mans and Ribeiro, 2008).

Search models for MR were generated using the structures of these proteins. Using OmCI as an example, an initial model was built using Chainsaw in CCP4 (Stein, 2008). The sequence of CIRp-A1 was mapped onto the structure of OmCI, with non-conserved residues pruned to the gamma atom. Chainsaw deleted 3 residues, retained 28 conserved residues and mutated 115 residues (**Figure 5.8A**). Following Chainsaw, the α -helix and some of the loops between the β -sheets were manually removed in Coot (Emsley et al., 2010) to further improve the search model (**Figure 5.8B**). Structures of moubatin and TSGP4 were trimmed in a similar manner to generate search models for MR.

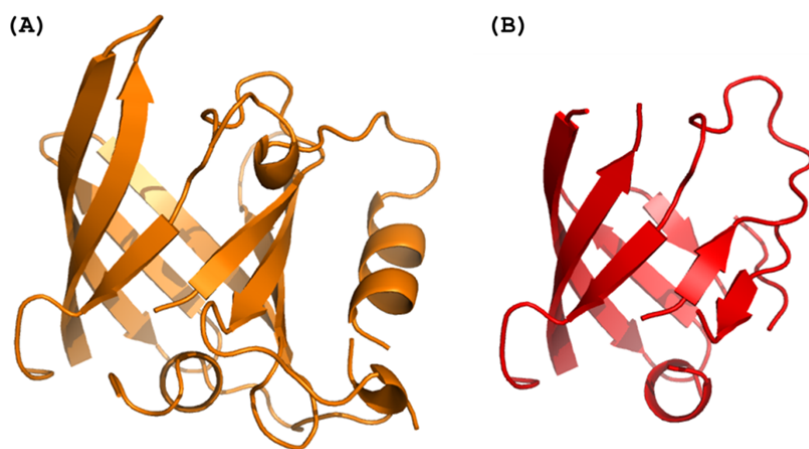


Figure 5.8. Generation of a MR search model for CIRp-A1. (A) The search model was generated with Chainsaw using the structure of OmCI (PDB 3ZUI). (B) The search model was manually edited in Coot to remove the α -helix and some of the loops between the β -sheets. The models were visualised with PyMOL (Version 2.1, Schrödinger, LLC).

MR was carried out using MOLREP in CCP4 (Vagin and Teplyakov, 1997, 2010). It performs a rotational search to orient the search model in the target unit cell and then a translational search to position the oriented model within the unit cell. All solutions are given a score based on the correlation coefficient between the observed intensities and those generated from the solution, and the packing function which describes the steric overlap between molecules during packing. A contrast value is also calculated, which compares a score of a given solution to the average of other solutions. A solution with a contrast value greater than 2.5 is considered highly likely, whereas a value less than 1.3 is considered unlikely since it is not significantly different from the mean (Vagin and Teplyakov, 1997). The top solution from MR using OmCI as the search model had a contrast value of 2.05, indicating a possible solution. MR using moubatin and S116 had lower contrast values of 1.60 and 1.47, respectively (**Table 5.2**).

Table 5.2. Search models used for molecular replacement of CIRp-A1. MR with MOLREP was tried using different search models.

Protein	Sequence identity with CIRp-A1	MOLREP contrast value
OmCI	19%	2.05
Moubatin	16%	1.60
TSGP4	20%	1.47

Another evidence of a correct MR solution is the behaviour of refinement and the electron density. Therefore, the MR solution from OmCI was used for refinement, despite its contrast value being lower than 2.5. To assess the solution from MOLREP, restrained refinement was carried out using REFMAC5 within CCP4 (Murshudov et al., 2011). The refinement process is monitored using two statistical values: R-work and R-free. The R-work value assesses the agreement between the experimental X-ray data and model-derived structure factor amplitudes. With successful refinement, R-work should decrease with each round of model building and refinement. In order to minimise model bias, 5% of reflections are set aside and excluded from refinement, allowing calculation of the R-free value; R-free should be similar to R-work. After initial refinement of CIRp-A1, the R-work and R-free values decreased from 56.20% and 57.54%, to 46.62% and 51.55%, respectively. In addition, the electron density from the refinement and the positive residual densities in the Fo-Fc difference map suggested that the MR solution was correct (**Figure 5.9**).

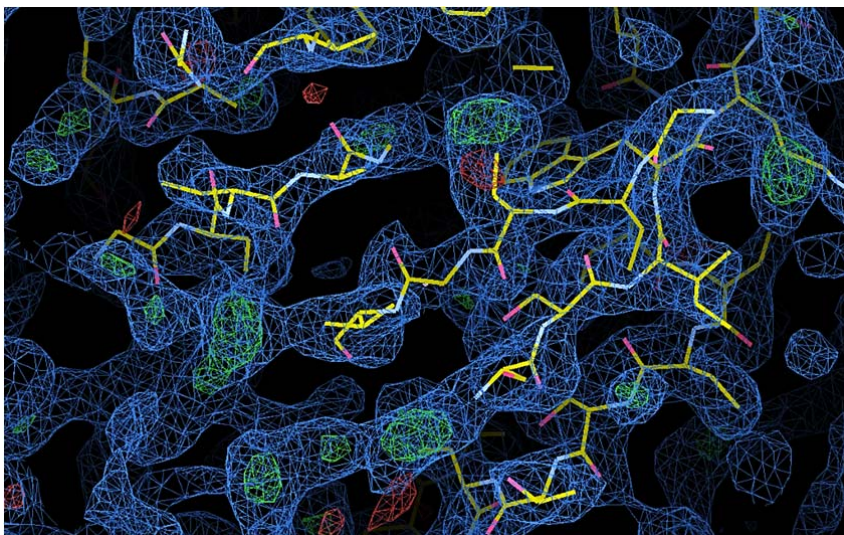


Figure 5.9. Initial refinement of CIRp-A1 structure after a solution was found by MOLREP. The map was viewed in Coot, with the 2Fo-Fc map (blue density) contoured at 1.2 rmsd and the Fo-Fc map (green = positive residual density; red= negative residual density) contoured at 3.0 rmsd. Pockets of residual density could be seen, into which the side chains of CIRp-A1 could be built in.

5.3.4 Refinement of CIRp-A1 structure

The structure of CIRp-A1 was built and refined through iterations of Buccaneer (Cowtan, 2006) and REFMAC5 (Murshudov et al., 2011). Due to the trimming of the search model used for MR, the initial model for CIRp-A1 was incomplete, so automatic tracing of the polypeptide chain into the electron density was attempted using Buccaneer (Cowtan, 2006). Buccaneer performs automated model building using the electron density likelihood function to identify connected C α positions in the electron density map. It builds the C α positions into chain fragments, joins the fragments together, and then docks the input sequence (Cowtan, 2006). To build a model of CIRp-A1, Buccaneer was initially carried out without an input model, using the map from the initial refinement with REFMAC5 after phasing. Following Buccaneer, rebuilding and refinement were carried out with Coot (Emsley et al., 2010) and REFMAC5 (Murshudov et al., 2011), respectively. After a few rounds of Buccaneer, an input model was provided to be extended. In each round of refinement, a new electron density map is calculated using phases from the refined model; this phase improvement results in a more accurate map into which the structure can be more accurately modelled. During rebuilding and refinement, residues were deleted, mutated, rotated or translated, guided by the 2Fo-Fc map and residual densities (**Figure 5.10**). ‘Real Space Refine Zone’ tool within Coot (Emsley et al., 2010) was used after making any changes to correct geometry errors and improve the fit to the map.

Final rounds of refinement were performed using PHENIX (Afonine et al., 2012). The refinement was continued until the final model and the 2Fo-Fc map were in good agreement as visualised in Coot (Emsley et al., 2010), and supported by final R-work and R-free values of 20.79% and 24.26%, respectively. The protein chemistry of the final model was validated using MolProbity (Chen et al., 2010), and the refinement statistics for the final model of CIRp-A1 are summarised in **Table 5.3**.

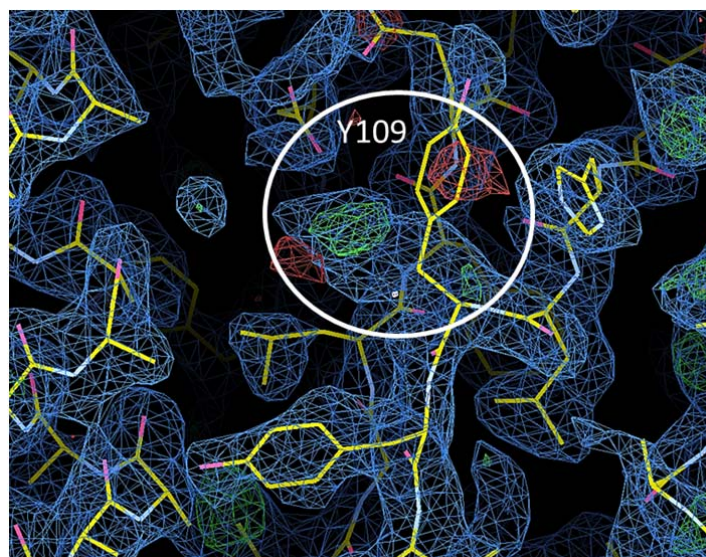


Figure 5.10. Rebuilding and refinement of CIRp-A1 structure. An example of the rebuilding and refinement process for CIRp-A1. The map was viewed in Coot, with the 2Fo-Fc map (blue density) contoured at 1.0 rmsd and the Fo-Fc map (green = positive residual density; red= negative residual density) contoured at 3.0 rmsd. The positive and negative residual densities around the side chain direct the correct orientation of Y109.

Table 5.3. Refinement statistics for the final model of CIRp-A1.

	CIRp-A1
Residues seen in structure	E21-Q176
Resolution range	45.08-1.96 (2.11-1.96)
R-work (%)	20.79 (28.48)
R-free (%)	24.26 (29.87)
Ramachandran outliers (%)	0.0
Ramachandran favoured (%)	96.8
Rotamer outliers (%)	0.0
Clashscore	6.92
MolProbity score	1.58

5.3.5 Crystal structure of CIRp-A1

The structure of CIRp-A1 reveals a lipocalin-like fold (**Figure 5.11A**). Lipocalins are characterised by an eight-stranded anti-parallel β -barrel and a C-terminal α -helix. Lipocalins are highly represented in tick salivary glands (Beaufays et al., 2008). As observed in the structures of other lipocalins (Adam et al., 2008; Paesen et al., 1999), the helix of CIRp-A1 is connected to the barrel by a disulphide bond (C121-C156) while another disulphide bond links the most C-terminal cysteine residue to the loop between the first two β -strands of the barrel (C51-C179).

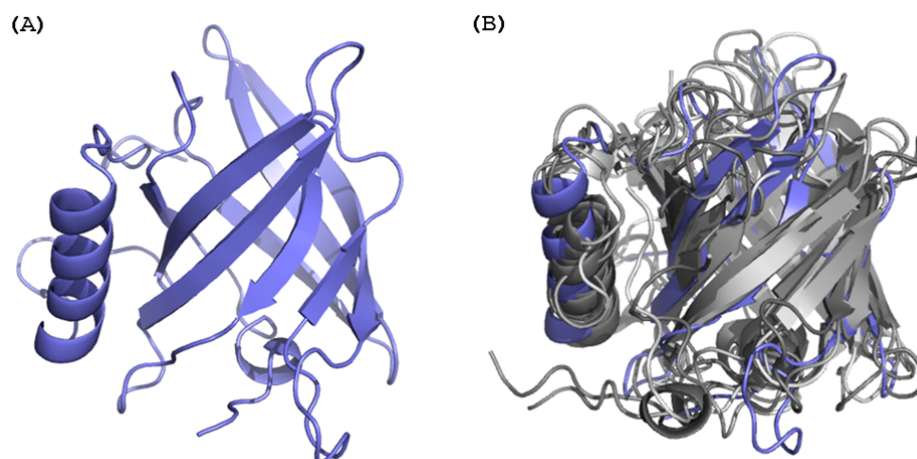


Figure 5.11. Crystal structure of CIRp-A1. (A) The final model of CIRp-A1 in cartoon representation shows a lipocalin-like fold, characterised by an eight-stranded anti-parallel β -barrel and an α -helix. (B) The crystal structure of CIRp-A1 (purple) was compared with crystal structures of the top five results from the PDB25 search on the Dali server (Holm, 2019). Structural alignment shows that the overall protein fold is conserved between these proteins. In decreasing shades of grey are OmCI (PDB 5HCC), monotonin (PDB 3BRN), HBP (PDB 1QFT), E-RABP (PDB 1EPA) and Lili-Mip (PDB 4NYQ). Details about these proteins are outlined in Table 5.4. Images were produced with PyMOL (Version 2.1, Schrödinger, LLC).

The Dali server (Holm, 2019) was used to compare the structure of CIRp-A1 to structures in the Protein Data Bank (PDB). The top five results are shown in **Table 5.4**; all five proteins are lipocalins. Despite low sequence identity (as low as 3% for rat E-RABP), the overall protein fold is well conserved between CIRp-A1 and these proteins (**Figure 5.11B**), as has been observed among lipocalins (Adam et al., 2008). Lipocalins typically have a central cavity within the β -barrel where small and hydrophobic molecules can bind (Adam et al., 2008). Structures of the proteins in **Table 5.4** were all solved with a ligand. For example, the tick proteins OmCI, monotonin and RaHBP (*Rhipicephalus appendiculatus* histamine-binding protein) can bind leukotriene B₄, serotonin and histamine, respectively (Mans et al., 2008; Paesen et al., 1999; Roversi et al., 2013). By sequestering pro-inflammatory molecules in the β -barrel, these tick lipocalins likely suppress the host inflammatory response at the blood feeding site (Beaufays et al., 2008). In comparison to published structures of tick lipocalins, the middle of the β -barrel of CIRp-A1 is relatively packed with side chains (**Figure 5.12**), so it is unlikely to bind other molecules. This is in agreement with the results from native mass spectrometry analysis of CIRp-A1, where no bound species were identified (**Section 4.2.3**).

Table 5.4. Dali server search results with CIRp-A1 structure. The top five results from the PDB25 search on the Dali server (Holm, 2019) are shown. Sequence identity with CIRp-A1 is shown.

PDB	Dali Z-score	rmsd	% identity	Protein/function	Reference
5HCC-C	16.9	2.1	11	OmCI, C5-binding tick complement inhibitor	(Jore et al., 2016)
3BRN-A	16.5	2.2	16	Monotonin, tick salivary protein that binds to serotonin	(Mans et al., 2008)
1QFT-A	15.7	2.3	10	Tick histamine-binding protein (HBP)	(Paesen et al., 1999)
1EPA-B	14.0	2.4	3	Rat epididymal retinoic acid binding protein (E-RABP)	(Newcomer, 1993)
4NYQ-A	12.6	2.7	5	Cockroach lipocalin-like milk protein (Lili-Mip) that binds to lipids	(Banerjee et al., 2016)

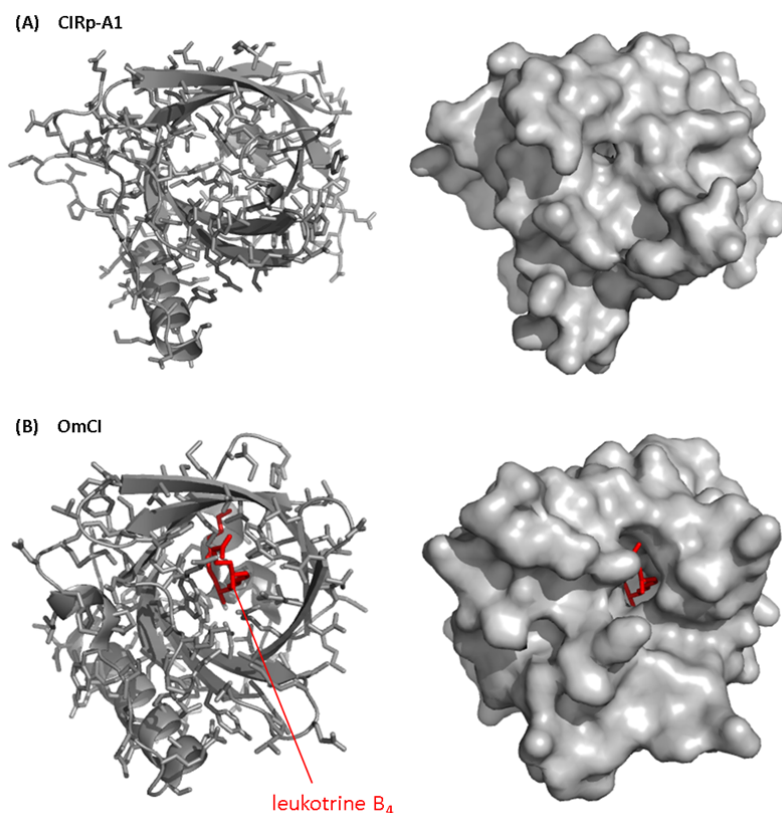


Figure 5.12. Potential ligand binding site on CIRp-A1. (A) The β -barrel of CIRp-A1, where lipocalins can sequester small, hydrophobic molecules, is relatively packed with side chains. (B) In comparison, the β -barrel of OmCI has a central cavity to sequester small molecules, such as leukotriene B₄ (PDB 3ZUO). Images were produced with PyMOL (Version 2.1, Schrödinger, LLC).

5.4 Structure determination of CIRp-A6

5.4.1 Crystallisation trials with CIRp-A6

Crystallisation trays were set up with refolded CIRp-A6 using the sitting drop vapour diffusion method. Broad screens were set up with a drop size of 0.3 μl at 75% (v/v) and 50% (v/v) protein:mother liquor ratios. The drops were left to equilibrate at 21 °C over a well containing 75 μl of mother liquor. Crystals grew overnight in 50% (v/v) protein:mother liquor ratio in the condition 0.1 M Sodium HEPES, pH 8.2, 50% (v/v) PEG500 MME (**Figure 5.13**). The concentration of CIRp-A6 was 21.5 mg/ml.

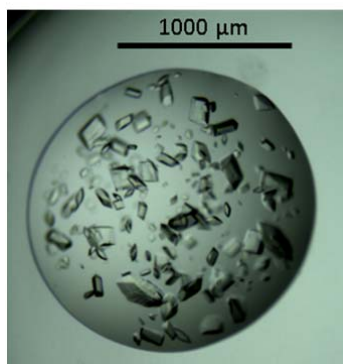


Figure 5.13. Crystals of CIRp-A6. The drop contained 0.225 μl of CIRp-A6 at 21.5 mg/ml and 0.075 μl of mother liquor (0.1 M Sodium HEPES, pH 8.2, 50% (v/v) PEG500 MME).

5.4.2 CIRp-A6 data collection and processing

Crystals of CIRp-A6 were harvested by Prof. Susan Lea for data collection. All crystals were cryoprotected in mother liquor as it contained 50% (v/v) PEG500 MME, and were flash frozen in liquid nitrogen. Data collection was performed by Susan Lea at the I04-1 beamline of Diamond Light Source. Crystals were exposed to an X-ray beam of 0.9159 Å wavelength and two datasets were collected. Datasets were processed by the Diamond automated pipeline using XIA2 in the 3dii or DIALS mode (Winter, 2010). Data processing procedures are discussed in **Section 5.3.2**. Both datasets were assigned to the orthorhombic *I*222 space group with the same unit cell dimensions. The processing statistics are summarised in **Table 5.5**.

Table 5.5. Processing statistics for CIRp-A6 datasets.

	CIRp-A6_1	CIRp-A6_2
Auto processing	XIA2 3dii	XIA2 DIALS
Space group	<i>I</i> 222	<i>I</i> 222
Cell dimensions		
a, b, c (Å)	52.01, 71.32, 110.15	51.79, 70.91, 109.79
α , β , γ (°)	90, 90, 90	90, 90, 90
Resolution	55.08-1.91	41.82-1.41
R _{merge} (overall/inner/outer)	0.247/0.071/1.800	0.090/0.076/0.952
R _{pim} (overall/inner/outer)	0.108/0.031/0.740	0.049/0.042/0.573
Unique reflections (overall/inner/outer)	16268/221/1181	39010/500/2728
Multiplicity (overall/inner/outer)	6.4/5.9/6.9	4.1/4.1/3.6
Completeness (overall/inner/outer)	99.9/99.6/99.9	99.3/99.2/95.9
I/ σ I (overall/inner/outer)	5.3/17.5/1.0	7.8/17.7/1.4
CC _{1/2} (overall/inner/outer)	0.988/0.997/0.580	0.975/0.991/0.353

5.4.3 Structure determination of CIRp-A6 by molecular replacement

Matthews Coefficient of 2.43 and solvent content of 49.46% calculated in CCP4 suggested one copy of CIRp-A6 per asymmetric unit. I obtained the structure solution for CIRp-A6 via MR using CIRp-A1 as the search model. CIRp-A1 and CIRp-A6 share 45% sequence identity. MR was carried out using Phaser MR in CCP4 (McCoy et al., 2007). Phaser MR is based on the maximum likelihood function, which calculates the probability of obtaining the observed structure factor amplitudes given the model. During MR, the search model is first rotated, and then translated within the unit cell, until there is agreement between the observed and calculated structure factor amplitudes. The different solutions are assessed using Rotation Function Z-score (RFZ), Translation Function Z-score (TFZ) and log-likelihood gain (LLG) score. The Z-scores indicate how much better

the solution is compared to the mean. The LLG indicates the extent to which the solution gives a better prediction of the data than a random selection of atoms. Phaser also reports the number of clashes during packing. The TFZ score of the solution for CIRp-A6 was 18.1, which suggested a highly likely solution. To assess the solution, restrained refinement was carried out using REFMAC5 within CCP4 (Murshudov et al., 2011). After refinement, the R-work and R-free values decreased from 52.32% and 52.86%, to 41.23% and 45.87%, respectively. The electron density resulting from the refinement showed density consistent with the missing sequence, when viewed in Coot (Emsley et al., 2010) (**Figure 5.14**), so I moved forward with further refinement and model building.

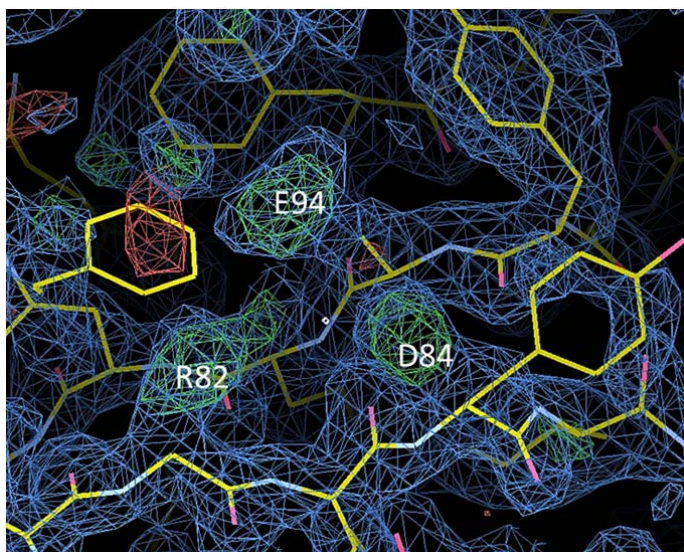


Figure 5.14. Initial refinement of CIRp-A6 structure after a solution was found by Phaser MR. The map was viewed in Coot, with the 2Fo-Fc map (blue density) contoured at 1.0 rmsd and the Fo-Fc map (green = positive residual density; red= negative residual density) contoured at 3.0 rmsd. Pockets of residual density are evident into which the side chains of CIRp-A6 could be built in. Examples of side chains are highlighted.

5.4.4 Refinement of CIRp-A6 structure

The structure of CIRp-A6 was built and refined through cycles of automated model building by Buccaneer (Cowtan, 2006) and refinement by REFMAC5 (Murshudov et al., 2011). The sequence of CIRp-A6 was provided to Buccaneer for model building. Initially, Buccaneer was carried out without an input model, using the map generated from the initial REFMAC5 refinement after phasing. After a few rounds, an input model was

provided to be extended. Following refinement by REFMAC5 (Murshudov et al., 2011), rebuilding of the model was done in Coot (Emsley et al., 2010). An example of refinement of CIRp-A6 is shown in **Figure 5.15**: the density for a loop was visible after one round of model building and refinement (**Figure 5.15A**); after several rounds, the loop could be modelled into the improved density (**Figure 5.15B**). The improved map showed positive residual densities which guided the next rounds of refinement.

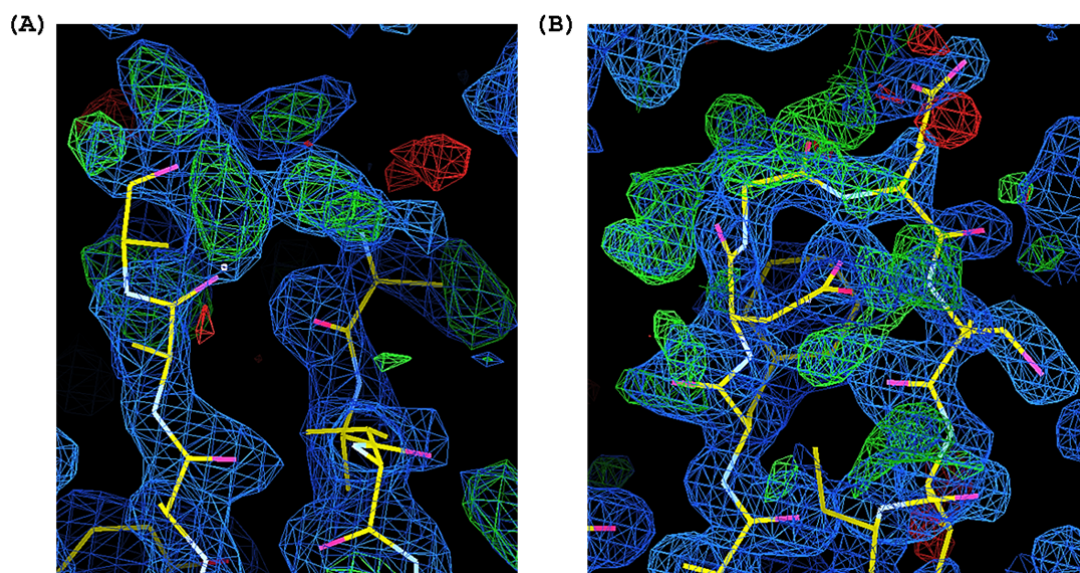


Figure 5.15. Refinement of CIRp-A6 structure. An example of the refinement process for CIRp-A6. The map was viewed in Coot, with the 2Fo-Fc map (blue density) contoured at 1.2 rmsd and the Fo-Fc map (green = positive residual density; red= negative residual density) contoured at 3.0 rmsd. (A) The positive residual densities are visible for a loop after the first round of model building by Buccaneer and refinement by REFMAC5. (B) After several rounds of model building and refinement, the density was improved and the loop could be built. Positive residual densities (green) indicate the model requires further refinement.

Final rounds of refinement were performed using PHENIX (Afonine et al., 2012). The final model was supported by R-work and R-free values of 21.70% and 24.69%, respectively. The refinement statistics for the final model of CIRp-A6, including validation of protein chemistry by MolProbity (Chen et al., 2010), are summarised in **Table 5.6**.

Table 5.6. Refinement statistics for the final model of CIRp-A6.

	CIRp-A6
Residues seen in structure	K4-K165
Resolution range	55.08-1.91 (2.03-1.91)
R-work (%)	21.70 (30.27)
R-free (%)	24.69 (30.22)
Ramachandran outliers (%)	0.6
Ramachandran favoured (%)	97.5
Rotamer outliers (%)	0.0
Clashscore	6.51
MolProbity score	1.46

5.4.5 Crystal structure of CIRp-A6

The structure of CIRp-A6 displays a lipocalin-like fold (**Figure 5.16A**) and is homologous to the structure of CIRp-A1 (**Figure 5.16B**). The four cysteine residues are conserved between CIRp-A6 and CIRp-A1; the disulphide bonds might be important for the tertiary structure of lipocalins. Non-conserved residues between CIRp-A6 and CIRp-A1, which scored less than 0.5 in the Gonnet PAM 250 matrix used by Clustal Omega (Sievers et al., 2014), are primarily located within the loops between the β -strands of the barrel (**Figure 5.16C**). For lipocalins, it had been postulated that the four variable loops connecting the β -strands at the open end of the β -barrel (in the view of **Figure 5.16A**, these four loops are at the top of the barrel) give rise to antigenic variation (Skerra, 2000). In the case of CIRp-A1 and CIRp-A6, the sequence variations exist within the loops at both ends of the barrel. In AP haemolysis assays, CIRp-A6 showed complement inhibitory activity in rat serum, but not in human serum, whereas CIRp-A1 was active in human serum, but not in rat serum (**Section 4.3.2**). As the sequence differences are located within the loops, these loops might contribute to host specificity. Detailed analysis of the structure will be carried out at the end of this chapter (**Section 5.8**).

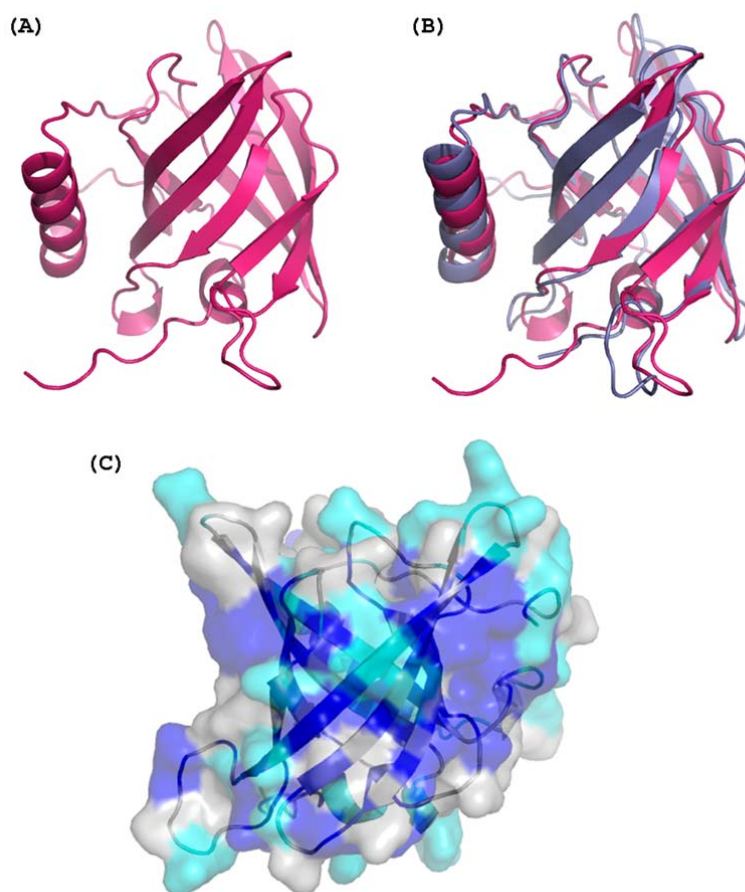


Figure 5.16. Crystal structure of CIRp-A6. (A) The final model of CIRp-A6 shows a lipocalin-like fold, characterised by an eight-stranded anti-parallel β -barrel and an α -helix. (B) The overlay of structures of CIRp-A6 (magenta) and CIRp-A1 (purple) shows structural homology between the two proteins. (C) Cartoon and surface representation of CIRp-A6 are shown. Fully conserved residues between CIRp-A6 and CIRp-A1 were coloured blue; strongly similar residues based on the Gonnet PAM 250 scoring matrix (score > 0.5) were coloured cyan; and non-conserved residues (score < 0.5) were coloured white. The non-conserved residues are primarily located within the loops connecting the β -strands. Images were produced with PyMOL (Version 2.1, Schrödinger, LLC).

5.5 Structure determination of CIRp-A4

5.5.1 Crystallisation trials with CIRp-A4

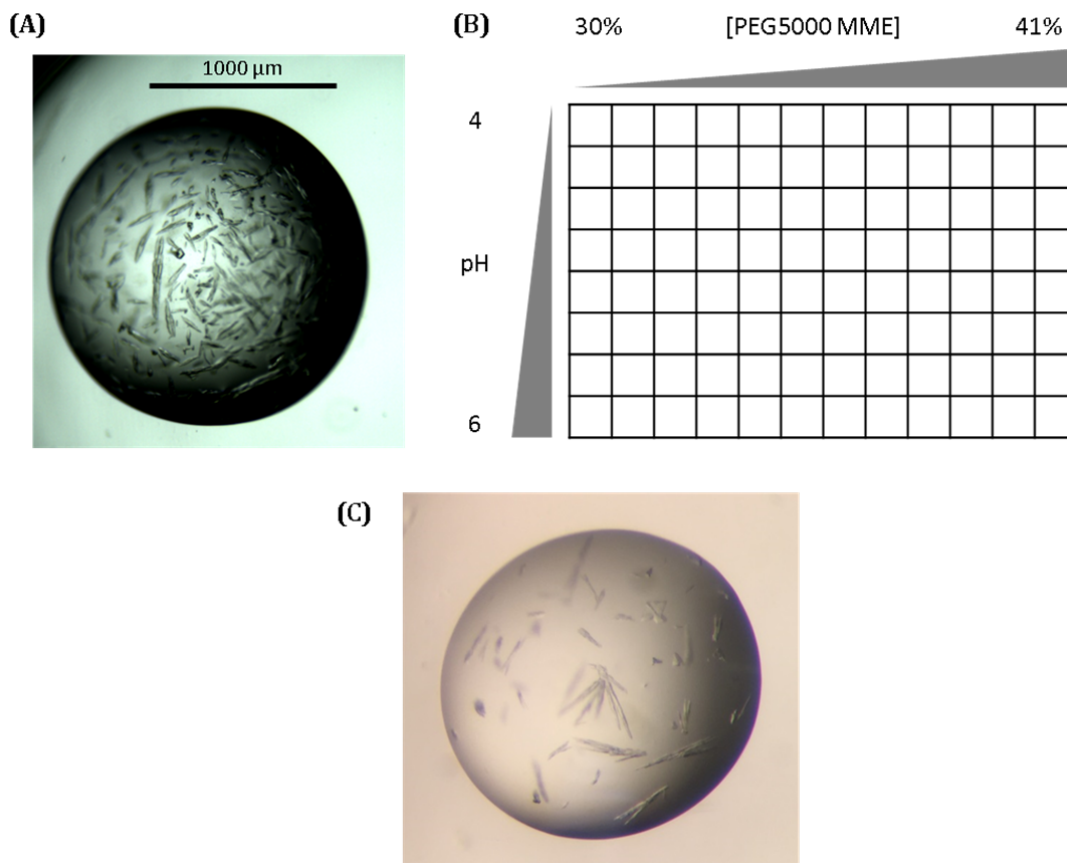


Figure 5.17. Crystallisation trials of CIRp-A4. (A) Initial crystal hit in Stura screen. The drop contained 0.15 μl of CIRp-A4 at 10.4 mg/ml and 0.15 μl of mother liquor (0.1 M sodium acetate, pH 5, 36% (w/v) PEG5000 MME). (B) Optimisation trays were set up based on the initial hit conditions, with respect to pH and precipitant concentration. (C) Crystals obtained from optimisation.

Crystallisation trays were set up with refolded CIRp-A4 using the sitting drop vapour diffusion method. Broad screens were set up with a drop size of 0.3 μl at 75% (v/v) and 50% (v/v) protein:mother liquor ratios. The drops were left to equilibrate at 21 $^{\circ}\text{C}$ over a well containing 75 μl of mother liquor. Crystals were obtained with CIRp-A4 at 10.4 mg/ml in 0.1 M sodium acetate, pH 5, 36% (w/v) PEG5000 MME (**Figure 5.17A**); however, these crystals did not have very sharp edges. To get better crystals, optimisation trays were set up based on the initial crystallisation condition with respect to pH and

precipitant concentration (**Figure 5.17B**). The stock solutions were 1 M sodium acetate at pH 4 and pH 6, and 50% (w/v) PEG5000 MME. The optimisation trays were set up with CIRp-A4 at 4.12 mg/ml. The crystals from optimisation trays did not have sharper edges and did not look much better than the original crystals (**Figure 5.17C**).

5.5.2 Limited proteolysis with CIRp-A4

Limited proteolysis by proteases could aid crystallisation as the proteases might cleave off flexible parts of the protein. Small-scale limited proteolysis was carried out with CIRp-A4 to assess whether stable fragments could be achieved via proteolysis. Three proteases were tried – chymotrypsin, subtilisin and trypsin. For each reaction, 10 µl of CIRp-A4 (at 1 mg/ml) and 10 µl of protease were used. Serial dilution of the proteases was generated, to test the proteases at different protease:protein (w/w) ratios. After mixing CIRp-A4 and the protease, the reaction was incubated at room temperature for 1 h, before SDS-PAGE analysis (**Figure 5.18**). Chymotrypsin (**Figure 5.18A**) and subtilisin (**Figure 5.18B**) cleaved CIRp-A4 into many fragments, so these proteases would be unsuitable to generate a stable product for crystallisation. Trypsin added at protease:protein ratio of 1:10 resulted in a relatively clean product, 3-4 kDa lower than full-length CIRp-A4 (**Figure 5.18C**).

Trypsin digest was carried out on a larger scale at protease:protein ratio of 1:10. For the reaction, CIRp-A4 was used at 1 mg/ml and an equal volume of trypsin at 0.1 mg/ml was added. After incubation at room temperature for 1 h, PMSF was added to stop the reaction; PMSF was added at a final concentration of 1 mM. To purify CIRp-A4 before crystallisation trials, SEC was performed on a S75 16/60 column; the running buffer was 50 mM Tris, 100 mM NaCl, pH 8 (data not shown).

Crystallisation trials were set up as before using trypsin-treated CIRp-A4 at 16.8 mg/ml. Crystals were obtained in the same condition as the full-length protein (0.1 M sodium acetate, pH 5, 36% (w/v) PEG5000 MME). The crystals looked better than those of the full-length protein as they had sharper edges, but were quite clustered (**Figure 5.19**).

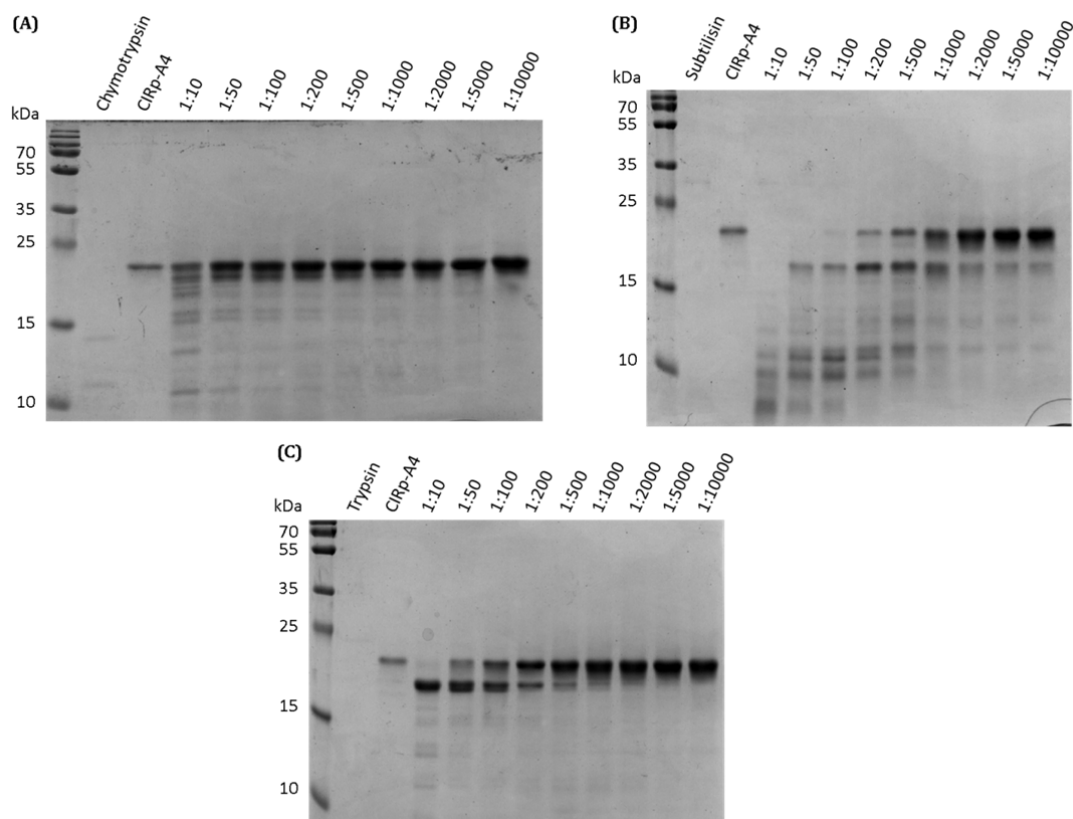


Figure 5.18. Limited proteolysis with CIRp-A4. (A) chymotrypsin, (B) subtilisin, and (C) trypsin were used for limited proteolysis with CIRp-A4. Reactions were then analysed by reducing 15% SDS-PAGE. The protease:protein (w/w) ratios of the reactions are shown.

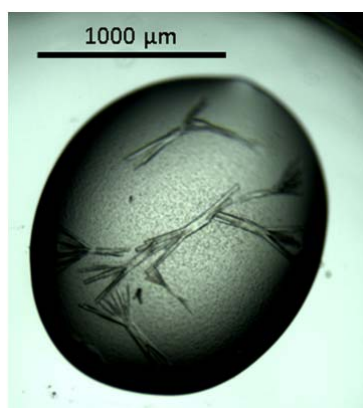


Figure 5.19. Crystals of CIRp-A4 treated with trypsin. The drop contained 0.15 μl of trypsin-treated CIRp-A4 at 16.8 mg/ml and 0.15 μl of mother liquor (0.1 M sodium acetate, pH 5, 36% (w/v) PEG5000 MME).

5.5.3 Crystallisation trials with truncated CIRp-A4 constructs

To obtain better crystals, I designed different constructs of CIRp-A4 by cloning. Based on the sequence alignment between CIRp-A4 and CIRp-A1, the C-terminus was truncated to Q160 (**Figure 5.20**). Two different N-termini were tried – N1 which was the same as the full-length protein and E6 which matched what was observed in the structure of CIRp-A1. Thus, two truncated CIRp-A4 constructs were cloned, expressed and purified: CIRp-A4(1-160) and CIRp-A4(6-160). These proteins were purified by refolding using the same protocol as for full-length CIRp-A4 (**Section 5.2.2**).

CIRp-A4	1	-----NDWNP	ESFAIDEFMN	TTDDIWVLNT	TQQNPQACKK	35
CIRp-A1	1	EDQETDFSST	DGAELIAKEP	EVYPIDQFMN	N-TEIWFVNT	49
			: * : * : * : * : *	: * : * : * : * : *		
CIRp-A4	36	DKKHNITENG	IYFFRSHKEN	GQIKTQTLFG	EFIHFSEEEK	85
CIRp-A1	50	DKSKSMTQTA	TSFVRSHVKN	GNIIEENLVG	NFTYFNDKEK	99
		** . : . : * : . .	* . * * * : *	* : * : . . * :	* : * . : . : * * :	
CIRp-A4	86	SSGVHAEHLY	YSSDKKCGL	VQVFAK---D	QNVWTELVRV	131
CIRp-A1	100	SSGVYAEHLY	YVSEDKKCGL	FQVFAHVNDK	TTIWRDVRVS	149
		*** : * * * * *	* * * * * * * *	. * * * * :	. : * : * * *	
CIRp-A4	132	AGCRREYEAY	VKEIKGKKN	TSPYSDDCQI	KV	163
CIRp-A1	150	LNCTKEFDEY	VKLVA--TS	KSPYTSECQ-	--	176
		. * : * : * * *	: . . : *	. * * * : . : * *		

Figure 5.20. Constructs of CIRp-A4. Sequence alignment of CIRp-A4 and CIRp-A1 by Clustal Omega is shown. The N-terminus and C-terminus of CIRp-A4 constructs are indicated by blue triangle and red arrow, respectively.

Crystallisation trials were set up as before using the truncated CIRp-A4 constructs. Crystals were obtained with CIRp-A4(6-160) at 13.1 mg/ml in two different conditions. CIRp-A4(6-160)_1 crystals were plate-like and crystallised in 0.2 M ammonium sulphate, 30% (w/v) PEG 4000 (**Figure 5.21A**). CIRp-A4(6-160)_2 crystals were also plate-like, but much smaller than CIRp-A4(6-160)_1 crystals. These crystallised in 0.1 M SPG, pH 4, 25% (w/v) PEG 1500 (**Figure 5.21B**). They looked quite similar to the crystals obtained with trypsin-treated CIRp-A4 (**Figure 5.19**).

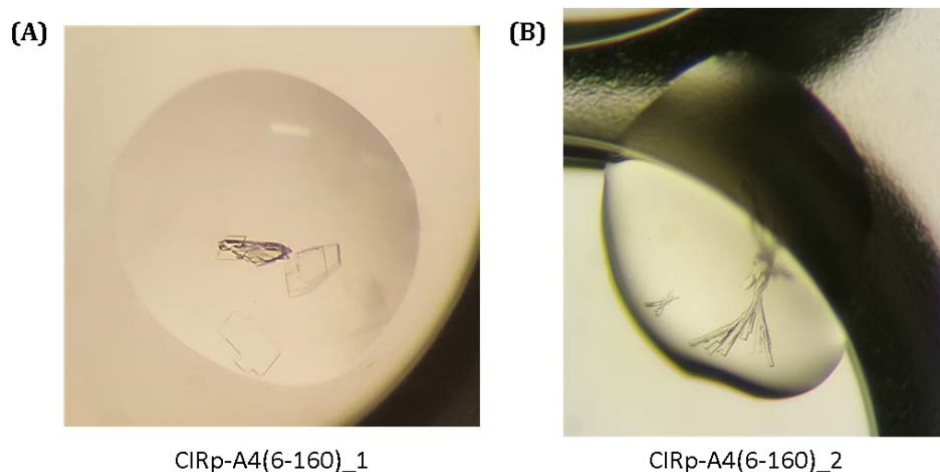


Figure 5.21. Crystals of CIRp-A4(6-160). (A) The drop contained 0.225 μl of CIRp-A4(6-160) at 13.1 mg/ml and 0.075 μl of mother liquor (0.2 M ammonium sulphate, 30% (w/v) PEG 4000). (B) The drop contained 0.225 μl of CIRp-A4(6-160) at 13.1 mg/ml and 0.075 μl of mother liquor (0.1 M SPG, pH 4, 25% (w/v) PEG 1500).

5.5.4 CIRp-A4 data collection and processing

Crystals of CIRp-A4(6-160) were harvested by Prof. Susan Lea for data collection. CIRp-A4(6-160)_1 crystals were cryoprotected in mother liquor (0.2 M ammonium sulphate, 30% (w/v) PEG 4000) supplemented with 10% (v/v) PEG400 and were flash frozen in liquid nitrogen. CIRp-A4(6-160)_2 crystals were cryoprotected in mother liquor (0.1 M SPG, pH 4, 25% (w/v) PEG 1500) supplemented with 20% (v/v) PEG400 and were flash frozen in liquid nitrogen. Data collection was performed by Prof. Susan Lea at the I04-1 beamline of Diamond Light Source, using an X-ray beam of 0.9762 Å wavelength. A dataset for CIRp-A4(6-160)_1 crystals was collected and processed by autoPROC (Vonrhein et al., 2011). autoPROC uses XDS for indexing and integration (Kabsch, 2010), and AIMLESS for scaling and merging (Evans and Murshudov, 2013). Data processing procedures are discussed in **Section 5.3.2**. The dataset was assigned to the monoclinic $P12_11$ space group. The processing statistics are summarised in **Table 5.7**.

Table 5.7. Processing statistics for CIRp-A4(6-160) dataset.

CIRp-A4(6-160)_1	
Auto processing	autoPROC
Space group	<i>P</i> 12 ₁ 1
Cell dimensions	
a, b, c (Å)	68.37, 150.30, 68.43
α , β , γ (°)	90, 91.83, 90
Resolution	68.33-2.11
R _{merge} (overall/inner/outer)	0.143/0.062/1.211
R _{pim} (overall/inner/outer)	0.093/0.040/0.759
Unique reflections (overall/inner/outer)	78899/2543/11657
Multiplicity (overall/inner/outer)	3.4/3.2/3.5
Completeness (overall/inner/outer)	99.0/98.0/99.9
I/ σ I (overall/inner/outer)	5.1/13.8/1.0
CC _{1/2} (overall/inner/outer)	0.991/0.993/0.471

5.5.5 Structure determination of CIRp-A4 by molecular replacement

Matthews Coefficient and solvent content calculations were performed in CCP4. The most common solvent content of a crystal is around 43% (Matthews, 1968) and the mean Matthews Coefficient value for proteins is 2.69 (Kantardjieff and Rupp, 2003). Given the monomeric mass of CIRp-A4 and the unit cell dimensions, the highest probability was for 8 copies of CIRp-A4 in the asymmetric unit, with a solvent content of 46.83% (**Table 5.8**).

Table 5.8. Estimation of the number of copies of CIRp-A4 in the asymmetric unit. The Matthews Coefficient and solvent content were calculated in CCP4 from the unit cell dimensions and the molecular weight of CIRp-A4.

Number of molecules per asymmetric unit	Matthews Coefficient	Solvent content (%)	Probability
1	18.49	93.35	0.00
2	9.25	86.71	0.00
3	6.16	80.06	0.00
4	4.62	73.41	0.00
5	3.70	66.77	0.03
6	3.08	60.12	0.10
7	2.64	53.47	0.25
8	2.31	46.83	0.35
9	2.05	40.18	0.21
10	1.85	33.53	0.04

Self rotation and self translation analysis can also provide information about the number of monomers in the asymmetric unit. The self rotation function shows if the molecules in the asymmetric unit are related by rotational symmetry. To construct the self rotation function, the Patterson map is first rotated from the z axis by θ degrees followed by ϕ degrees from the x axis, and the last rotation takes place around the final axis by χ degrees. Peaks above the background noise are present when the maps overlap in the presence of crystallographic and/or non-crystallographic symmetry. Self rotation function analysis by MOLREP within CCP4 (Vagin and Teplyakov, 1997) suggested multiple copies of the protein, as indicated by peaks in the stereographic projections that were not explained by the crystal symmetry (**Figure 5.22**). The rotational axis was almost aligned with the crystallographic axis (**Figure 5.22** and **Table 5.9**). MOLREP also checks Patterson for self translation; this returned a peak at (0.652, 0.500, 0.471) in fractional coordinates, which almost aligns with the 2_1 crystallographic axis.

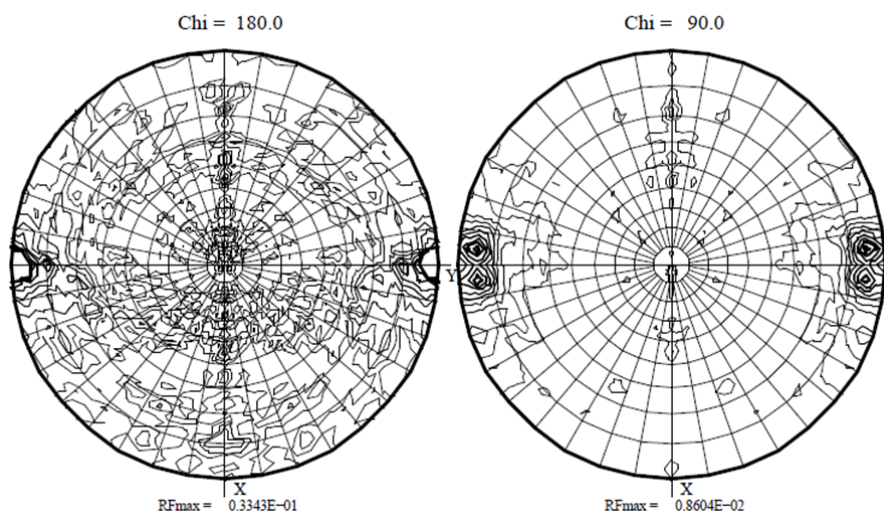


Figure 5.22. Stereographic projections of the self rotation function of CIRp-A4(6-160) dataset. Self rotation function was calculated by MOLREP with an integration radius of 40 Å in the resolution range 15.0-4.0 Å. The chi (χ)= 180° and chi (χ)= 90° sections of the self rotation function are shown.

Table 5.9. Self rotation function analysis of CIRp-A4(6-160) dataset using MOLREP. The top ten results from MOLREP Self Rotation Function analysis are shown.

	theta	phi	chi
1	0.00	0.00	0.00
2	81.93	-90.00	180.00
3	87.38	-91.83	179.92
4	98.65	91.04	179.77
5	88.00	-92.73	180.00
6	93.66	-88.48	179.89
7	97.45	-88.48	179.80
8	83.87	-92.38	179.89
9	96.92	94.16	179.80
10	83.76	-86.66	180.00

The phase problem was solved by molecular replacement using the structure of CIRp-A1; CIRp-A4 and CIRp-A1 share 51% sequence identity. The search model for MR was created using Chainsaw in CCP4 (Stein, 2008). Using the sequence alignment of CIRp-A1 and CIRp-A4, Chainsaw creates a model by keeping the conserved side chains, but pruning non-conserved residues to the gamma atom. Chainsaw resulted in 78 conserved residues, 74 mutated residues and 4 deleted residues. Compared to using CIRp-A1 as the search model for MR, using the Chainsaw-created model reduces bias.

MR was carried out using Phaser MR in CCP4 (McCoy et al., 2007). For the initial MR, the number of copies of the protein to search for was set to 4. Four copies of the search model were successfully placed by Phaser, with good TFZ scores (Phaser discussed in **Section 5.4.3**). The packing of the solution was also checked visually in Coot (Emsley et al., 2010) (**Figure 5.23A**). Using the solution with 4 copies, Phaser MR was repeated, searching for additional 4 copies to pack all 8 copies within the asymmetric unit. Phaser found a packing solution containing all 8 copies (**Figure 5.23B**). The TFZ scores of the different copies are shown in **Table 5.10**.

Table 5.10. TFZ scores from the molecular replacement of CIRp-A4 by Phaser MR. CIRp-A1 was used as the search model for MR. TFZ scores for the different copies of the protein in the asymmetric unit are shown.

Copy number	TFZ score
1	4.4
2	14.6
3	30.6
4	26.6
5	20.2
6	23.3
7	25.8
8	6.0

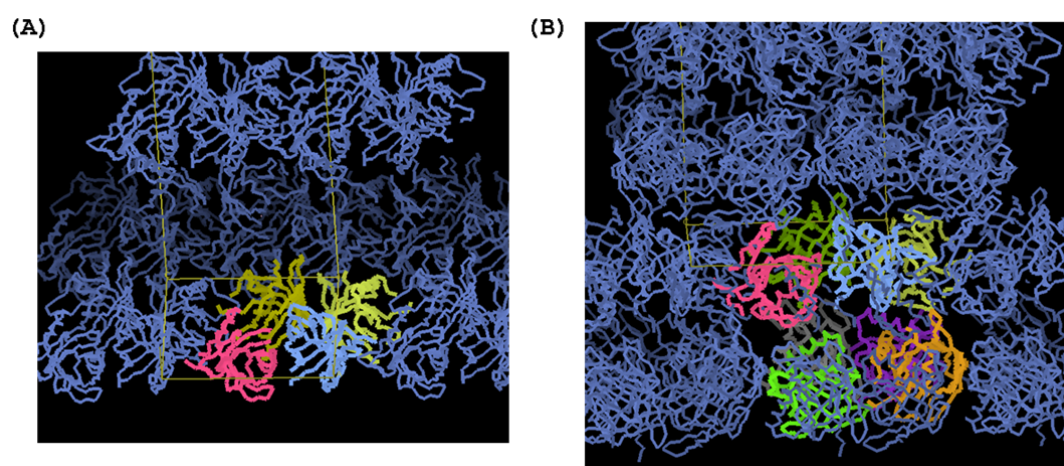


Figure 5.23. Structure determination of CIRp-A4 by molecular replacement. Phaser MR was used for the molecular replacement of CIRp-A4 using CIRp-A1 as the search model. (A) Four copies of the model were placed first. (B) Using the solution from (A), further four copies were searched for, completing the eight copies within the asymmetric unit. Solutions were visualised in Coot.

The solution from MR was analysed for non-crystallographic symmetry (NCS) (**Figure 5.24**). A 2-fold NCS could clearly be seen in the first four copies (chains A-D, **Figure 5.24B**). This NCS was also expected in the next four copies (chains E-H, **Figure 5.24C**); however, the 8th copy (chain H) looked out of place. Additionally, when analysed in Coot, the 8th copy was clearly misfit in the electron density map (**Figure 5.25A**). Therefore, I completely deleted this copy from the solution. Then, I used the modified solution containing 7 copies of the search model to run refinement with REFMAC5 (Murshudov et al., 2011). For refinement, automatically generated local NCS restraints were used. NCS restraints can reduce the number of independently refined parameters. The refinement resulted in R-work and R-free values of 38.77% and 42.59%, respectively, and

the electron density was consistent with the missing sequence. The refined solution was used to rebuild the model with 7 copies manually in Coot (Emsley et al., 2010). Then, both the modified model containing 7 copies and a monomer search model (chain A) were used for Phaser MR to place the 8th copy. This second attempt was successful with a much better TFZ value of 45.9; the electron density map also looked much better (**Figure 5.25B**).

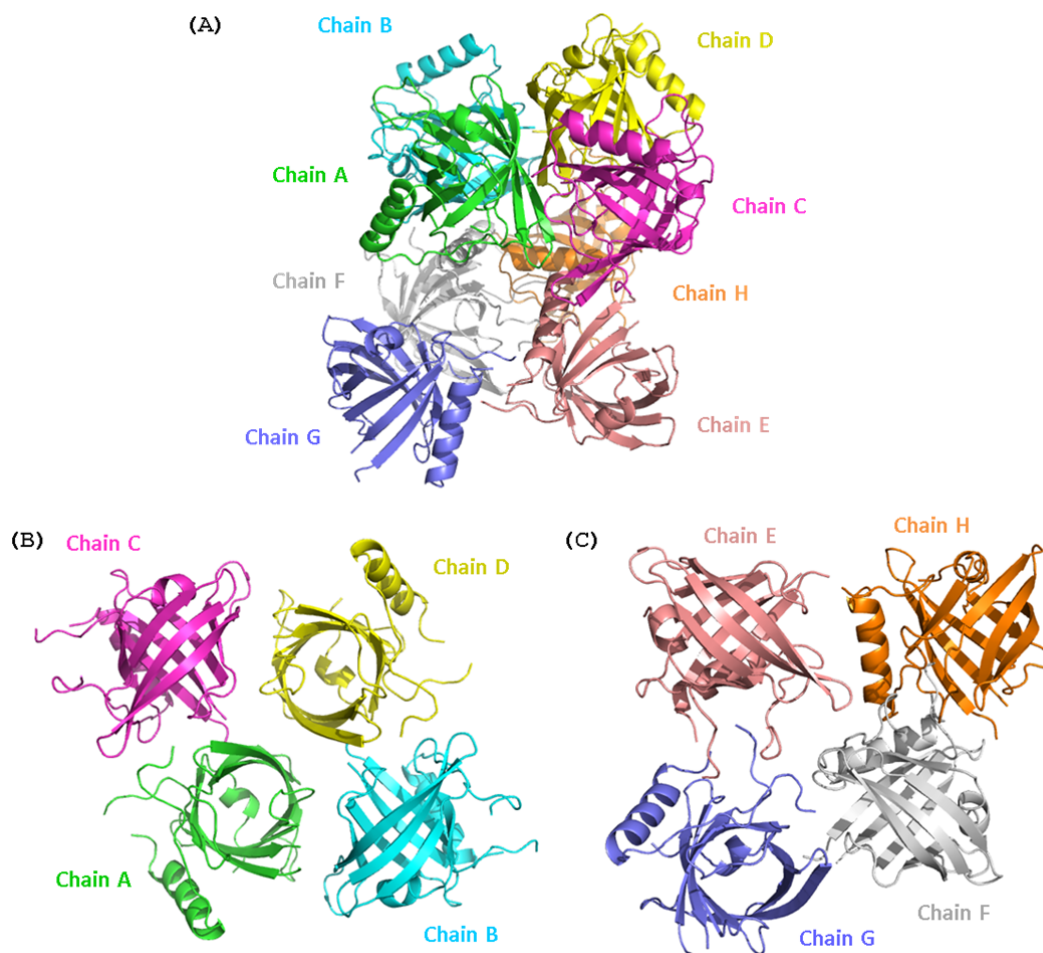


Figure 5.24. MR solution after placing eight copies of the search model. (A) Eight copies of the protein were placed into the asymmetric unit (chains A-H). (B) The ‘top layer’ containing chains A-D displays non-crystallographic symmetry. (C) The ‘bottom layer’ containing chains E-H shows that chain H (8th copy) does not fit with the non-crystallographic symmetry indicated by the top layer in (B). Images were produced with PyMOL (Version 2.1, Schrödinger, LLC).

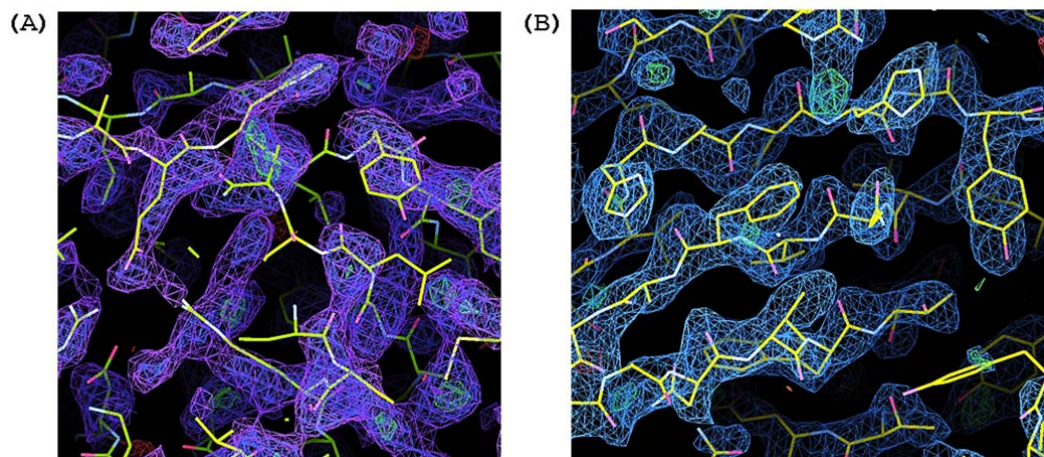


Figure 5.25. Electron density map of the 8th copy of the search model for CIRp-A4 following molecular replacement. The solutions from Phaser MR were visualised in Coot. (A) The 8th copy of the search model was badly fit into the 2Fo-Fc map (purple) contoured at 1.1 rmsd. (B) Second MR attempt resulted in a better solution for the 8th copy of the model. The 2Fo-Fc map (blue) was contoured at 1.6 rmsd.

5.5.6 Refinement of CIRp-A4 structure

Once all 8 copies were placed into the density, refinement was continued using Coot (Emsley et al., 2010) and PHENIX (Afonine et al., 2012). As the search model for MR was generated using Chainsaw, some of the side chains were missing. Using the 2Fo-Fc map and the Fo-Fc difference map resulting from refinement, side chains were manually built in using Coot. The structure of CIRp-A4 was built by iterative rounds of model building in Coot and refinement using PHENIX. For refinement in PHENIX, torsion-based NCS restraints were switched on.

Model building for CIRp-A4 was aided by the ‘NCS Ghost Control’ feature of Coot (Emsley et al., 2010). When rebuilding one of the eight copies in the model, the other seven copies could be visualised as ‘ghosts’ to guide manual model building. For example, in **Figure 5.26**, Chain F was set as the master chain, but the other seven chains were visualised by switching on the ‘NCS Ghost Control’. This indicated that PHENIX misplaced the side chain of a glutamate residue to where the backbone of the loop should be built (**Figure 5.26A,B**). By looking at the other chains, which had this loop built already, the backbone of the loop of Chain F could be built into the density. Further refinement also improved the electron density map, enabling the placement of side chains (**Figure 5.26C**).

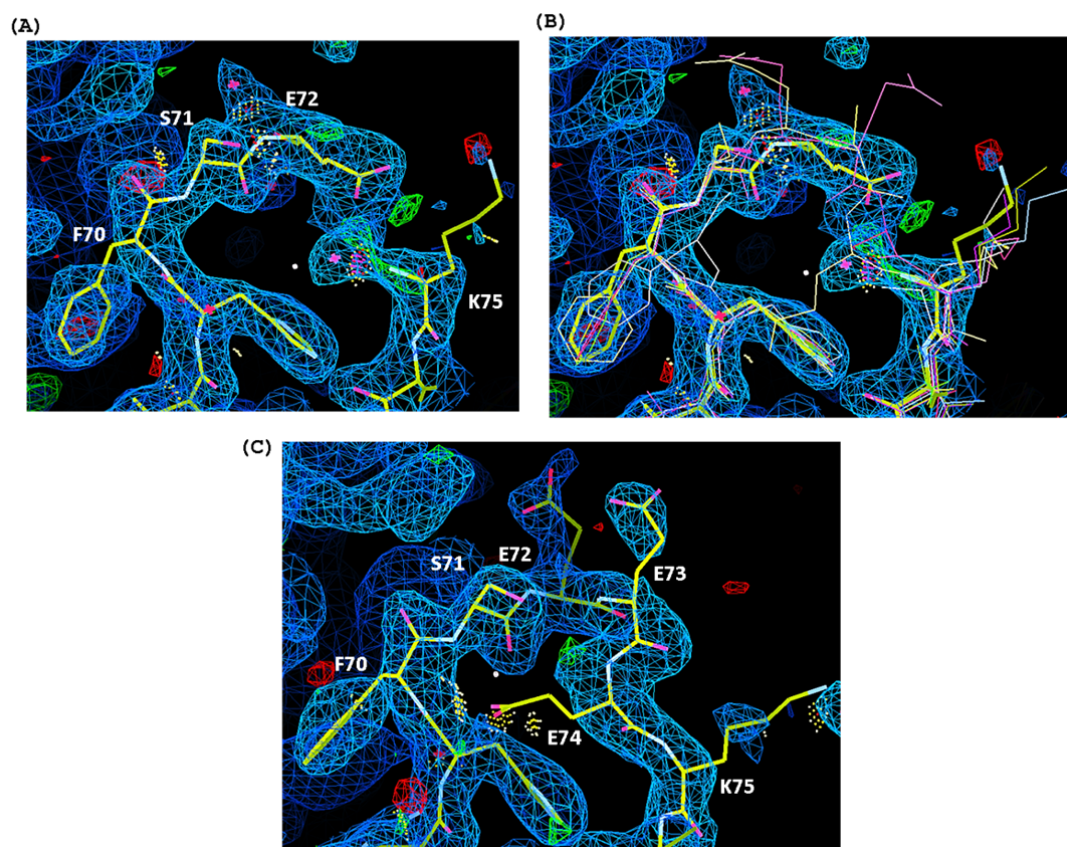


Figure 5.26. Refinement of CIRp-A4 structure. An example of the refinement process for building the structure of CIRp-A4 is shown. The map was viewed in Coot, with the 2Fo-Fc map (blue density) contoured at 1.0 rmsd and the Fo-Fc map (green = positive residual density; red = negative residual density) contoured at 3.0 rmsd. (A) The loop within Chain F was incomplete during the initial rounds of refinement. (B) NCS Ghost Control feature was switched on to overlay the other chains as 'ghosts', which indicated the misplacement of the side chain of E72. The NCS Ghost Control guided the building of the backbone of the loop. (C) After several rounds of refinement and model building, the loop could be built completely.

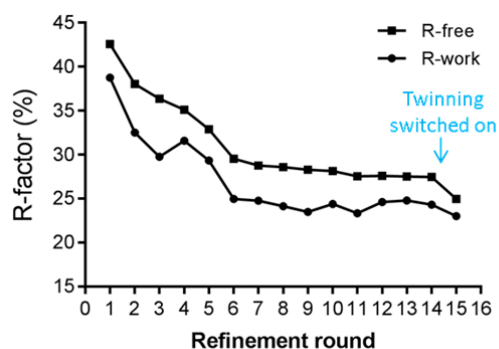


Figure 5.27. R-factors after each round of refinement of CIRp-A4 model. R-free and R-work values following refinement were plotted. Twin refinement was used for the last round of refinement.

The refinement was continued until the final model and the 2Fo-Fc map were in good agreement as visualised in Coot (Emsley et al., 2010). After rounds of refinement, R-work and R-free values stalled at 24.30% and 27.47%, respectively (**Figure 5.27**), which were high for the resolution of the dataset. Two of the dimensions of the unit cell were very similar ($a=c$, **Table 5.7**), which could cause twinning. Therefore, for the last round of refinement in PHENIX, twin refinement was carried out using the twin law (l,-k,h) as obtained from Xtriage (Adams et al., 2010).

The presence of twinning was identified by Xtriage based on the intensity statistics. A set of intensities from a single crystal is characterised by a small amount of very weak reflections and a small amount of very strong reflections. In a twinned crystal, reflection intensities are added up by the twin law. The probability that two extreme intensities combine is small (two very weak or two very strong reflections), which means that twinned crystals produce a smaller fraction of very weak or very strong reflections (Dauter, 2003). Therefore, the intensity distribution of a twinned dataset will differ from the expected Wilson statistics (Wilson, 1949), and will have a sharper distribution around the mean. The second moment of the intensity distribution of the dataset $\langle I^2 \rangle / \langle I \rangle^2$ is reported by Xtriage. $\langle I^2 \rangle / \langle I \rangle^2$ is expected to be 2.0 and 1.5 for untwinned and twinned data, respectively (Stanley, 1972; Yeates, 1997); the CIRp-A4 dataset had $\langle I^2 \rangle / \langle I \rangle^2$ value of 1.907 (**Table 5.11**). The Wilson ratio $\langle F \rangle^2 / \langle I \rangle$ (Wilson, 1949) is another measure of the intensity distribution. Untwinned and twinned data are expected to have $\langle F \rangle^2 / \langle I \rangle$ values of 0.785 and 0.885, respectively (Stanley, 1972); the CIRp-A4 dataset had $\langle F \rangle^2 / \langle I \rangle$ value of 0.822 (**Table 5.11**).

In some cases, the presence of pseudo-translational symmetry or anisotropy gives rise to weak and strong classes of reflections, and influences the intensity statistics in such a way that twinning cannot readily be detected. Therefore, the $|L|$ statistic or the L-test was developed to be a more robust statistic for the detection of twinning (Padilla and Yeates, 2003). The L-test is based on the local intensity distribution and analyses local pairs of reflection intensities. Untwinned and twinned data are expected to have $\langle |L| \rangle$ values of 0.500 and 0.375, respectively (Padilla and Yeates, 2003); the CIRp-A4 dataset had $\langle |L| \rangle$ value of 0.431 (**Table 5.11**). Additionally, untwinned and twinned data are expected to have $\langle L^2 \rangle$ values of 0.333 and 0.200, respectively (Padilla and Yeates, 2003); the CIRp-

A4 dataset had $\langle L^2 \rangle$ value of 0.256 (**Table 5.11**). The results of the intensity statistics analyses are summarised in **Table 5.11** and a plot of the L-test is shown in **Figure 5.28**. The analyses by Xtriage (Adams et al., 2010) indicated that the intensities are significantly different from expected for normal data. Based on the twin law (l,k,h), Britton analysis (Fisher and Sweet, 1980) and H-test (Yeates, 1988, 1997) estimated a twin fraction of 8.3% and 8.8%, respectively.

Modelling the twinning improved refinement, and the final model of CIRp-A4 was supported by R-work and R-free values of 23.00% and 24.97%, respectively (**Figure 5.27**). In addition, the protein chemistry of the model was validated using MolProbity (Chen et al., 2010). The refinement statistics for the final model of CIRp-A4 are summarised in **Table 5.12**.

Table 5.11. Xtriage analysis of CIRp-A4 dataset. The presence of twinning was indicated by Xtriage based on the moments of intensities distribution and moments of L. The theoretical values for untwinned and twinned crystals are shown.

	Untwinned	Twinned	CIRp-A4
$\langle I^2 \rangle / \langle I \rangle^2$	2.0	1.5	1.907
$\langle F \rangle^2 / \langle I \rangle$	0.785	0.885	0.822
$\langle L \rangle$	0.500	0.375	0.431
$\langle L^2 \rangle$	0.333	0.200	0.256

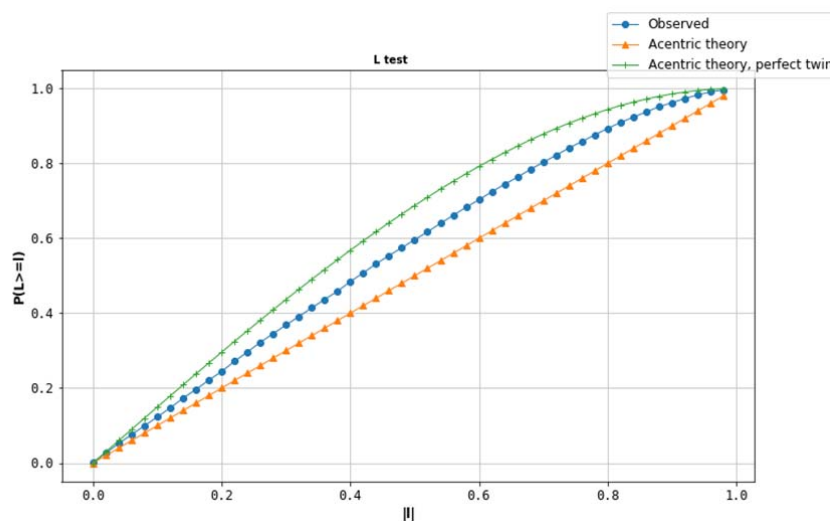


Figure 5.28. Plot of the L test to analyse twinning in CIRp-A4 dataset. The plot was the result of the L-test as a part of twinning analysis by Xtriage. The plot shows expected curves from untwinned (orange) and twinned (green) crystals. The analysis indicated twinning in CIRp-A4 crystals (blue).

Table 5.12. Refinement statistics for the final model of CIRp-A4.

	CIRp-A4
Residues seen in structure	E6-Q160
Resolution range	62.25-1.89 (1.96-1.89)
R-work (%)	23.00 (36.01)
R-free (%)	24.97 (55.90)
Ramachandran outliers (%)	0.6
Ramachandran favoured (%)	97.35
Rotamer outliers (%)	2.11
Clashscore	12.81
MolProbity score	1.99

5.5.7 Crystal structure of CIRp-A4

The final model of the asymmetric unit containing eight copies of CIRp-A4 is shown in **Figure 5.29A**. As expected, CIRp-A4 also displays a lipocalin-like fold (**Figure 5.29B,C**). The structure will be discussed in **Section 5.8**.

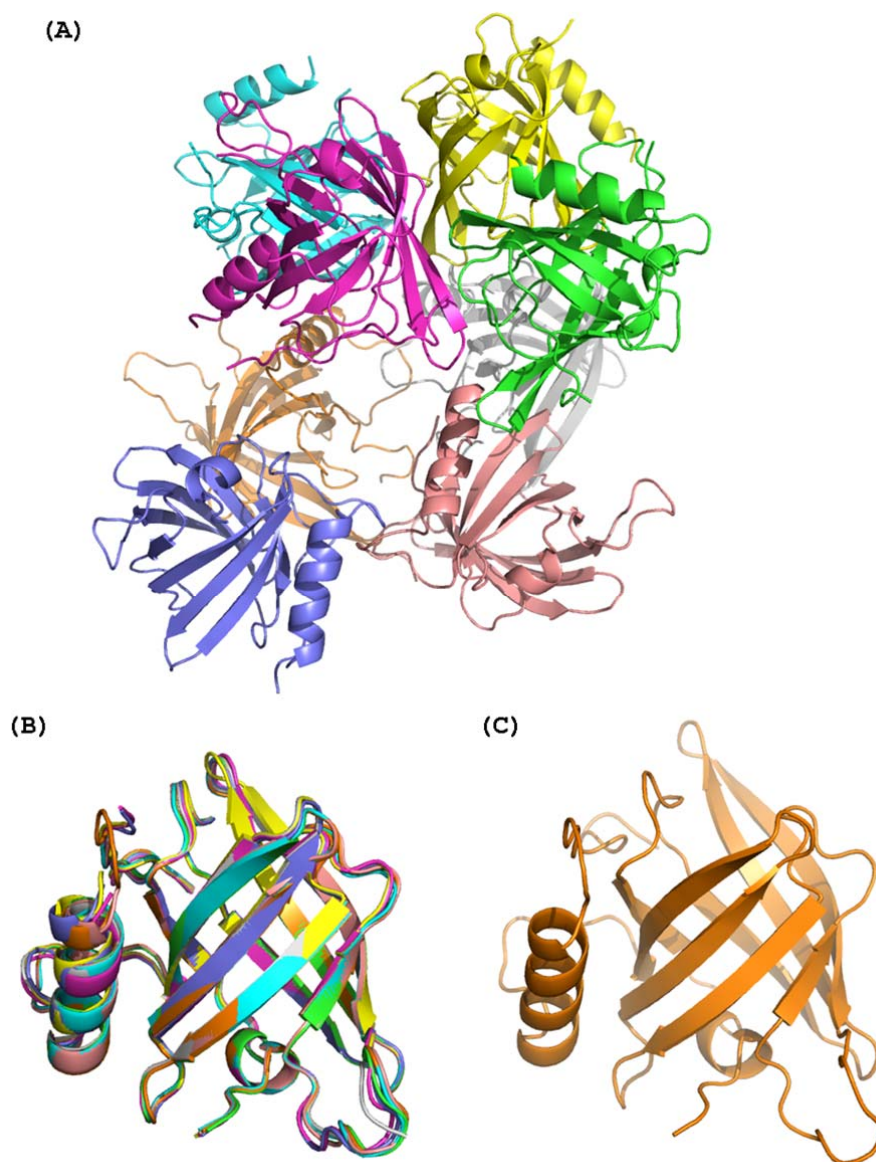


Figure 5.29. Crystal structure of CIRp-A4. (A) The asymmetric unit of the crystal contains eight copies of CIRp-A4. Each copy is coloured differently. (B) Overlay of the eight copies in the asymmetric unit. (C) The final model of CIRp-A4 shows a lipocalin-like fold, characterised by an eight-stranded anti-parallel β -barrel and an α -helix. Chain F from the asymmetric unit is shown here as a representative. Images were produced with PyMOL (Version 2.1, Schrödinger, LLC).

5.6 Structure determination of CIRp-A2

5.6.1 Crystallisation trials with CIRp-A2

Crystallisation trials were set up with refolded CIRp-A2 using the sitting drop vapour diffusion method. Broad screens were set up with a drop size of 0.3 μl at 75% (v/v) and 50% (v/v) protein:mother liquor ratios. The drops were left to equilibrate at 21 $^{\circ}\text{C}$ over a well containing 75 μl of mother liquor. Crystals were observed in multiple conditions (**Figure 5.30**, **Table 5.13**) with CIRp-A2 at 9.6-9.8 mg/ml. The crystals displayed different morphologies; for example, CIRp-A2_1 crystals were needle-like, whereas CIRp-A2_2 crystals looked relatively cubic.

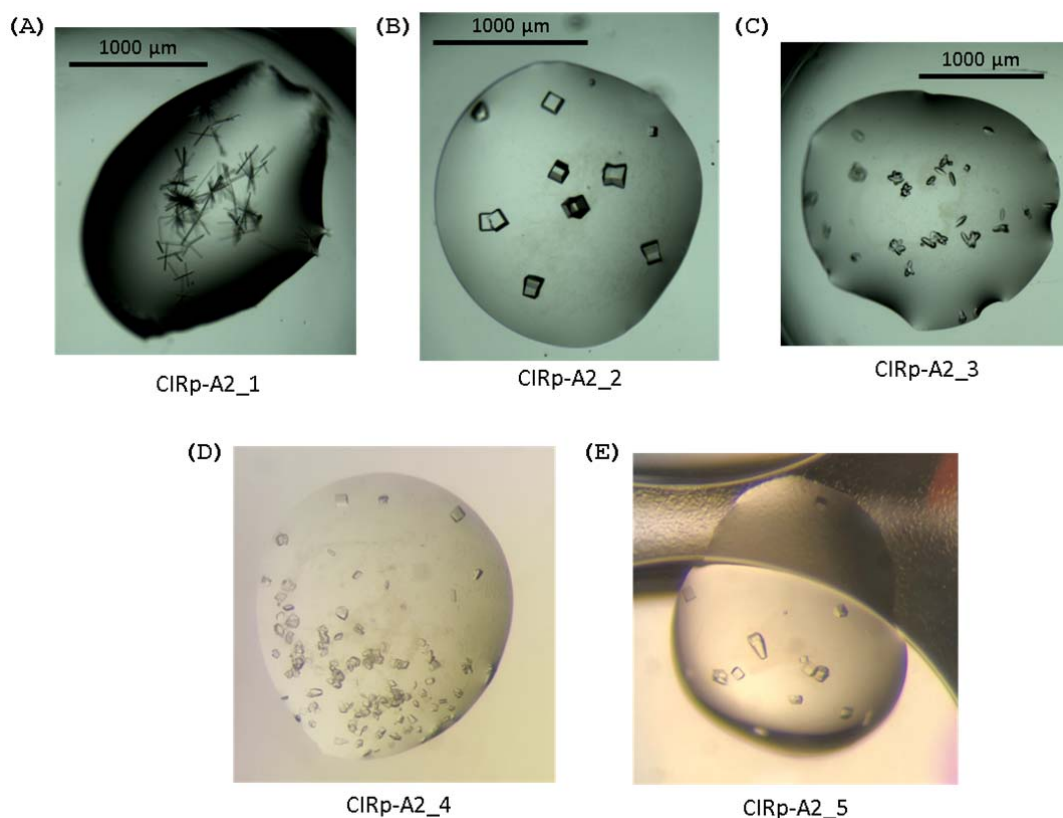


Figure 5.30. Crystals of CIRp-A2. (A)–(E) Crystals of various shapes and sizes were obtained from crystallisation trials with CIRp-A2 at 9.6-9.8 mg/ml. The crystallisation conditions are outlined in Table 5.13.

Table 5.13. Crystallisation and cryoprotection conditions for CIRp-A2 crystals.

Crystal	Crystallisation condition	Cryoprotectant
CIRp-A2_1	0.005 M cadmium chloride hemi(pentahydrate), 0.1 M sodium acetate, pH 5.5, 20% (w/v) PEG 4000	20% (v/v) PEG400 in mother liquor
CIRp-A2_2	0.1 M sodium acetate, pH 5, 36% (w/v) PEG5000 MME	20% (v/v) PEG400 in mother liquor
CIRp-A2_3	0.1 M sodium cacodylate, pH 6.5, 45 % (w/v) PEG 2000 MME	10% (v/v) PEG400 in mother liquor
CIRp-A2_4	0.005 M nickel(II) chloride hexahydrate, 0.1 M sodium acetate, pH 6, 30% (w/v) PEG4000	20% (v/v) PEG400 in mother liquor
CIRp-A2_5	0.2 M ammonium acetate, 0.1 M sodium acetate, pH 4.6, 30% (w/v) PEG4000	10% (v/v) PEG400 in mother liquor

Table 5.14. Processing statistics for CIRp-A2 datasets.

	CIRp-A2 1	CIRp-A2 2	CIRp-A2 3	CIRp-A2 4	CIRp-A2 5
Auto processing	autoPROC	XIA2 3dii	GrenADeS	autoPROC	autoPROC
Space group	$P2_12_12_1$	$P4_12_12$	$P422$	$C222$	$P4_12_12$
Cell dimensions a, b, c (Å)	40.12, 54.33, 79.73	38.95, 38.95, 185.91	39.33, 39.33, 200.06	55.06, 55.05, 91.76	39.67, 39.67, 95.52
α, β, γ (°)	90, 90, 90	90, 90, 90	90, 90, 90	90, 90, 90	90, 90, 90
Resolution	44.90-1.83	61.97-2.10	40.01-1.95	45.88-2.52	36.64-2.29
R_{merge} (overall/inner/outer)	0.077/0.047/ 0.571	0.162/0.080/ 1.777	0.189/0.075/ 0.699	0.094/0.061/ 0.845	0.096/0.058/ 0.466
R_{pim} (overall/inner/outer)	0.043/0.025/ 0.336	0.043/0.025/ 0.446	0.080/0.032/ 0.288	0.041/0.026/ 0.352	0.047/0.028/ 0.203
Unique reflections (overall/inner/outer)	14869/868/ 764	9190/152/ 669	11660/289/ 1026	4965/288/ 255	3738/233/ 197
Multiplicity (overall/inner/outer)	4.0/3.9/3.7	15.2/10.5/ 16.4	6.5/5.9/6.1	6.3/5.9/6.8	5.3/4.9/5.9
Completeness (overall/inner/outer)	93.2/98.1/ 100.0	100.0/99.9/ 100.0	94.9/98.6/ 90.4	100.0/100.0/ 100.0	97.7/96.7/ 100.0
$I/\sigma I$ (overall/inner/outer)	11.0/24.0/2.1	10.9/25.8/1.7	6.5/16.1/1.2	12.6/23.6/2.3	11.3/19.7/3.2
$CC_{1/2}$ (overall/inner/outer)	0.997/0.996/ 0.777	0.998/0.998/ 0.558	0.988/0.998/ 0.756	0.997/0.998/ 0.598	0.993/0.993/ 0.915

5.6.2 CIRp-A2 data collection and processing

Crystals of CIRp-A2 were harvested by Prof. Susan Lea for data collection. The cryoprotectants used for flash freezing in liquid nitrogen are outlined in **Table 5.13**. Data collection for CIRp-A2_2 crystals was performed by Prof. Susan Lea at the I04-1 beamline of Diamond Light Source, using an X-ray beam of wavelength 0.9159 Å. Other crystals were shot at the MASSIF1 beamline of ESRF by automatic data collection, using an X-ray beam of wavelength 0.966 Å. Datasets were processed using XIA2 3dii (Kabsch, 2010; Winter, 2010), GrenADeS (Monaco et al., 2013) or autoPROC (Vonnrhein et al., 2011). The processing statistics for CIRp-A2 datasets are summarised in **Table 5.14**.

5.6.3 Twinning of CIRp-A2 crystals

The CIRp-A2 datasets were analysed by PHENIX Xtriage (Adams et al., 2010) to assess the presence of twinning. Xtriage reports several analyses of the intensity statistics (discussed in **Section 5.5.6**). Untwinned and twinned data are expected to have $\langle I^2 \rangle / \langle I \rangle^2$ values of 2.0 and 1.5, respectively (Stanley, 1972; Yeates, 1997). CIRp-A2 crystals had $\langle I^2 \rangle / \langle I \rangle^2$ values close to theoretically twinned crystals, with the exception of CIRp-A2_1 crystals (**Table 5.15**). Untwinned and twinned data are expected to have $\langle F \rangle^2 / \langle I \rangle$ values of 0.785 and 0.885, respectively (Stanley, 1972). Again, with the exception of CIRp-A2_1 crystals, the other crystals had $\langle F \rangle^2 / \langle I \rangle$ values close to theoretically twinned crystals (**Table 5.15**). Untwinned and twinned data are expected to have $\langle |L| \rangle$ values of 0.500 and 0.375, respectively (Padilla and Yeates, 2003). CIRp-A2_2 to CIRp-A2_5 crystals had $\langle |L| \rangle$ values ranging from 0.287 to 0.387, whereas CIRp-A2_1 crystals had $\langle |L| \rangle$ value of 0.482 (**Table 5.15**). Additionally, untwinned and twinned data are expected to have $\langle L^2 \rangle$ values of 0.333 and 0.200, respectively (Padilla and Yeates, 2003). CIRp-A2_2 to CIRp-A2_5 crystals had $\langle L^2 \rangle$ values ranging from 0.127 to 0.212, whereas CIRp-A2_1 crystals had $\langle L^2 \rangle$ value of 0.318 (**Table 5.15**).

Table 5.15. Xtriage analysis of CIRp-A2 datasets. The presence of twinning was assessed using the moments of intensities distribution and moments of L. The theoretical values for untwinned and twinned crystals are shown.

Dataset	$\langle I^2 \rangle / \langle I \rangle^2$	$\langle F^2 \rangle / \langle I \rangle$	$\langle L \rangle$	$\langle L^2 \rangle$
	Untwinned 2.0 Twinned 1.5	Untwinned 0.785 Twinned 0.885	Untwinned 0.500 Twinned 0.375	Untwinned 0.333 Twinned 0.200
CIRp-A2_1	1.938	0.791	0.482	0.318
CIRp-A2_2	1.578	0.880	0.350	0.180
CIRp-A2_3	1.793	0.851	0.387	0.212
CIRp-A2_4	1.438	0.904	0.314	0.148
CIRp-A2_5	1.425	0.914	0.287	0.127

The intensity statistics of CIRp-A2 datasets are summarised in **Table 5.15**. For CIRp-A2_2 to CIRp-A2_5 crystals, the analyses by Xtriage (Adams et al., 2010) indicated that the intensity statistics are unusual and are significantly different than is expected from reasonable, untwinned data. On the other hand, CIRp-A2_1 crystals did not seem twinned from the Xtriage analysis, and therefore, structure determination was carried out using this dataset.

5.6.4 Structure determination of CIRp-A2

The crystal structure of CIRp-A2 using the dataset from CIRp-A2_1 crystals was solved by Dr. Steven Johnson. The structure solution was obtained by MR using CIRp-A1 as the search model; CIRp-A1 and CIRp-A2 share 48% sequence identity. Final rounds of refinement were performed using PHENIX (Afonine et al., 2012). The final round of refinement resulted in R-work and R-free values of 19.23% and 22.94%, respectively. The refinement statistics and MolProbity analysis (Chen et al., 2010) for the final model of CIRp-A2 are summarised in **Table 5.16**.

Table 5.16. Refinement statistics for the final model of CIRp-A2.

CIRp-A2	
Residues seen in structure	E6-Q160
Resolution range	44.90-1.83 (1.90-1.83)
R-work (%)	19.23 (32.59)
R-free (%)	22.94 (35.24)
Ramachandran outliers (%)	0.65
Ramachandran favoured (%)	97.39
Rotamer outliers (%)	0.69
Clashscore	4.34
MolProbity score	1.33

5.6.5 Crystal structure of CIRp-A2

The structure of CIRp-A2 shows a lipocalin-like fold, similar to CIRp-A1 and other homologs. The structure will be analysed in detail at the end of this chapter (**Section 5.8**).

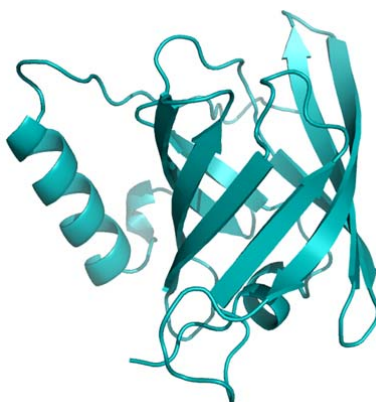


Figure 5.31. Crystal structure of CIRp-A2. The final model of CIRp-A2 shows a lipocalin-like fold. The image was produced with PyMOL (Version 2.1, Schrödinger, LLC).

5.7 Crystallisation attempts with CIRp-A5

5.7.1 Crystallisation trials with CIRp-A5

Crystallisation trials were set up with refolded CIRp-A5 using the sitting drop vapour diffusion method. Broad screens were set up with a drop size of 0.3 μ l at 75% (v/v) and 50% (v/v) protein:mother liquor ratios. The drops were left to equilibrate at 21 °C over a well containing 75 μ l of mother liquor. Trays were set up using CIRp-A5 at various concentrations (**Table 5.17**). From looking at the drops, CIRp-A5 seemed less soluble than other homologs; many drops contained heavy precipitates at 13 mg/ml. In comparison, crystals for CIRp-A1 and CIRp-A6 were obtained at 18 mg/ml and 22 mg/ml, respectively. No crystals were observed after a few weeks.

Table 5.17. Crystallisation trials with CIRp-A5.

Protein concentration	Broad screens
13 mg/ml	Stura FootPrint
10 mg/ml	Stura FootPrint
6 mg/ml	Stura FootPrint, PACT premier, JCSG- <i>plus</i>
5 mg/ml	Stura FootPrint, Structure Screen 1+2, PACT premier, JCSG- <i>plus</i> , Clear Strategy I&II, MIDAS <i>plus</i> , Morpheus, PGA

5.7.2 Limited proteolysis with CIRp-A5

Small-scale limited proteolysis was carried out with CIRp-A5 to assess whether stable fragments could be achieved via proteolysis. Three proteases were tried – chymotrypsin, subtilisin and trypsin. Limited proteolysis reactions were set up as in **Section 5.5.2**. SDS-PAGE analysis showed that all three proteases were capable of cleaving CIRp-A5 at low concentrations; even at 1:10,000 protease:CIRp-A5 ratio, cleavage products were observed on the gel (**Figure 5.32**). None of the proteases resulted in a stable product, as they seemed to cleave CIRp-A5 into many fragments. Therefore, limited proteolysis would be unsuitable to generate stable constructs of CIRp-A5 for crystallisation.

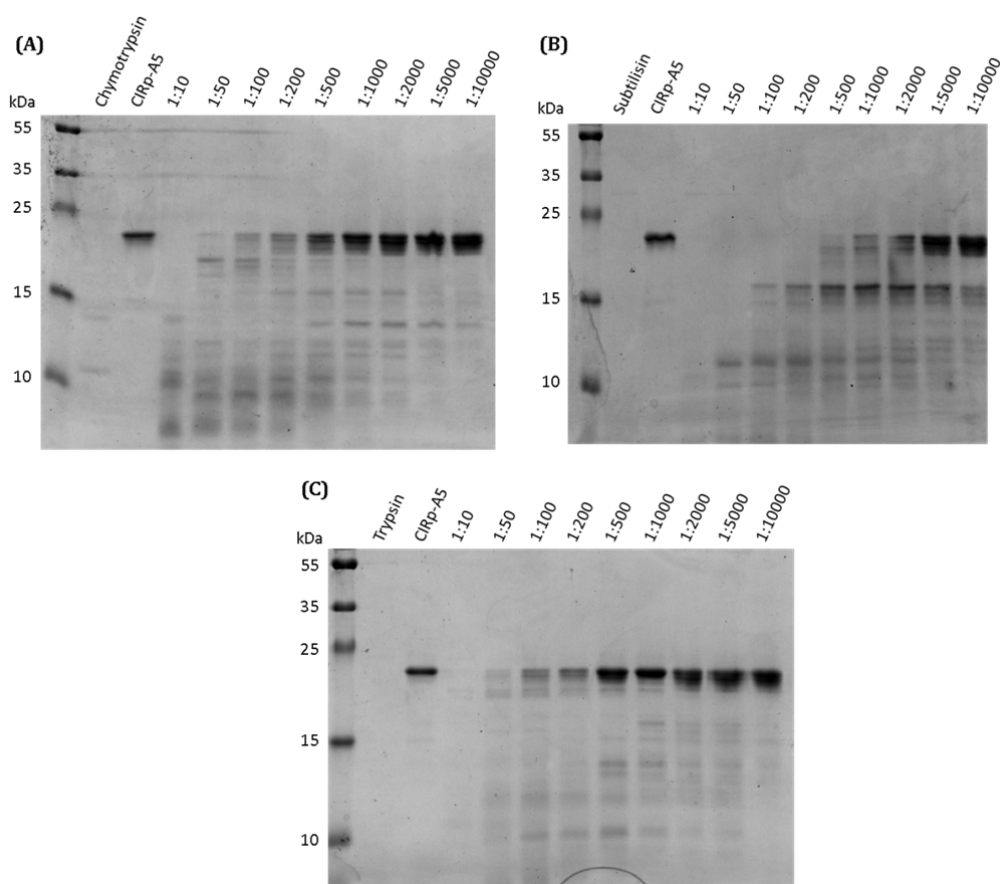


Figure 5.32. Limited proteolysis with CIRp-A5. (A) chymotrypsin, (B) subtilisin, and (C) trypsin were used for limited proteolysis with CIRp-A5, before analysis with reducing 15% SDS-PAGE. The protease:protein (w/w) ratios of the reactions are shown.

5.7.3 Crystallisation trials with truncated CIRp-A5 constructs

Since limited proteolysis did not yield suitable constructs of CIRp-A5 for crystallisation, I designed different constructs by cloning. Based on the sequence alignment of CIRp-A5 and CIRp-A1, the C-terminus was truncated to Q174 (**Figure 5.33**). Based on the structures of CIRp-A1 and CIRp-A6, various N-termini were tried, as this region was observed to be disordered in the solved structures. Three different constructs were cloned, expressed and purified: CIRp-A5(1-174), CIRp-A5(14-174) and CIRp-A5(19-174). These proteins were purified by refolding using the same protocol as for full-length CIRp-A5 (**Section 5.2.2**).

CIRp-A5	1	▼	EDQETDFSST	DGAELIAKEP	EVYPIDQFMN	NTEIWVENTT	QPDPPNCKKD	50
CIRp-A1	1		EDQETDFSST	DGAELIAKEP	EVYPIDQFMN	NTEIWVENTT	QPDPPNCKKD	50
			*****	*****	*****	*****	*****	
CIRp-A5	51		KSKSMTQTAT	SFVRSHVKN	NIIEENLVGN	FTYFNDKEKV	YDGIYISGES	100
CIRp-A1	51		KSKSMTQTAT	SFVRSHVKN	NIIEENLVGN	FTYFNDKEKV	YDGIYISGES	100
			*****	*****	*****	*****	*****	
CIRp-A5	101		SGVYAEHLYY	VSEDKKCGLV	QVFAK---DQ	NVWTELVRVG	HPNYG-SLDA	146
CIRp-A1	101		SGVYAEHLYY	VSEDKKCGLF	QVFAHVNDKT	TIWRDVRVSG	RPEEGVPLEL	150
			*****	*****	*****	*****	*****	
CIRp-A5	147		ECRREYEAYV	KEIKGKKNST	SPYSDDCQIK	V		177
CIRp-A1	151		NCTKEFDEYV	KLVNA--TSK	SPYTSECQ--	--		176
			*****	*****	*****	*****	*****	

Figure 5.33. Constructs of CIRp-A5. Sequence alignment of CIRp-A5 and CIRp-A1 by Clustal Omega is shown. The N-terminus and C-terminus of CIRp-A5 constructs are indicated by blue triangle and red arrow, respectively.

Crystallisation trials were set up with the truncated CIRp-A5 constructs as before. Several concentrations were tried for each construct (**Table 5.18**). These constructs seemed more soluble than full-length CIRp-A5. Compared to the full-length CIRp-A5, for which heavy precipitates were observed for trays at 13 mg/ml, trays were set up at concentrations up to 18 mg/ml for the shorter constructs. Nonetheless, no crystals were observed after a few weeks.

Table 5.18. Crystallisation trials with different CIRp-A5 constructs.

Construct	Protein concentration	Broad screens
CIRp-A5(1-174)	16 mg/ml	Stura FootPrint, JCSG- <i>plus</i>
CIRp-A5(14-174)	10 mg/ml	Stura FootPrint, JCSG- <i>plus</i> , PACT premier, Structure Screen 1+2, Clear Strategy I&II, PGA, Morpheus
CIRp-A5(14-174)	14 mg/ml	PACT premier, Structure Screen 1+2, Clear Strategy I&II, PGA,
CIRp-A5(19-174)	7 mg/ml	Stura FootPrint
CIRp-A5(19-174)	18 mg/ml	Stura FootPrint
CIRp-A5(19-174)	9 mg/ml	Structure Screen 1+2, Clear Strategy I&II, JCSG- <i>plus</i> , PACT premier, LMB, MIDAS <i>plus</i> , Morpheus

5.8 Analysis of crystal structures of CIRp-A1 and homologs

There is overall structural homology between CIRp-A1 and homologs (**Figure 5.34**). One small region of difference is the loop connecting the 7th and 8th β -strands of the barrel. CIRp-A2 and CIRp-A4 have a slightly shorter loop (by three amino acid residues) compared to CIRp-A1 and CIRp-A6 (**Figure 5.35**). CIRp-A2 and CIRp-A4 did not show complement inhibitory activity against any of the animals tested in AP haemolysis assays (**Section 4.3.2**), whereas CIRp-A1 and CIRp-A6 were active in human and rat, respectively. The length of the loop, however, does not seem to determine whether the homolog is functionally active, since CIRp-A5, which also has a shorter loop, showed complement inhibitory activity in monkey serum (**Section 4.3.2**). Additionally, there is no obvious sequence similarity within this loop region between the active homologs (**Figure 5.35**).



Figure 5.34. Crystal structures of CIRp-A1 and homologs. The overlay of structures of CIRp-A1 (purple), CIRp-A2 (teal), CIRp-A4 (orange) and CIRp-A6 (magenta) shows overall structural homology between the four proteins. The region of difference is highlighted with a black circle, where the loops of CIRp-A2 and CIRp-A4 are shorter than those of CIRp-A1 and CIRp-A6. Images were produced with PyMOL (Version 2.1, Schrödinger, LLC).

therefore, should be positively charged under physiological conditions. Mutagenesis studies or the structure of properdin-CIRp-A1 complex would be required to investigate whether the binding of properdin and CIRp-A1 involves charge-charge interactions.

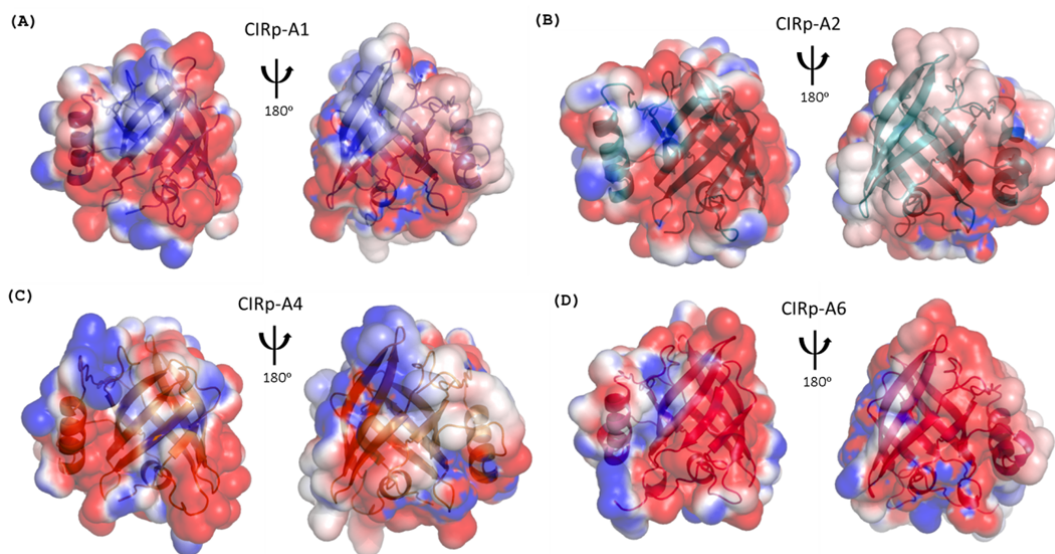


Figure 5.36. The electrostatic surfaces of CIRp-A1 and homologs. Positive (blue) and negative (red) electrostatic potential regions were mapped onto the surface representations of (A) CIRp-A1, (B) CIRp-A2, (C) CIRp-A4, and (D) CIRp-A6. The two orientations (left and right) are related by a 180° rotation around the vertical axis. The electrostatic potentials were calculated with the APBS program in PyMOL (Jurrus et al., 2018) (protein dielectric = 2.0; solvent dielectric = 78.0; ion concentration = 150 mM). Images were produced with PyMOL (Version 2.1, Schrödinger, LLC).

5.9 Discussion

For crystallisation, CIRp-A1 and homologs were expressed in bacteria and purified by refolding (**Section 5.2.1**). Secondary structure formation in the refolded proteins was confirmed by CD spectroscopy, and more importantly, complement inhibitory activity was confirmed in AP WIESLAB assay (**Figure 5.2**). The fact that CIRp-A1 expressed in *E.coli* was still active suggests that the glycosylation of insect-purified CIRp-A1 might not be important for functional activity. The homolog CIRp-A3 was the only one that could not be refolded, at least using the same protocol as for CIRp-A1 and other homologs. CIRp-A3 has nine predicted N-linked glycosylation sites, the majority of which were confirmed to be occupied by native mass spectrometry (**Section 4.2.3**). CIRp-A3 was the only homolog of CIRp-A1 which also inhibited AP haemolysis by human serum (**Section 4.3.2**). The conserved regions between CIRp-A1 and CIRp-A3 are not predicted to be glycosylated (**Figure 4.4**), further suggesting that the sugars may not be important for complement inhibitory function. Salp20, also a properdin-binding tick complement inhibitor, was previously proposed to bind properdin via N-linked glycosylation (Tyson et al., 2008). In the case of CIRp-A3, the glycans might be required for correct protein folding or for aiding solubilisation, for example, by masking hydrophobic patches on the surface. A homology model of CIRp-A3, generated with the SWISS-MODEL server (Waterhouse et al., 2018) and the structure of CIRp-A1, shows that the predicted N-linked glycosylation sites are located in proximity to hydrophobic patches (**Figure 5.37**).

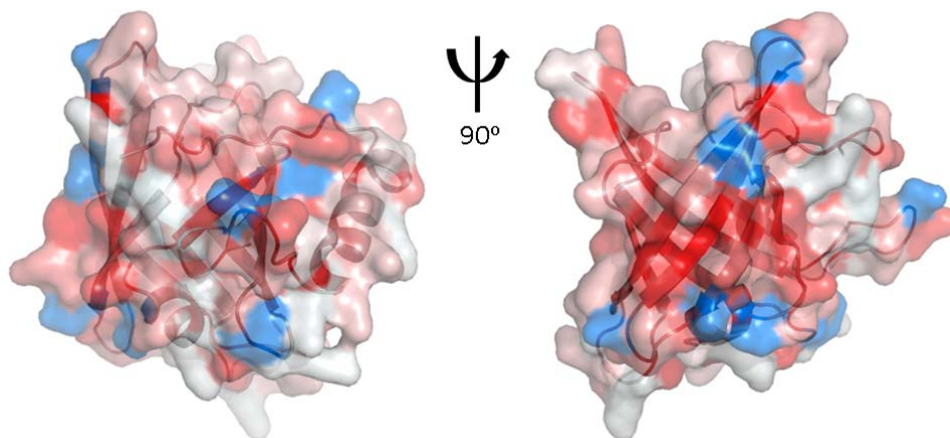


Figure 5.37. Hydrophobic surface analysis of CIRp-A3. A structure model of CIRp-A3 was generated with the SWISS-MODEL server by homology modelling with CIRp-A1 (Waterhouse et al., 2018). The hydrophobic surface (red) was modelled using the “color_h” script which colours the protein molecule according to the Eisenberg hydrophobicity scale (Eisenberg et al., 1984). Predicted N-linked glycosylation sites from the NetNGlyc 1.0 Server are coloured in blue. Images were produced with PyMOL (Version 2.1, Schrödinger, LLC).

The refolded CIRp-A1 and homologs were used for crystallisation trials. I have solved the crystal structure of CIRp-A1 (**Figure 5.11**), which reveals a lipocalin-like fold. The characteristic protein fold of lipocalins – eight-stranded β -barrel and C-terminal α -helix – is conserved amongst the members of the lipocalin family despite low sequence identity (Adam et al., 2008). As observed in the structures of other lipocalins (Paesen et al., 1999), the disulphide bonds were conserved in the structure of CIRp-A1; these disulphide bonds might be important for the tertiary structure of CIRp-A1. The only difference in the structure of CIRp-A1 from typical lipocalins is the lack of a central cavity within the β -barrel where small and hydrophobic molecules can bind (Adam et al., 2008). Tick lipocalins use this cavity to sequester pro-inflammatory molecules (Beaufays et al., 2008). In comparison to published structures of tick lipocalins, the middle of the β -barrel of CIRp-A1 is relatively packed with side chains (**Figure 5.12**), so it is unlikely to bind other molecules. No bound species were identified from native mass spectrometry analysis of CIRp-A1 (**Section 4.2.3**).

I have also solved the crystal structures of the homologs CIRp-A6 (**Figure 5.16**), CIRp-A4 (**Figure 5.29**) and CIRp-A2 (**Figure 5.31**). All homologs display conserved overall protein fold and the four cysteine residues are also conserved. The middle of the β -barrel of the homologs is also relatively packed with side chains, therefore the homologs are

also unlikely to sequester small molecules (data not shown). The non-conserved residues between CIRp-A1 and CIRp-A6 were primarily located within the loops between the β -strands of the barrel (**Figure 5.16C**). As these loops had previously been proposed to contribute to antigenic variation in lipocalins (Skerra, 2000), they could be giving rise to the different activities of CIRp-A1 and CIRp-A6 in AP haemolysis assays (**Section 4.3.2**).

When the structures of CIRp-A1 and homologs were compared, the overall protein fold was conserved (**Figure 5.34**); however, the loop connecting the 7th and 8th β -strands of the β -barrel was three amino acids shorter in CIRp-A2 and CIRp-A4 than in CIRp-A1 and CIRp-A6 (**Figure 5.29C**). Due to the lack of sequence identity within and around this loop in the homologs which displayed complement inhibitory activity in AP haemolysis assays (**Section 4.3.2**), the sequence might not be important for activity. The length of the loop and/or the overall protein fold around this region might be more important, especially if these proteins utilise backbone interactions to bind properdin. The use of backbone interactions would enable the inhibitors to maintain complement inhibitory activity despite high sequence divergence, as observed with the RaCI family (Jore et al., 2016). Additionally, the overall surface charge of the inhibitors might be important for properdin binding, as properdin is a positively charged molecule across different species. The homologs which displayed complement inhibitory activity in AP haemolysis assays (CIRp-A1, CIRp-A6) were more negatively charged than the ones that did not (CIRp-A2, CIRp-A4) (**Figure 5.36**). In summary, mutagenesis and interaction studies would be required to investigate the functional relevance of the β 7- β 8 loop region and the surface charges. These are discussed in **Chapter 6**.

CHAPTER 6: CHARACTERISATION OF THE INTERACTION BETWEEN PROPERDIN AND CIRP-A1

6.1 Introduction

In **Chapter 5**, I described the structure determination of a novel set of properdin-binding tick complement inhibitors. The structures of CIRp-A1 and its homologs displayed the characteristic lipocalin fold (Adam et al., 2008). Based on their structures and results of inhibition assays from **Chapter 4**, I hypothesised that a loop might contribute to host specificity and complement inhibitory activity. Alternative pathway haemolysis assays using human and other animal sera showed that CIRp-A1 and homologs have different complement inhibitory activity profiles in different animals (**Section 4.3.2**). I have solved the crystal structures of CIRp-A1 and CIRp-A6, which were active in human and rat, respectively, and CIRp-A2 and CIRp-A4, which were inactive in the animals tested. Structure comparison of the active homologs (CIRp-A1 and CIRp-A6) and the inactive homologs (CIRp-A2 and CIRp-A4) showed that there was conservation of the overall lipocalin protein fold, but the loop connecting the 7th and 8th β -strands of the β -barrel was shorter in the inactive homologs. In this chapter, the functional relevance of this loop is investigated via mutagenesis studies. Additionally, the binding site on properdin is investigated via pulldown assays. Towards understanding the molecular basis of properdin inhibition by these inhibitors, the effect of CIRp-A1 on properdin-C3b binding is also analysed, and crystallisation trials of the properdin-CIRp-A1 complex were carried out. For purification and crystallisation trials of the complex, engineered monomeric properdin FPc and FPc Δ 3 were used (Pedersen et al., 2019).

6.2 Probing the binding site on CIRp-A1 by mutagenesis

6.2.1 Analysis of the crystal structures of CIRp-A1 and homologs for the design of mutants

Analysis of the crystal structures of CIRp-A1 and homologs was discussed in detail in **Section 5.8**. In brief, the comparison of structures and sequences of the CIRp-A1 family revealed that the active homologs (CIRp-A1, CIRp-A3, CIRp-A6) have a slightly longer

loop between the 7th and 8th β -strands of the β -barrel, compared to the inactive homologs (CIRp-A2, CIRp-A4) (**Figure 6.1**); the activities of the homologs were assessed using different animal sera in AP haemolysis assays (**Section 4.3.2**). Sequence comparison of all six CIRp-A1 homologs shows that there is a lack of sequence conservation within and around the β 7- β 8 loop of the active homologs, suggesting that the sequence of the loop might not be important for complement inhibitory activity. Instead, the length of the loop and the overall protein fold might be more important if CIRp-A1 utilises backbone interactions to bind properdin. This binding mode has been observed with the RaCI family of C5-binding tick complement inhibitors (Jore et al., 2016).



Figure 6.1. Overlay of structures of CIRp-A1 homologs. The overlay of structures of CIRp-A1 (purple), CIRp-A6 (magenta), CIRp-A4 (orange) and CIRp-A2 (teal) shows a difference in length of one of the loops of the β -barrel. The image was produced with PyMOL (Version 2.1, Schrödinger, LLC).

To investigate whether the aforementioned loop contributes to the inhibitory activity of CIRp-A1, I carried out mutagenesis studies. For the mutants, residues within the loop were either substituted with alanine residues or deleted to mimic the shorter loop. In parallel, CIRp-A5 mutagenesis was also carried out. CIRp-A5 shares 82% sequence identity with CIRp-A1, but CIRp-A5 was only active in monkey serum (**Section 4.3.2**), despite human and monkey properdin sharing 93% sequence identity. As CIRp-A5 has a shorter β 7- β 8 loop compared to CIRp-A1 (**Figure 6.2**), I added three amino acid residues into the loop of CIRp-A5, to see whether the mutant of CIRp-A5 with a longer loop might be functionally active. The mutants of CIRp-A1 and CIRp-A5 are summarised in **Table 6.1**.

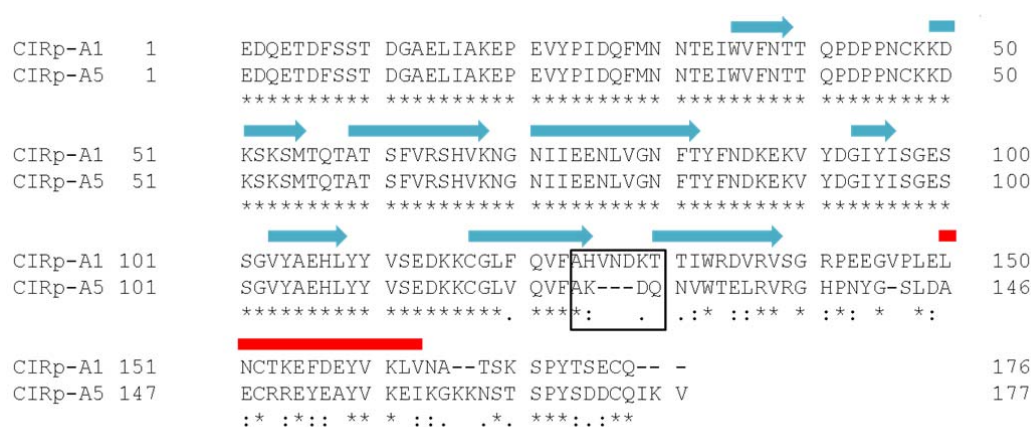


Figure 6.2. Design of CIRp-A1 and CIRp-A5 mutants. Sequence alignment of CIRp-A1 and CIRp-A5 by Clustal Omega is shown. Based on the structure of CIRp-A1, secondary structure elements are indicated: α -helix with a red rectangle and β -strand with a blue arrow. Mutants were designed for the β 7- β 8 loop region highlighted with a black box. Residues within this loop of CIRp-A1 were substituted with alanine, or deleted. Based on the sequence alignment, CIRp-A5 has a shorter loop compared to CIRp-A1; for CIRp-A5 mutant, the ‘extra’ residues VND were added into CIRp-A5.

Table 6.1. Mutants of CIRp-A1 and CIRp-A5. Mutants of CIRp-A1 and CIRp-A5 were designed based on the structures of CIRp-A1 and homologs.

Mutant	Mutations	Description
CIRp-A1 mutant A	N127A/D128A/K129A	Alanine substitution of loop residues
CIRp-A1 mutant B	V126_D128del	Deletion of loop residues (V126, N127, D128)
CIRp-A5 mutant	K125_D126insVND	Insertion of CIRp-A loop residues between K125 and D126

6.2.2 Purification of CIRp-A1 and CIRp-A5 mutants by refolding

Cloning of the mutants was carried out by Gibson Assembly using the NEBuilder HiFi DNA Assembly Master Mix (NEB) (protocol in **Section 2.2.1**). The plasmids were then transformed into *E. coli* BL21 (DE3) for expression and purification. The mutants were purified using the same refolding protocol as CIRp-A1 (**Section 5.2.1**). All mutants displayed similar SEC profiles to wild-type proteins – void peak around 0.35 CV and protein peak around 0.55 CV (**Figure 6.3**). For all mutants, there were still contaminants present after SEC when analysed by SDS-PAGE; therefore, SEC was repeated to achieve purified proteins for activity assays (data not shown).

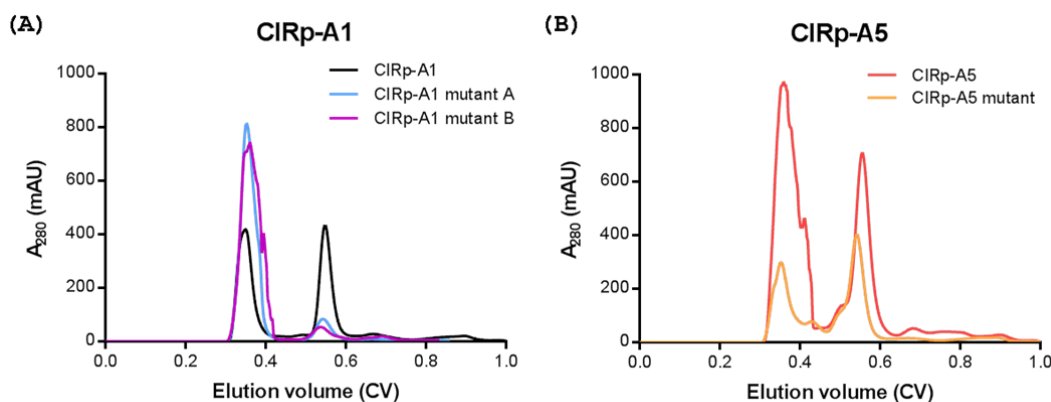


Figure 6.3. SEC profiles of refolded CIRp-A1 and CIRp-A5 mutants. S75 26/60 size exclusion profiles of mutants of (A) CIRp-A1 and (B) CIRp-A5. Mutants were purified by SEC following the refolding protocol. The running buffer for SEC was 50 mM Tris, 150 mM NaCl, pH 8.

6.2.3 CD analysis to assess secondary structure of CIRp-A1 and CIRp-A5 mutants

CD analysis was performed with the mutants and compared with wild-type proteins to assess protein folding (**Figure 6.4**). The proteins were dialysed against 10 mM sodium phosphate buffer, pH 8. The CD spectra of the mutant proteins overlaid nicely with the CD spectra of the wild-type protein, suggesting that all mutants were properly folded.

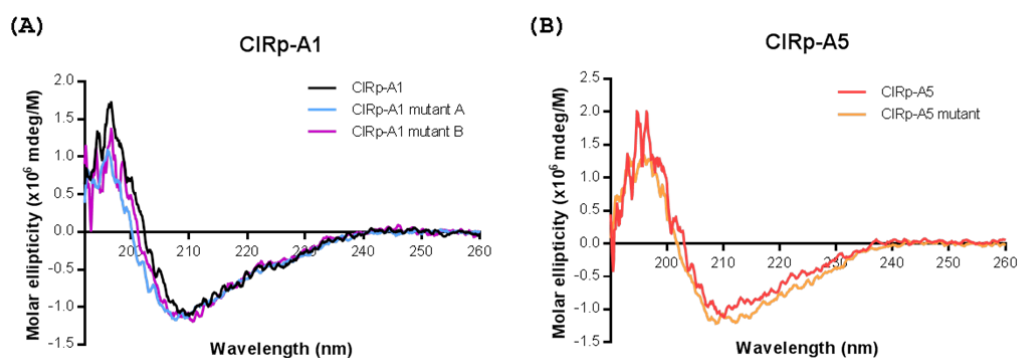


Figure 6.4. CD analysis of CIRp-A and CIRp-A5 mutants. Purified mutants were analysed by CD spectroscopy to assess protein folding. The CD buffer was 10 mM sodium phosphate buffer, pH 8. When compared to wild-type proteins, all mutant proteins were similarly folded.

6.2.4 Measuring complement inhibition by CIRp-A1 and CIRp-A5 mutants in alternative pathway WIESLAB assay

AP WIESLAB assay was carried out to assess the complement inhibitory activities of CIRp-A1 and CIRp-A5 mutants. Both CIRp-A1 mutants showed decreased activity compared to the wild-type (**Figure 6.5**). CIRp-A1 mutant A (alanine substitution) and CIRp-A1 mutant B (deletion) had 2-fold and 10-fold higher IC₅₀ values compared to the wild-type, respectively (**Table 6.2**). Since the alanine mutant is less affected than the deletion mutant, the loop length seems to be important for activity. However, adding the three extra residues into CIRp-A5 did not change the activity of CIRp-A5 (**Figure 6.5**), indicating that it is not a simple correlation. Although there was a decrease in activity, shortening the loop did not completely abolish the activity of CIRp-A1, which indicates that there are some other elements required for its activity. CIRp-A1 and CIRp-A5 are conserved up until the β 7- β 8 loop, suggesting that the C-terminal region of the proteins contributes to host specificity (**Figure 6.2**). Analysis of the residues before and after the three extra residues of the β 7- β 8 loop in CIRp-A1 homologs (**Figure 5.35**) showed that there is no correlation between the types of residues and whether the homolog has an insert. Further analyses of the sequences and structures of CIRp-A1 homologs, combined with mutagenesis studies, would be required to map the binding site for properdin on CIRp-A1, but were not undertaken due to time constraints.

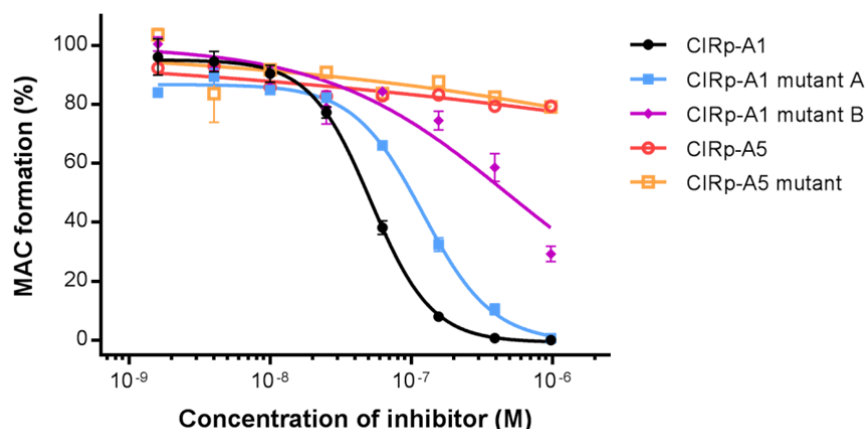


Figure 6.5. AP WIESLAB assay with CIRp-A1 and CIRp-A5 mutants. AP WIESLAB assay was carried out to assess the complement inhibitory activities of mutants of CIRp-A1 and CIRp-A5. CIRp-A1 mutant B had reduced activity compared to wild-type CIRp-A1. Values were normalised for no-serum samples (0% MAC formation) and no-additive samples (100% MAC formation). Error bars, s.e.m. (n = 3). Curve fitting was carried out in GraphPad Prism using a dose response inhibition (variable slope) model.

Table 6.2. Inhibition parameters of CIRp-A1 and mutants. Curve fitting using a dose response dose response inhibition (variable slope) model and statistical analyses were performed using GraphPad Prism to obtain IC₅₀ values. 95% CI represents the 95% confidence intervals for the obtained IC₅₀ values. R square is a statistical measure of how close the data are to the fitted curve.

Protein	IC ₅₀ (M)	95% CI (M)	R square
CIRp-A1	5.2 x 10 ⁻⁸	4.89 x 10 ⁻⁸ to 5.47 x 10 ⁻⁸	0.999
CIRp-A1 mutant A	1.2 x 10 ⁻⁷	1.00 x 10 ⁻⁷ to 1.51 x 10 ⁻⁷	0.998
CIRp-A1 mutant B	4.8 x 10 ⁻⁷	2.67 x 10 ⁻⁷ to 1.19 x 10 ⁻⁶	0.907

6.3 Probing the binding site on properdin with pulldown assays

6.3.1 Small-scale transient expression and purification of TSR domains of properdin in CHO cells

To probe the binding site for CIRp-A1 on properdin, I recombinantly expressed and purified various combinations of properdin TSR domains in CHO cells (**Figure 6.6**), and used these in pulldown assays. For small-scale transient expression, the TSR domains were cloned into pcDNA3.1(+) vector (as discussed in **Section 3.7.1**). For transfection of a 30 ml CHO cell culture in a T-175 flask, 7.5 µg of plasmid DNA was added to 1.5 ml of Opti-MEM, and incubated for 2 min. 60 µl of PEI (1 mg/ml in PBS, filter-sterilised) was then added to the DNA/Opti-MEM mixture, and incubated for 10 min. During the incubation, cells were washed with 30 ml of PBS and 30 ml of supplemented DMEM was added to the flask. The DNA/PEI/Opti-MEM mixture was added dropwise to the cells, and then left at 37 °C. The supernatants were harvested four days after transfection for small-scale purification.

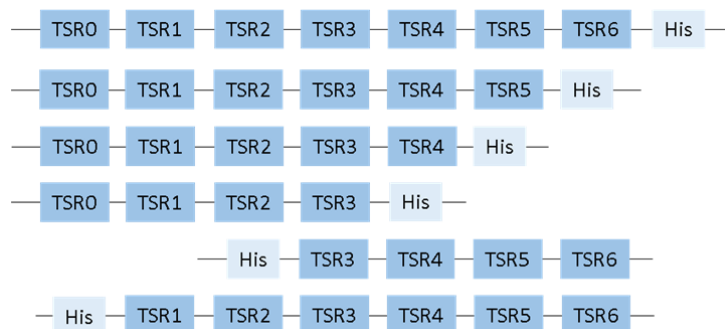


Figure 6.6. Properdin constructs for CHO transient expression and purification. Different combinations of TSR domains were expressed and purified from CHO cells, to be used in pulldown assays with CIRp-A1.

For small-scale purification, 200 µl of cComplete His-Tag Purification Resin (Roche) was added to 20 ml of filtered supernatant. The resin was left to bind to properdin overnight at 4 °C. The resin was then collected by centrifugation and transferred to a microcentrifuge tube. It was washed with PBS, pH 7.4, and then the protein was eluted with PBS containing 250 mM imidazole, pH 7.4. For all constructs shown in **Figure 6.6**, expression was confirmed by Western blot with α -properdin antibody (**Figure 6.7A**).

6.3.2 Pulldown assays to assess binding of TSR domains to immobilised CIRp-A1

For pulldown assays, CIRp-A1 was immobilised onto Pierce NHS-Activated Magnetic Beads (Thermo Scientific). Coupling of the beads was performed following the manufacturer's instructions. 50 µl of the beads were placed into a microcentrifuge tube. Using a magnetic stand, the beads were collected and the supernatant was discarded. 1 ml of wash buffer A (ice-cold 1 mM hydrochloric acid) was added to the tube and then mixed by shaking. After discarding the supernatant, 500 µl of protein solution (1 mg/ml) was immediately added to the beads and mixed by shaking. For the negative control (empty beads), 500 µl of PBS, pH 7.4, was added. The coupling was left to take place overnight on a rotary wheel at 4 °C. Following coupling, the beads were washed twice with 1 ml of wash buffer B (0.1 M glycine, pH 2) and then once with 1 ml of water. The beads were then incubated with 1 ml of quenching buffer (3 M ethanolamine, pH 9) on a rotary wheel for 1 h at room temperature. The beads were then washed once with 1 ml of water and three times with 1 ml of storage buffer (PBS, pH 7.4). They were re-suspended in 50 µl of storage buffer and stored at 4 °C until use.

For pulldown assays, 25 µl of the CIRp-A1 beads were used per reaction. The binding/wash buffer was 25 mM Tris, 200 mM NaCl, 0.05% (v/v) Tween 20, pH 7.5. For each reaction, 500 µl of the purified TSR domains from small-scale transient expression described above (**Section 6.3.1**) were used. The positive control reaction was with 100 µl of serum and 400 µl of binding buffer. For all constructs, a negative control was carried out using empty beads. The reactions were incubated on a rotary wheel for 1 h at room temperature. Following incubation, the beads were washed five times with 500 µl of wash buffer. They were then re-suspended in 50 µl of 1x non-reducing SDS-PAGE loading dye.

Western blot analysis of the reactions with α -properdin antibody showed that CIRp-A1 could bind to serum properdin, but not TSR0-3, TSR0-4 and TSR0-5, which suggested that CIRp-A1 binds to TSR6 or only binds to full-length properdin, for example, by binding to vertices formed by oligomers. Subsequent pulldown assays showed that CIRp-A1 binds to recombinantly expressed fragments containing TSR6 (TSR1-6 and TSR3-6) (**Figure 6.7B**). This suggests that CIRp-A1 binds to TSR6 domain of properdin and that the vertices are not necessary for CIRp-A1 binding as TSR3-6 should not form them.

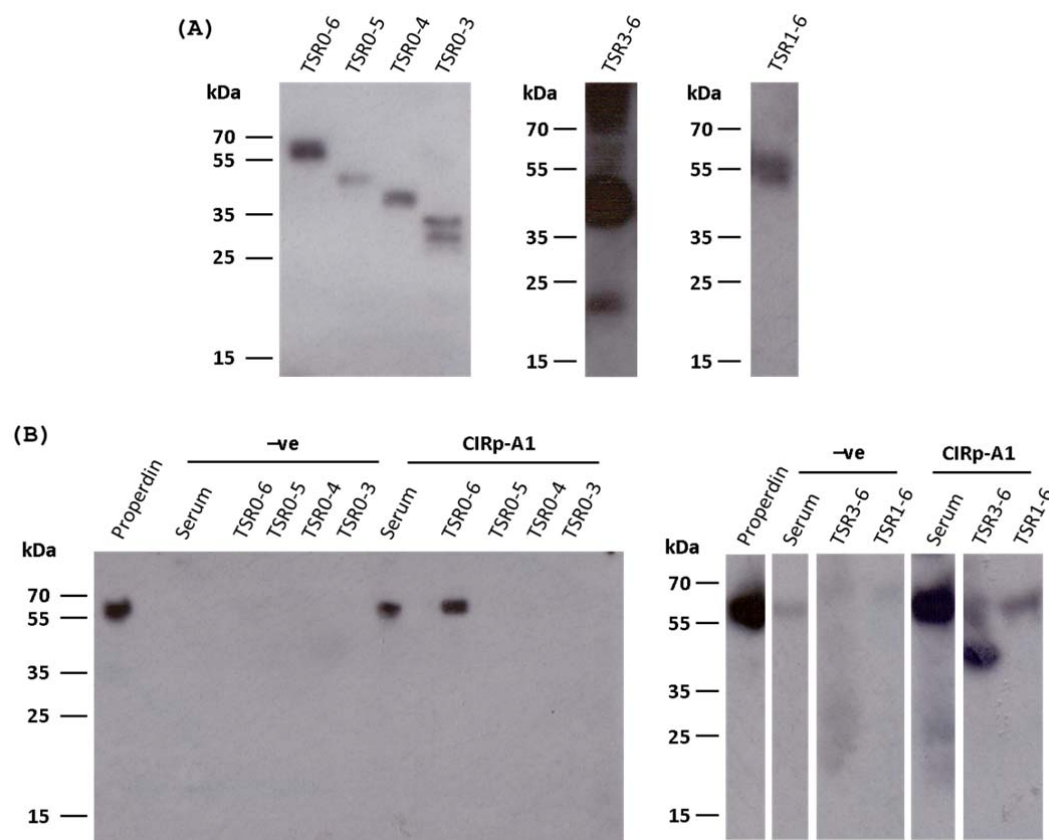


Figure 6.7. Pulldown assays using immobilised CIRp-A1 to probe the binding site for CIRp-A1 on properdin. (A) Western blots with α -properdin antibody confirm expression of different properdin constructs. (B) Pulldown assays followed by Western blots with α -properdin antibody show that CIRp-A1 only binds to recombinantly expressed fragments containing TSR6.

TSR6 domain had been indicated in binding to C3b. Recombinantly expressed properdin mutant lacking TSR6 (Higgins et al., 1995) or properdin TSR6 mutant (Y414D) from type III properdin deficiency patients (Fredrikson et al., 1996) could not bind C3b. Structural studies had indicated that TSR5 interacts with C3b (Pedersen et al., 2017) and TSR6 has a large loop which interacts with TSR5 (Pedersen et al., personal communication). Therefore, CIRp-A1 binding to TSR6 could disrupt the conformation of TSR5, affecting C3b binding. Alternatively, CIRp-A1 might disrupt the oligomerisation of properdin by binding to TSR6 domain. TSR6 had been implicated in oligomerisation via mutagenesis (Higgins et al., 1995) and structural studies (Alcorlo et al., 2013; Pedersen et al., 2017). Higher order oligomers had been shown to be more functionally active than lower order oligomers (Blatt et al., 2016b; Pangburn, 1989).

6.4 Biophysical analyses of properdin-CIRp-A1 interaction

6.4.1 SEC-MALS to investigate binding ratio of properdin and CIRp-A1

SEC-MALS experiments were used to investigate the binding ratio of properdin and CIRp-A1. SEC-MALS was performed by Dr. Steven Johnson. Serum properdin (purification described in **Section 3.3**) was analysed by SEC-MALS to test whether CIRp-A1 was inhibiting properdin by monomerising it. Superdex 200 10/300 GL column was used and the running buffer was PBS, pH 7.4. The overlapping of the peaks of the properdin oligomers made it difficult to interpret the masses accurately, but the major peak is likely a trimer and the minor peak a dimer. Both peaks shifted in the presence of CIRp-A1 (**Figure 6.8**), indicating that CIRp-A1 bound to all oligomeric forms of properdin. This suggests that the binding of CIRp-A1 is not prevented by the formation of oligomers and that CIRp-A1 does not make the oligomers fall apart.

As the binding ratio could not be accurately calculated from serum properdin, the pseudo-monomeric recombinant properdin constructs FPc and FPc Δ 3 (Pedersen et al., 2019) were used for SEC-MALS. The purification of these constructs is described in **Section 3.7.6**. Additionally, purified FPc Δ 2,3 (pseudo-monomeric properdin lacking TSR2 and TSR3) was acquired from the lab of Gregers Andersen, and this construct was also analysed by SEC-MALS. The calculated masses from SEC-MALS of FPc-CIRp-A1, FPc Δ 3-CIRp-A1 and FPc Δ 2,3-CIRp-A1 complexes suggest that properdin and CIRp-A1 bind in 1:1 ratio (**Figure 6.9**), and that TSR2 and TSR3 domains are not required for binding CIRp-A1, consistent with the results of the pulldown assays in **Section 6.3.2**.

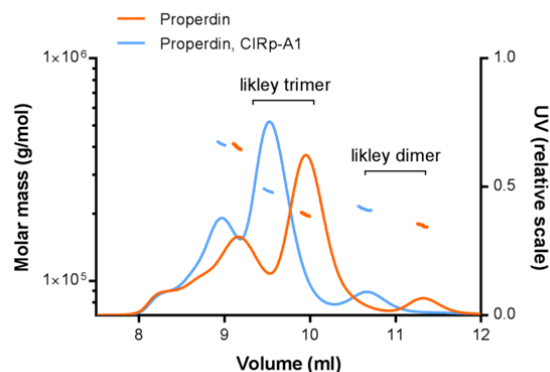


Figure 6.8. SEC-MALS analysis of serum properdin with CIRp-A1. Serum properdin was analysed by SEC-MALS using a Superdex 200 10/300 GL column in the absence (orange) or presence (blue) of CIRp-A1. CIRp-A1 could bind to all oligomeric forms of properdin. The running buffer for SEC-MALS was PBS, pH 7.4

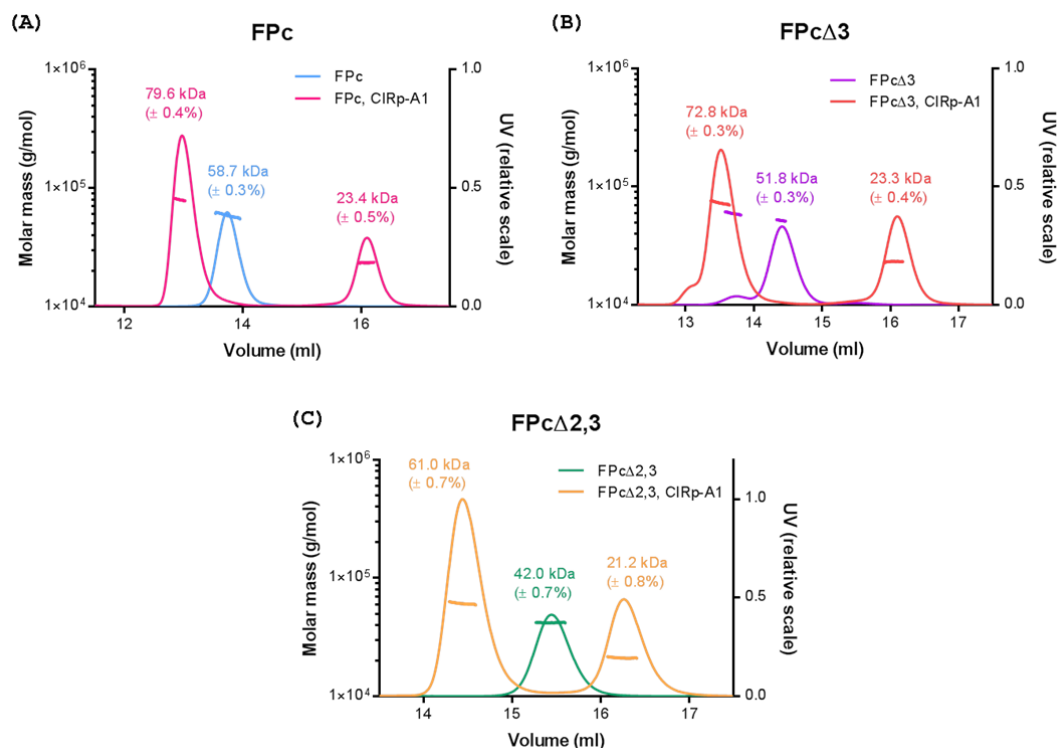


Figure 6.9. SEC-MALS analysis of FPc constructs with CIRp-A1. The molecular masses of monomeric FPc, FPcΔ3 and FPcΔ2,3 were analysed by SEC-MALS in the absence or presence of CIRp-A1. CIRp-A1 was added at 3-5 times molar excess of properdin. The calculated masses of FPc-CIRp-A1, FPcΔ3-CIRp-A1 and FPcΔ2,3-CIRp-A1 complexes suggest a 1:1 binding ratio between properdin and CIRp-A1. Superdex 200 10/300 GL column was used and the running buffer was PBS, pH 7.4.

6.4.2 SPR to determine binding affinity between properdin and CIRp-A1

Surface plasmon resonance (SPR) experiments were carried out to determine the binding affinity of properdin and CIRp-A1. In a SPR experiment, one of the binding partners (ligand) is immobilised on the surface, while the other (analyte) is in solution and passed over the surface. The interaction is detected via changes in the refractive index in the vicinity of a thin gold layer on the surface (Nguyen et al., 2015).

For SPR, pseudo-monomeric properdin FPc and FPc Δ 3 were used (Pedersen et al., 2019). SPR experiments were performed at 25 °C. The protocol for SPR experiments is described in **Section 2.4.4**. In brief, properdin was immobilised onto a Series S Sensor Chip CM5 (GE Healthcare) via amine coupling. Coupling was carried out at a flow rate of 10 μ l/min. The chip surface was activated with a 1:1 mixture of EDC and NHS for 5 min, and then properdin was covalently immobilised in acetate buffer, pH 5. Any remaining sites were blocked by injecting 1 M ethanolamine for 10 min.

SPR experiments were carried out at a flow rate of 30 μ l/min. The running buffer was 10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.005% (v/v) Tween 20, pH 7.4. CIRp-A1 was flowed over immobilised properdin FPc or FPc Δ 3. The following concentrations of CIRp-A1 were used: 1200 nM, 600 nM, 300 nM, 150 nM, 75 nM, 37.5 nM and 18.75 nM. CIRp-A1 was injected for 120 s, followed by 300 s dissociation and 300 s ‘regeneration’ with the running buffer. All injection series were repeated three times. Reference channel without immobilized protein was used for background subtraction.

Data analysis was performed using the BIAevaluation Software (GE Healthcare). An example of a series of curves is shown in **Figure 6.10**. The most commonly used binding model is the 1:1 Langmuir binding model, which describes a 1:1 interaction and assumes that the binding reactions are equivalent and independent at all binding sites. As properdin and CIRp-A1 bind in 1:1 ratio, as determined by SEC-MALS, the Langmuir binding model was tried for curve fitting. The fit can be assessed by the chi-square (χ^2) value which is a measure of the closeness of the fitted curve to the experimental data. The residual plot is also used in parallel to assess the fit, which shows the difference in the response unit (RU) between the experimental and calculated values for each data point.

Ideally, the residuals should scatter randomly around zero over a narrow range that is acceptable as short-term noise for the experiment conducted.

Curve fitting using 1:1 Langmuir binding model gave K_D of 284 nM (**Figure 6.10**) with χ^2 value of 7.34. Although the χ^2 value was reasonable, the residual plot showed large variations in residuals. Therefore, K_D could not be concluded from this model with confidence. Alternatively, K_D can be determined from equilibrium analysis. The response at equilibrium (R_{eq}) was plotted against CIRp-A1 concentration, and curve fitting was carried out using the steady state affinity model. Equilibrium analysis of CIRp-A1 on immobilised FPc suggested K_D of 285 nM ($\chi^2 = 0.0245$) (**Figure 6.11**). It should be noted that equilibrium analysis must be performed using flat regions of the responses; however, when injecting CIRp-A1 over FPc, the responses did not reach equilibrium (**Figure 6.10**). Therefore, the equilibrium analysis and the derived K_D are inaccurate; however, this was the only analysis which could be conducted with the data. The curves might not be reaching equilibrium due to self-association of CIRp-A1. SPR experiments were also carried out with CIRp-A1 flowing over the FPc Δ 3-coated surface; however, the fit was also poor ($\chi^2 = 7.21$, large variations in residuals) and K_D could not be determined (**Figure 6.12**).

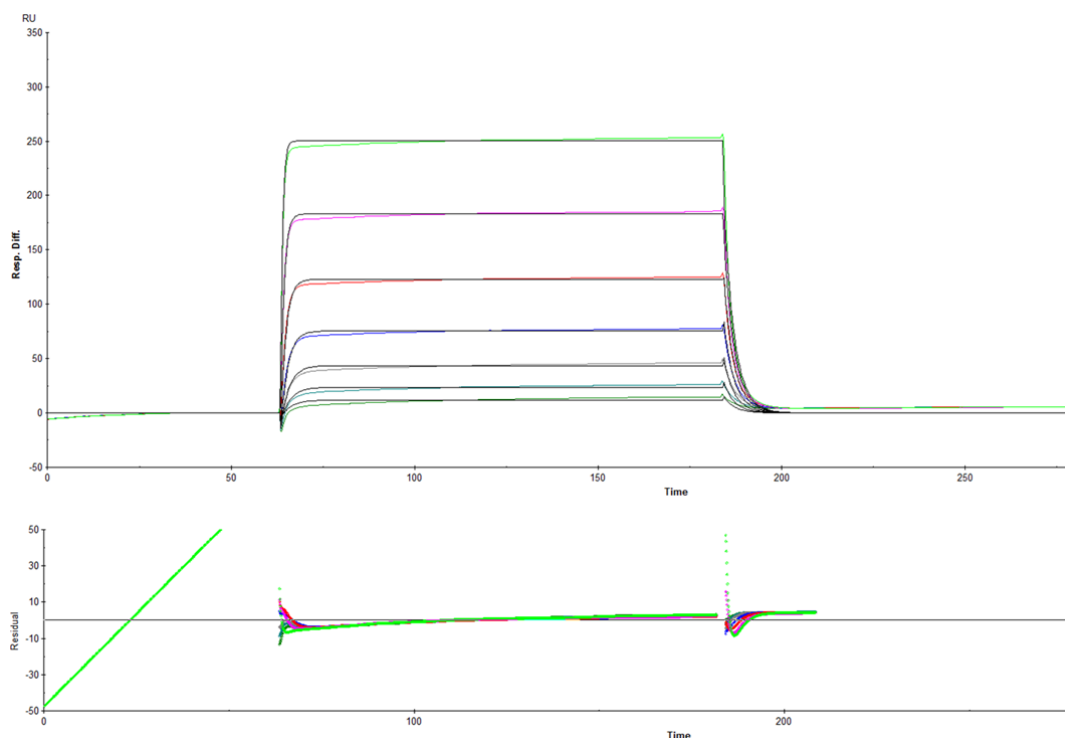


Figure 6.10. SPR analysis of CIRp-A1 on immobilised FPc, using 1:1 Langmuir binding model. CIRp-A1 was injected at concentrations ranging from 18.75 nM to 1200 nM. K_D calculation was attempted by fitting the data to a 1:1 Langmuir binding model (top). χ^2 value of 7.34 and the residual plot (bottom) indicated poor fit, and K_D could not be determined with confidence. Data analysis was performed using the BIAevaluation Software (GE Healthcare).

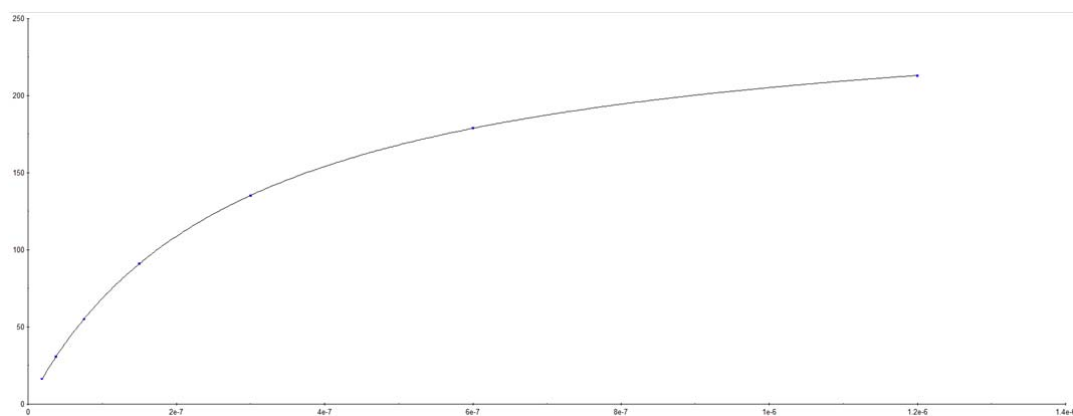


Figure 6.11. SPR analysis of CIRp-A1 on immobilised FPc, using equilibrium analysis. Steady state affinity fitting was performed after plotting R_{eq} from Figure 6.10 against analyte concentration. The calculated K_D was 285 nM ($\chi^2 = 0.0245$). Data analysis was performed using the BIAevaluation Software (GE Healthcare).

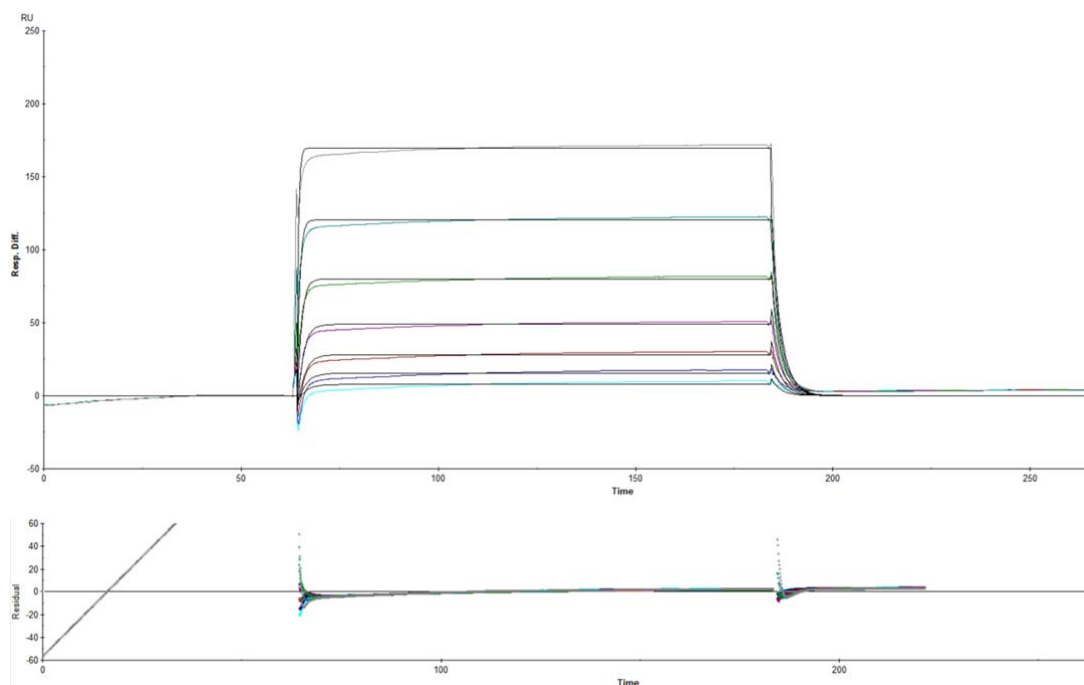


Figure 6.12. SPR analysis of CIRp-A1 on immobilised FPcΔ3, using 1:1 Langmuir binding model. CIRp-A1 was injected at concentrations ranging from 18.75 nM to 1200 nM. K_D calculation was attempted by fitting the data to a 1:1 Langmuir binding model (top). χ^2 value of 7.21 and the residual plot (bottom) indicated poor fit, and K_D could not be determined with confidence. Data analysis was performed using the BIAevaluation Software (GE Healthcare).

I also performed SPR experiments where FPc was injected over immobilised CIRp-A1. Coupling was done as described above. The running buffer was 10 mM HEPES, 150 mM NaCl, 5 mM $MgCl_2$, 0.005% (v/v) Tween 20, pH 7.2. The following concentrations of FPc were used: 600 nM, 300 nM, 150 nM, 75 nM, 37.5 nM and 18.75 nM. CIRp-A1 was injected for 120 s, followed by 3000 s dissociation and 300 s ‘regeneration’ with the running buffer. Curve fitting using the 1:1 Langmuir binding model resulted in poor fit ($\chi^2 = 216$) and K_D could not be determined from the data (**Figure 6.13**). Since properdin is a relatively long molecule for its size, diffusion could be a rate-limiting factor and the interaction could be limited by mass transport. Therefore, I also tried the 1:1 binding with mass transfer model for curve fitting; the fit was also poor ($\chi^2 = 76.8$) and K_D could not be determined (**Figure 6.14**).

Data suggests that SPR may not be a suitable technique to study the interaction between properdin and CIRp-A1. The immobilised ligand could display reduced binding activity to the analyte due to the way it is coupled. There are multiple sites (lysine residues) on the

ligand that can be used to couple to the sensor chip surface via amine coupling. However, if the protein-protein interaction site is close to a primary amine bound to the surface, it will be less accessible by the analyte. This could result in lower responses from the experiments. Additionally, coupling takes place at low pH, which could affect the protein fold and binding.

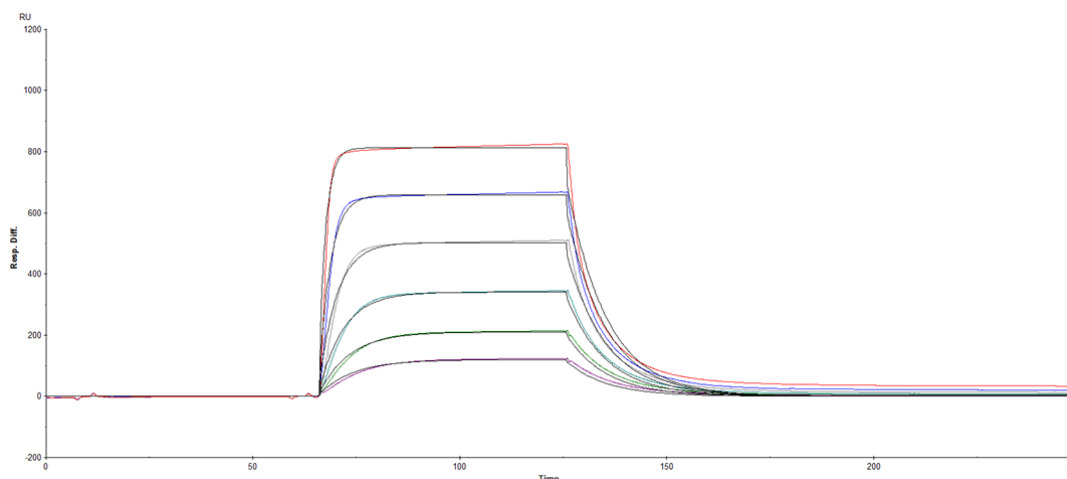


Figure 6.13. SPR analysis of FPc on immobilised CIRp-A1, using 1:1 Langmuir binding model. FPc was injected at concentrations ranging from 18.75 nM to 600 nM. K_D calculation was attempted by fitting the data to a 1:1 Langmuir binding model; however, due to poor fit ($\chi^2 = 216$), K_D could not be determined. Data analysis was performed using the BIAevaluation Software (GE Healthcare).

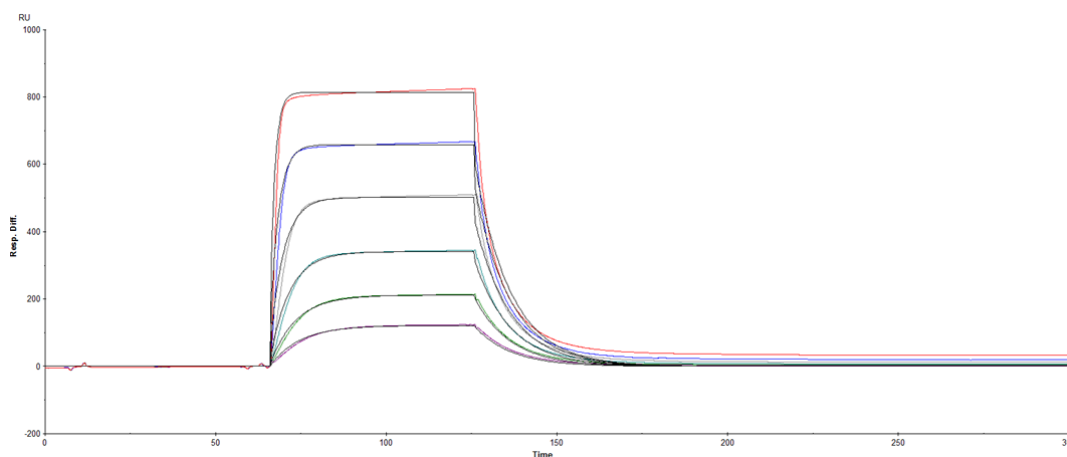


Figure 6.14. SPR analysis of FPc on immobilised CIRp-A1, using 1:1 binding with mass transfer model. FPc was injected at concentrations ranging from 18.75 nM to 600 nM. K_D calculation was attempted by fitting the data to a 1:1 binding with mass transfer model; however, due to poor fit ($\chi^2 = 76.8$), K_D could not be determined. Data analysis was performed using the BIAevaluation Software (GE Healthcare).

6.4.3 ITC to determine binding affinity between properdin and CIRp-A1

Isothermal titration calorimetry (ITC) was carried out to determine the binding affinity between properdin and CIRp-A1. ITC experiments were performed by Dr. David Staunton. In an ITC experiment, small volumes of one binding partner (ligand) are injected into a cell containing the other binding partner (sample) in solution. The resulting heat change upon binding is measured. The area under the peaks for each injection is measured and then plotted against the molar ratio of ligand to sample. The resulting titration curve can be analysed to determine the thermodynamic parameters of the interaction (Velazquez-Campoy et al., 2015).

Samples were prepared as outlined in **Section 2.4.5**; the running buffer was PBS, pH 7.4. For ITC experiments, the monomeric properdin FPc was used (Pedersen et al., 2017). Properdin was placed in the cell at 10 μ M and CIRp-A1 was titrated into the cell via multiple 2 μ l injections. In the raw data (**Figure 6.15A**), each peak corresponds to an injection of CIRp-A1 into the cell containing properdin and the area under the peak is proportional to the amount of heat released during binding (Damian, 2013). There was an interaction between CIRp-A1 and properdin but properdin was saturated after the first injection (**Figure 6.15A**), at a much lower molar ratio (1:5) than expected (1:1). The experiment was repeated with a higher concentration of properdin in the cell at 50 μ M, but again, properdin was saturated after two injections and at even lower molar ratio (**Figure 6.15B**). The protein concentrations must be accurate for ITC experiments. Since properdin contains glycosylated tryptophan residues (Hartmann, 2000), the absorption coefficient calculated based on the amino acid sequence could have been wrong. However, previous SEC-MALS experiments and protein conjugate analyses indicate that the theoretical extinction coefficient at 280 nm is not far off and the calculations of properdin concentrations should be correct.

ITC experiments were also carried out with CIRp-A1 placed in the cell at 10 μ M, while properdin was titrated into the cell. Looking at the raw data, the signals were very low and noisy, and therefore, it was difficult to interpret the data (**Figure 6.16**). It seems that ITC may not be a suitable technique to study the interaction between properdin and CIRp-A1. It could be that properdin and/or CIRp-A1 are not stable under the experimental conditions of the microcalorimeter. The stirring of the cell following injection could

cause denaturation or destabilisation of the proteins (Velazquez-Campoy et al., 2015) as the typical stirring rate in ITC experiments is 200-300 rpm. Stirring could be reduced to see if the signals improve. The proteins could also be sticking to the surfaces of the chamber, which may be prevented by using a different buffer. These troubleshooting experiments were not tried, however, due to time and cost constraints, and other binding studies were tried instead.

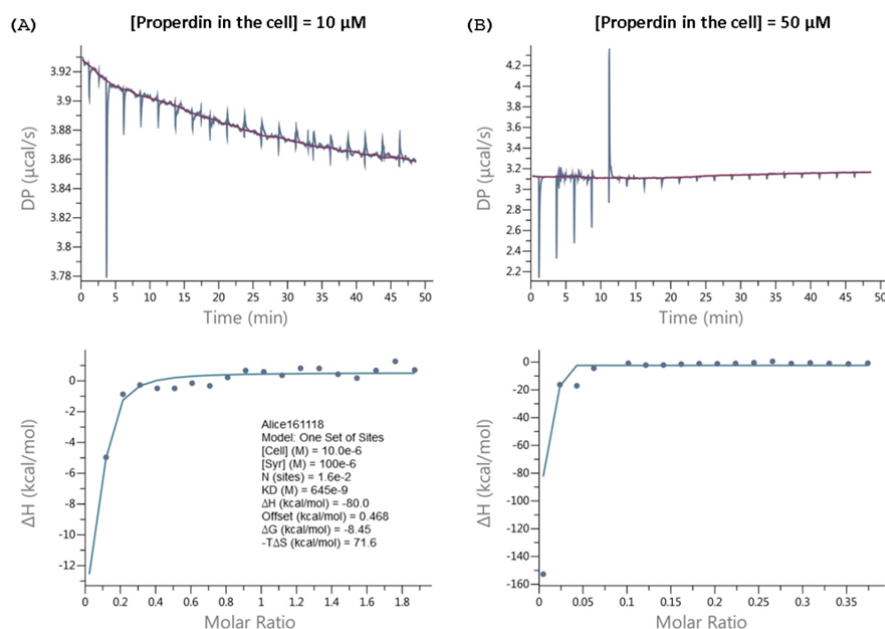


Figure 6.15. Analysis of properdin-CIRp-A1 interaction by ITC, with properdin placed in the cell. (A) Properdin concentration in the cell was 10 μ M. (B) Properdin concentration in the cell was 50 μ M. For both (A) and (B), the graph on the top shows the raw data where each peak corresponds to an injection of CIRp-A1 into the cell containing properdin, while the graph on the bottom is a plot of the area under the peak as a function of the molar ratio of CIRp-A1 to properdin. In both cases, properdin was saturated after a couple of injections.

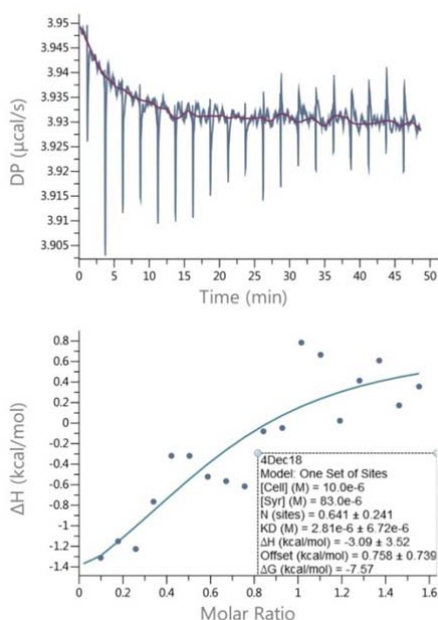


Figure 6.16. Analysis of properdin-CIRp-A1 interaction by ITC, with CIRp-A1 placed in the cell. CIRp-A1 concentration in the cell was 10 μM . The graph on the top shows the raw data where each peak corresponds to an injection of properdin into the cell containing CIRp-A1, while the graph on the bottom is a plot of the area under the peak as a function of the molar ratio of properdin to CIRp-A1. The data could not be interpreted due to low signal.

6.4.4 MST to determine binding affinity between properdin and CIRp-A1

Microscale thermophoresis (MST) can be used to determine the binding affinity between proteins. Thermophoresis describes the migration of molecules in response to a temperature gradient. The thermophoresis of a protein is expected to be different from the thermophoresis of a protein-protein complex, due to changes in the hydration shell, charge or size caused by the binding interaction. This change in thermophoresis due to binding can be used to determine equilibrium binding constants (Jerabek-Willemsen et al., 2011, 2014). In a MST experiment, one of the binding partners is fluorescently labelled. Initially, this labelled protein is homogeneously distributed inside a glass capillary. Then, an infrared (IR) laser is switched on, which generates a temperature gradient along the glass capillary. The movement of the labelled protein can be monitored by measuring fluorescence.

For MST experiments to determine the binding affinity between properdin and CIRp-A1, proteins were labelled as described in **Section 2.4.6**. The pseudo-monomeric properdin

FPc was used (Pedersen et al., 2017). The running buffer was PBS, 0.05% (v/v) Tween 20, pH 7.4. Labelled properdin was added to each reaction at a final concentration of 50 nM. A two-fold dilution series of CIRp-A1 was prepared, with the concentration ranging from 64 μ M to 2 nM. Reactions were mixed and incubated for 5 min at room temperature, before being loaded into glass capillaries for MST measurements. The LED power was set to 20%. For MST measurements, the IR laser was switched on for 30 s and then off for 5 s. Two MST powers of 20% and 40% were used. The obtained normalised fluorescence time trace is shown in **Figure 6.17**.

Data analyses to determine the dissociation constant, K_D , were performed using the NanoTemper Analysis Software. Three different settings can be chosen for data analysis: Temperature Jump (T-Jump), Thermophoresis, Thermophoresis with T-Jump (Mueller et al., 2017). During an MST experiment, the fluorescence of the heat spot within the sample-containing capillary is measured before, during and after the IR laser is switched on. The T-Jump of the fluorophore corresponds to the rapid change (less than 1 s) in fluorescence upon heating (**Figure 6.17A**). This rapid change is dependent on the fluorophore's local environment; therefore, it could be affected by binding in close vicinity to the fluorophore. On the other hand, thermophoresis describes the slower change in fluorescence over a longer time scale (**Figure 6.17B**) (Jerabek-Willemsen et al., 2014; Seidel et al., 2013). Alternatively, both T-Jump and Thermophoresis could be combined for analysis (**Figure 6.17C**).

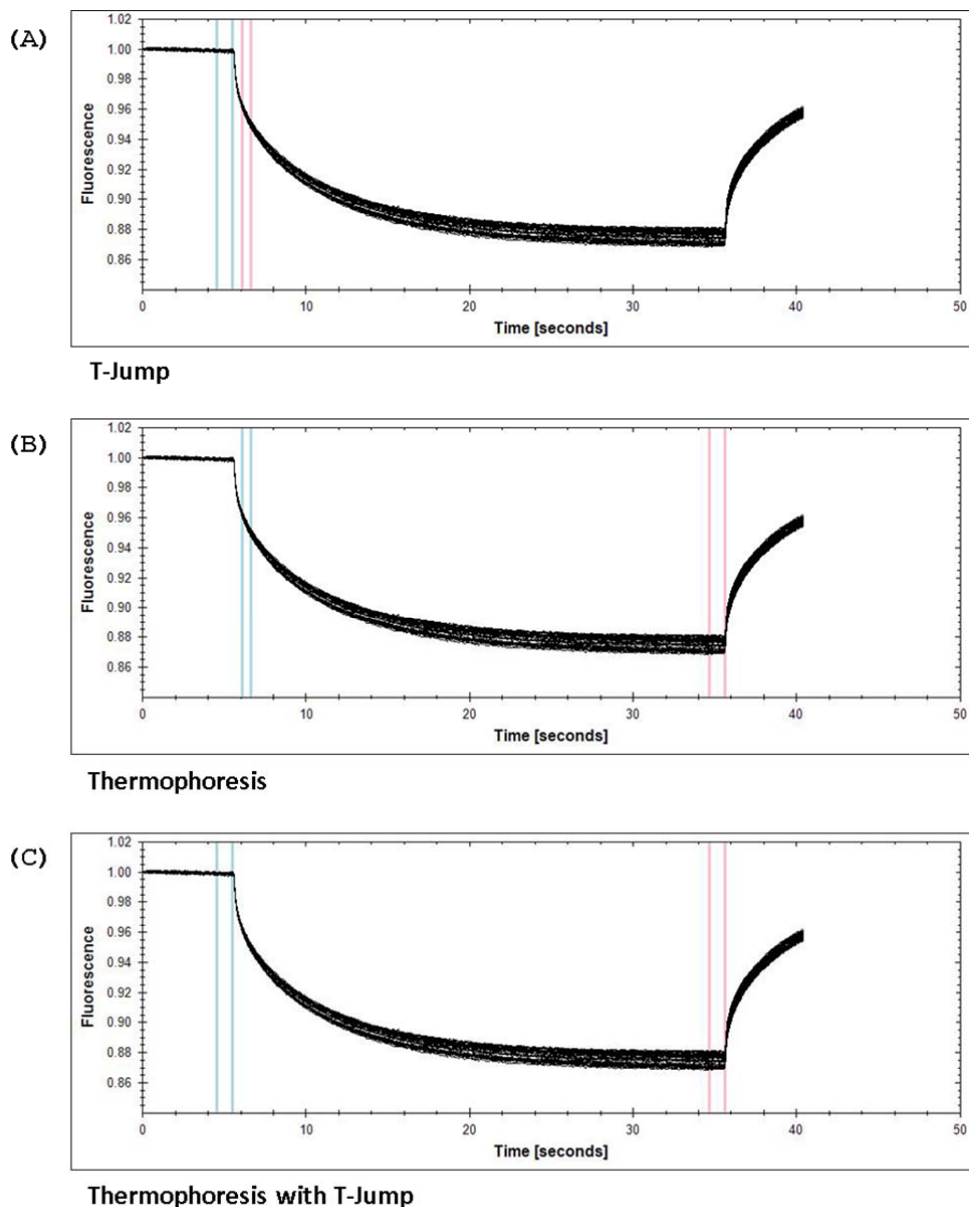


Figure 6.17. Different analysis modes of MST data. MST data could be interpreted using three different settings: (A) T-Jump, (B) Thermophoresis, or (C) Thermophoresis with T-Jump. Normalised fluorescence time traces are shown and the regions used for different analysis settings are indicated. F_{cold} and F_{hot} values are marked with blue and red lines, respectively. Analysis was performed using the NanoTemper Analysis Software.

For data analysis, the change in normalized fluorescence ($\Delta F_{\text{norm}} = F_{\text{hot}}/F_{\text{cold}}$) is used; F_{cold} and F_{hot} are the fluorescence values of defined regions of the fluorescence time trace marked by blue and red lines, respectively (**Figure 6.17**). ΔF_{norm} is then plotted against the concentration of the non-labelled protein (Jerabek-Willemsen et al., 2014). Binding was observed for properdin and CIRp-A1, and curve fitting was carried out according to

the law of mass action (Mueller et al., 2017), since monomeric FPc and CIRp-A1 were shown to bind in 1:1 ratio. The calculated K_D can vary depending on which analysis setting is chosen (Scheuermann et al., 2016), and this was observed for properdin and CIRp-A1 (**Table 6.3**). The K_D values obtained from analysing the T-Jump were the most consistent between MST power 20% and MST power 40% at 123 ± 13.4 nM and 112 ± 9.3 nM, respectively (**Figure 6.18**).

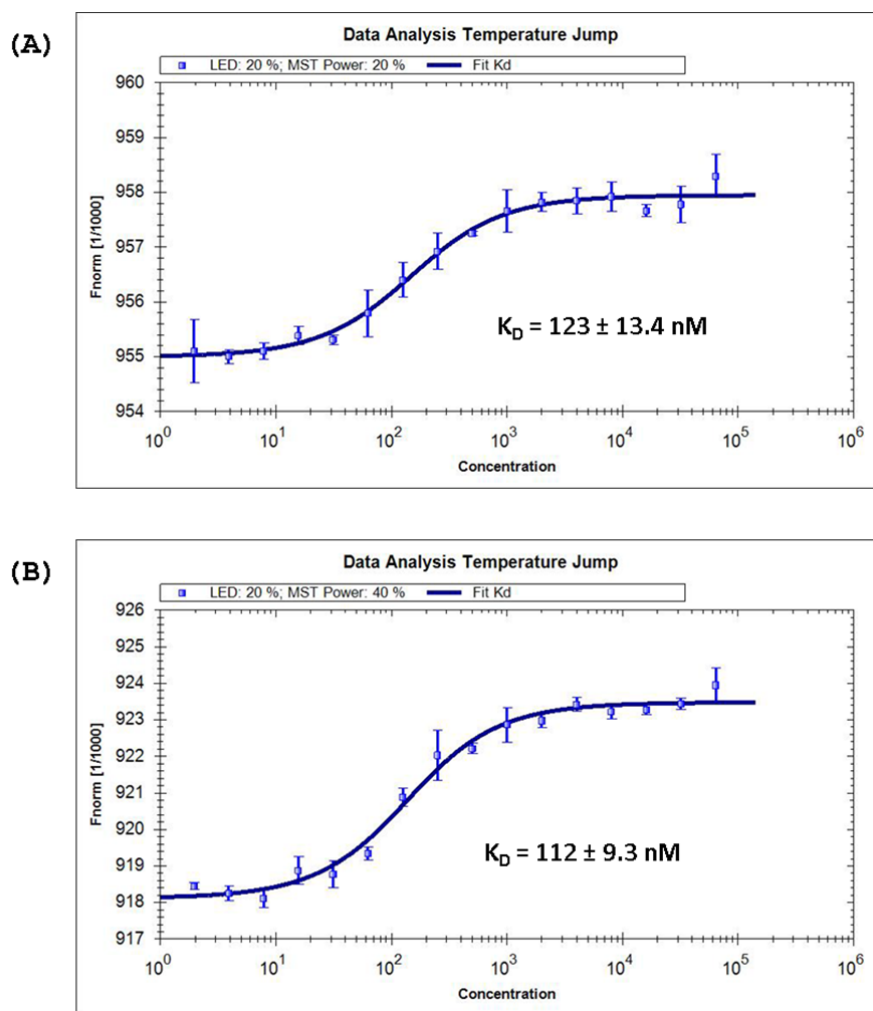


Figure 6.18. Determination of the binding affinity between properdin and CIRp-A1 by MST. K_D was calculated from the data obtained in Figure 6.14, using the T-Jump mode of data analysis. (A) At MST power 20%, the calculated K_D was 123 ± 13.4 nM. (B) At MST power 40%, the calculated K_D was 112 ± 9.3 nM. Analysis was performed using the NanoTemper Analysis Software.

Table 6.3. K_D values obtained from MST data analysis. Analysis was performed using the NanoTemper Analysis Software. Different analysis settings were tried.

Analysis mode	K_D from MST power 20%	K_D from MST power 40%
T-Jump	123 $\pm$ 13.4 nM	112 $\pm$ 9.3 nM
Thermophoresis	184 $\pm$ 30.6 nM	707 $\pm$ 134 nM
Thermophoresis with T-Jump	160 $\pm$ 20.2 nM	307 $\pm$ 42.7 nM

MST experiments were also carried out using labelled CIRp-A1 instead. The protocol was largely similar to above, with the following differences. The LED power was set to 25%, and a two-fold dilution series of unlabelled properdin was prepared, with the concentration ranging from 2 μ M to 0.06 nM. Labelled CIRp-A1 was added to each reaction at a final concentration of 100 nM. When CIRp-A1 was labelled, no binding was observed (**Figure 6.19**). As the fluorophore labelling occurs via amine coupling, the binding site on CIRp-A1 could be in the vicinity of a lysine residue. The structure of CIRp-A1 shows a lysine residue in proximity to the loop region that was investigated as a binding site in **Section 6.2 (Figure 6.20)**.

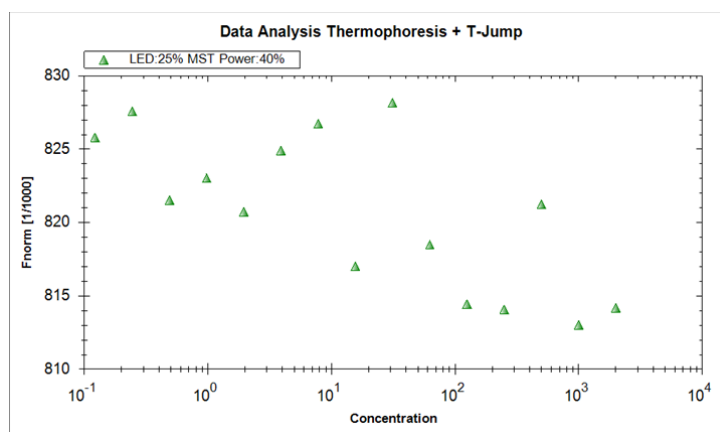


Figure 6.19. Data obtained from MST experiments using labelled CIRp-A1. In comparison to using labelled properdin, as shown in Figure 6.15, no binding was observed when using labelled CIRp-A1. The thermophoresis with T-Jump mode of analysis is shown here as an example. Analysis was performed using the NanoTemper Analysis Software.

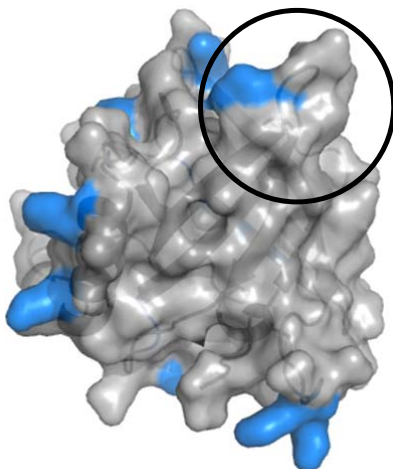


Figure 6.20. Structure of CIRp-A1 with lysine residues highlighted. Surface representation of CIRp-A1 is shown, with lysine residues in blue. The image was produced with PyMOL (Version 2.1, Schrödinger, LLC).

6.4.5 Pulldown assays to investigate the effect of CIRp-A1 on the interaction between properdin and C3b

To understand how CIRp-A1 affects ligand binding of properdin, the effect of CIRp-A1 on the interaction between properdin and C3b was investigated via pulldown assays. Biotinylated C3b was generated, as described in **Section 2.5.1**. The monomeric properdin FPc (Pedersen et al., 2017) was used for the following experiments.

The protocol for pulldown assays using Pierce Streptavidin Magnetic Beads (Thermo Scientific) was based on the manufacturer's instructions. The binding/wash buffer was 25 mM Tris, 200 mM NaCl, 0.1% (v/v) Tween 20, pH 7.5. For each reaction, 10 µg of biotin-C3b was mixed with different proteins (**Table 6.4**). CIRp-A1 was added at five times molar excess of properdin. The total reaction volume was made up to 300 µl with the binding buffer. Following incubation at room temperature for 1 h with mixing, 50 µl of beads were added to each reaction. The reactions were then incubated at room temperature for 1 h with mixing. The beads were then collected with a magnetic stand and the supernatant was discarded. The beads were washed five times with 300 µl of the wash buffer. For reactions 1-3, the beads were re-suspended in 50 µl of reducing 1x SDS-PAGE loading dye. For reaction 4, CIRp-A1 was added to the beads at the same final concentration as reaction 2, with the final volume made up to 300 µl with the binding

buffer. The mixture was incubated at room temperature for 1 h with mixing. After washing the beads five times with 300 μ l of the wash buffer, the beads were re-suspended in 50 μ l of reducing 1x SDS-PAGE loading dye. All samples were analysed by Western blot with α -properdin antibody. Properdin could no longer bind to C3b in the presence of CIRp-A1. Additionally, CIRp-A1 displaced properdin that was already bound to C3b (Figure 6.21).

Table 6.4. Reactions for pulldown assays using streptavidin magnetic beads and biotin-C3b.
The table shows the components added for each reaction in the pulldown assays.

Reaction	Components
1	Properdin
2	Biotin-C3b + properdin + CIRp-A1
3	Biotin-C3b + properdin
4	Same as reaction 1, then CIRp-A1 added later

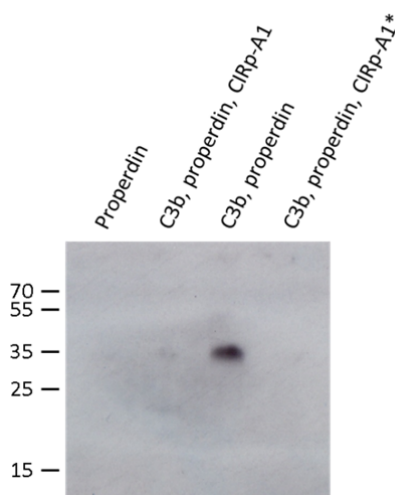


Figure 6.21. The effect of CIRp-A1 on the interaction between properdin and C3b. Pulldown assays using biotinylated C3b and streptavidin magnetic beads, followed by Western blot with α -properdin antibody show that properdin could not bind to C3b in the presence of CIRp-A1 (second lane) and CIRp-A1 displaced properdin already bound to C3b (fourth lane). CIRp-A1* indicates that it was added after C3b-properdin binding had already occurred.

6.5 Structural insights into the properdin-CIRp-A1 complex

6.5.1 Purification of properdin-CIRp-A1 complex using the HEK293F FPc and FPcΔ3 cell lines

For the purification of FPc-CIRp-A1 complex, the supernatant of the HEK293F FPc culture was harvested and filtered. The stable HEK293F cell line expressing FPc was provided by the lab of Gregers Andersen (described in **Section 3.7.6**). After adjusting the pH of the supernatant to pH 7, His-CIRp-A1 was added to the supernatant (5 mg per litre of culture) and mixed by stirring. The supernatant was then loaded onto a pre-equilibrated 5 ml Ni-NTA Superflow column; the running buffer was 50 mM HEPES, 300 mM NaCl, pH 7. The complex was eluted with the running buffer containing 300 mM imidazole. Following affinity chromatography, the complex was cleaved at 4 °C with both TEV and 3C proteases to cleave properdin and CIRp-A1, respectively; the proteases were added at protease:substrate ratio of 1:4. Reverse nickel purification was performed to remove the proteases (which contain a His tag) and uncleaved proteins (**Figure 6.22A**). This was followed by SEC using a S200 16/60 column; the running buffer was 10 mM HEPES, 75 mM NaCl, pH 7 (**Figure 6.22B**). Excess CIRp-A1 was separated by SEC (**Figure 6.22C**). The yield was typically around 3 mg of the complex using 1 litre culture of HEK293F FPc.

FPcΔ3-CIRp-A1 complex was purified using the same protocol as FPc-CIRp-A1 complex described above (data not shown). The running buffer for SEC was 20 mM Tris, 100 mM NaCl, pH 7. For this complex, Tris was used for SEC buffer, as Tris might be preferable for subsequent crystallisation trials. The stable HEK293F cell line expressing FPcΔ3 was provided by the lab of Gregers Andersen (described in **Section 3.7.6**).

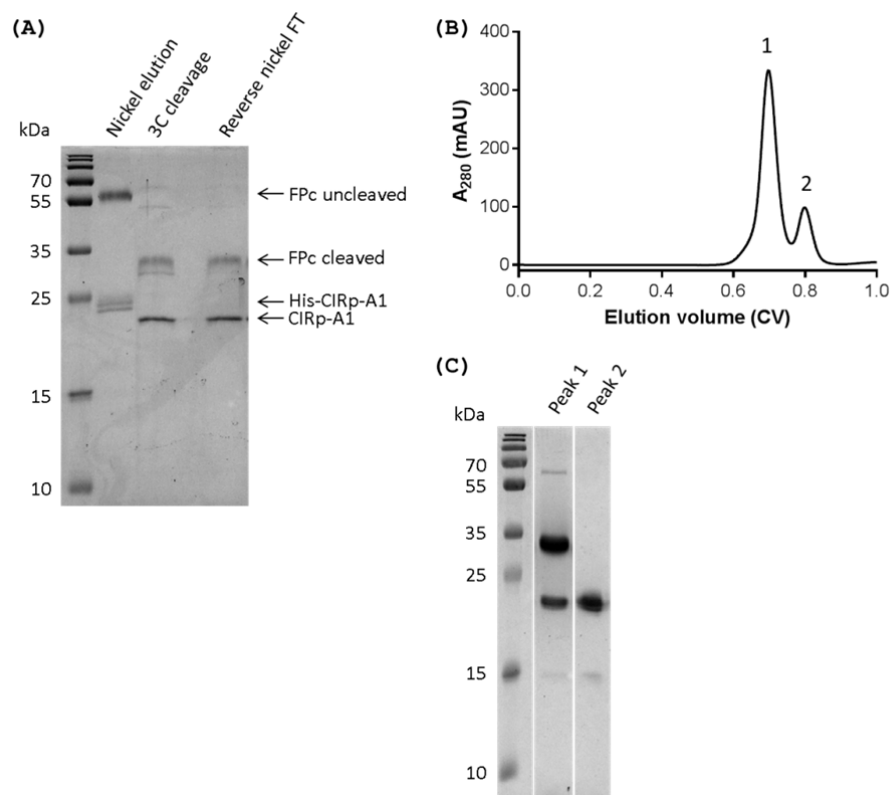


Figure 6.22. Purification of FPc-CIRp-A1 complex. (A) 15% SDS-PAGE analysis of FPc-CIRp-A1 following each purification step before SEC. (B) S200 16/60 size exclusion profile of FPc-CIRp-A1 showed two peaks. (C) 15% SDS-PAGE analysis of peak fractions from SEC shown in (B) showed FPc-CIRp-A1 complex in the first peak and CIRp-A1 in the second peak.

6.5.2 Crystallisation attempts of properdin-CIRp-A1 complex

Crystallisation trials were set up with FPc-CIRp-A1 complex using the sitting drop vapour diffusion method. Broad screens were set up with a drop size of 0.3 μ l or 0.2 μ l at 75% (v/v) and 50% (v/v) protein:mother liquor ratios, using the complex at different concentrations. Additionally, trays were put down using the complex at different salt concentrations. The properties of the different crystallisation screens are described in **Section 2.4.7**. No crystals were observed after weeks; phase separation was observed in many of the conditions.

Table 6.5. Crystallisation trials set up for FPc-CIRp-A1 complex. Different protein concentrations, sample salt concentrations, drop sizes and broad screens were used for crystallisation trials.

Protein concentration	Protein NaCl concentration	Drop size	Broad screens
15 mg/ml	150 mM	0.3 μ l	Structure Screen 1+2, Clear Strategy I&II, ProPlex, JCSG- <i>plus</i> , MIDAS <i>plus</i>
40 mg/ml	150 mM	0.3 μ l	Clear Strategy I&II, Structure Screen 1+2, JCSG- <i>plus</i>
25 mg/ml	75 mM	0.3 μ l	PACT premier, ProPlex, Clear Strategy I&II, JCSG- <i>plus</i>
30 mg/ml	75 mM	0.3 μ l	Stura FootPrint, PACT premier, Clear Strategy I, ProPlex, Structure Screen 1+2
40 mg/ml	75 mM	0.3 μ l	Stura FootPrint, PACT premier, Clear Strategy I&II, ProPlex, LMB
40 mg/ml	50 mM	0.2 μ l	Stura FootPrint, Clear Strategy I&II
40 mg/ml	500 mM	0.3 μ l	Clear Strategy I&II, JCSG- <i>plus</i>

Similar to crystallisation trials with FPc-CIRp-A1 complex described above, crystallisation trials with FPc Δ 3-CIRp-A1 were set up using broad screens. Trays were set up with a drop size of 0.3 μ l or 0.2 μ l at 75% (v/v) and 50% (v/v) protein:mother liquor ratios, using different protein concentrations. Small needles grew in 50% (v/v) protein: mother liquor ratio in 0.1 M sodium citrate, pH 4.5, 20% (w/v) PEG4000, using the complex at a concentration of 36 mg/ml (**Figure 6.23**). Optimisation trays were set up based on the initial conditions, with respect to pH and precipitant concentration. However, the needles could not be replicated, even when the original screen was used

Table 6.6. Crystallisation trials set up for FPc Δ 3-CIRp-A1 complex. Different protein concentrations, drop sizes and broad screens were used for crystallisation trials.

Protein concentration	Drop size	Broad screens
20 mg/ml	0.3 μ l	Stura FootPrint, Structure Screen 1+2, Clear Strategy I&II
25 mg/ml	0.3 μ l	PACT premier, JCSG- <i>plus</i> , ProPlex, Morpheus
36 mg/ml	0.2 μ l	Stura FootPrint, Structure Screen 1+2, ProPlex, LMB

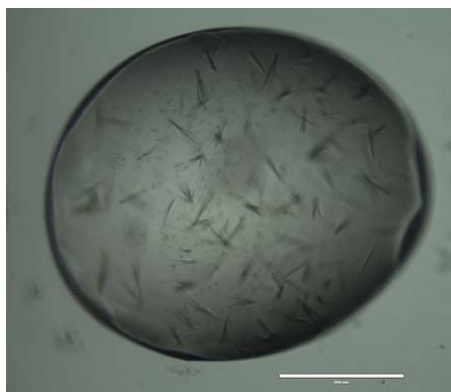


Figure 6.23. Crystals of FPcΔ3-CIRp-A1 complex. The drop contained 0.1 μ l of the complex at 36 mg/ml and 0.1 μ l of mother liquor (0.1 M sodium citrate, pH 4.5, 20% (w/v) PEG4000).

Crystallisation was also attempted using the crystallisation conditions for FPcΔ3 by (Pedersen et al., 2019). FPcΔ3 crystals were obtained in 1.5 M lithium sulphate, 0.1 M sodium acetate, pH 4.6. Trays were set up based on their condition with respect to pH and lithium sulphate concentration (**Figure 6.24A**). The stock solutions were 1 M sodium acetate at pH 3.5 and pH 5.4, and 2 M lithium sulphate. The trays were set up with the complex at 9-10 mg/ml. Several crystals were observed (**Figure 6.24B**). Crystals were cryoprotected in mother liquor (0.1 M sodium acetate, pH 3.9, 1.1 M lithium sulphate) supplemented with 20% (v/v) PEG400 and were flash frozen in liquid nitrogen. Data collection was performed by Prof. Susan Lea at Diamond Light Source. Data analysis by Dr. Steven Johnson showed that the complex did not co-crystallise and the crystals only contained FPcΔ3. This was perhaps not surprising as the condition contained very high salt concentration which could disrupt protein-protein interaction.

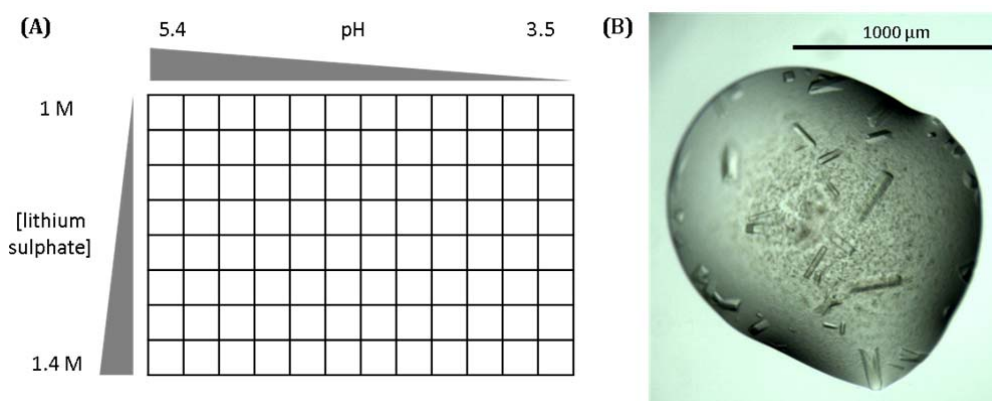


Figure 6.24. Crystallisation trials of FPcΔ3-CIRp-A1 complex. (A) Trays were set up based on the crystallisation conditions of FPcΔ3 by (Pedersen et al., 2019), with respect to pH and lithium sulphate concentration. (B) The drop contained 0.075 μ l of the complex at 9 mg/ml and 0.025 μ l of mother liquor (0.1 M sodium acetate, pH 3.9, 1 M lithium sulphate).

6.5.3 Limited proteolysis of FPc-CIRp-A1 complex

Since properdin is a long and flexible molecule, small-scale limited proteolysis was carried out with the purified FPc-CIRp-A1 complex to see whether flexible parts of the complex could be cleaved off, which might enable crystallisation. Three proteases were used for small-scale limited proteolysis – chymotrypsin, subtilisin and trypsin. For each reaction, 10 μ l of the complex (at 1 mg/ml) and 10 μ l of protease were used. Serial dilution of the proteases was generated to test the proteases at different protease:protein (w/w) ratios. After mixing the complex and the protease, the reaction was incubated at room temperature for 1 h, before SDS-PAGE analysis (**Figure 6.25**).

With chymotrypsin, only CIRp-A1 was cleaved by the enzyme at higher concentrations whereas properdin was resistant to cleavage (**Figure 6.25A**). Subtilisin cleaved CIRp-A1 and properdin into many fragments (**Figure 6.25B**), whereas the complex was resistant to cleavage by trypsin (**Figure 6.25C**). Therefore, limited proteolysis could not generate stable constructs of the complex that are suitable for crystallisation.

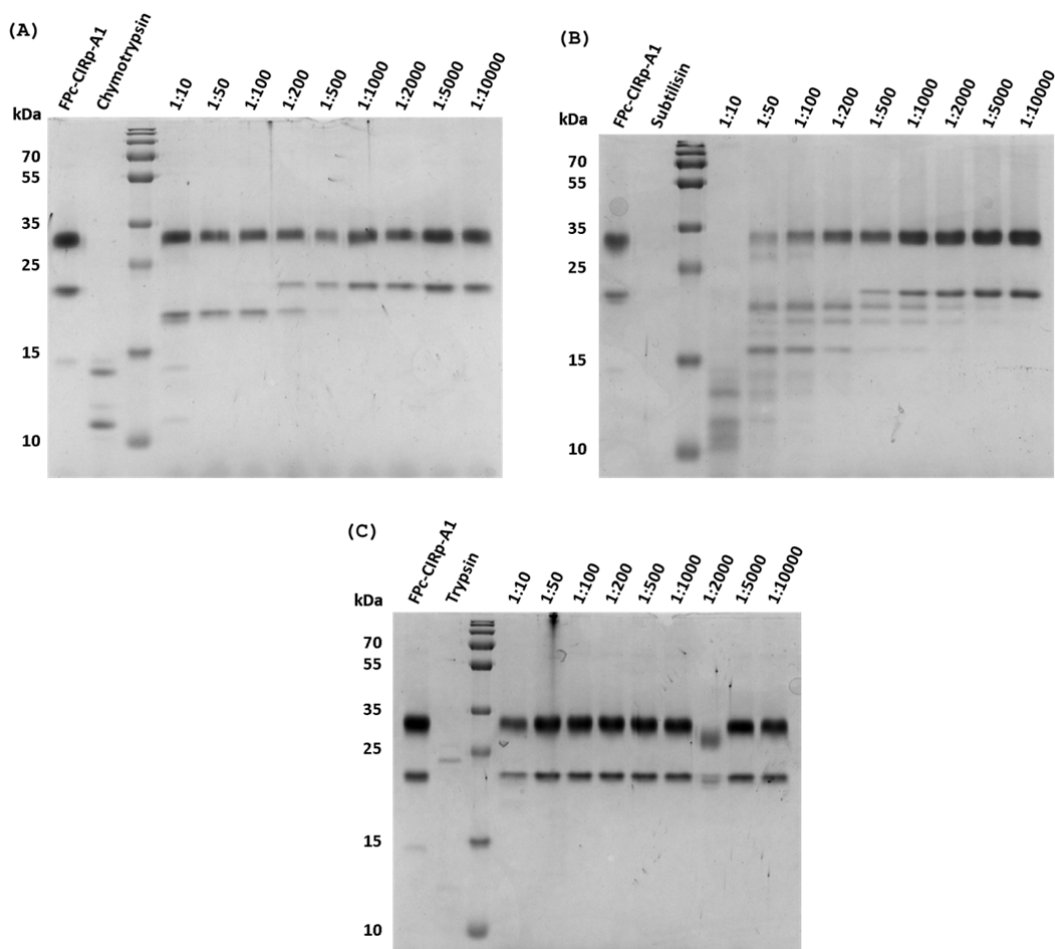


Figure 6.25. Limited proteolysis of FpC-CIRp-A1 complex. (A) chymotrypsin, (B) subtilisin, and (C) trypsin were used for limited proteolysis of FpC-CIRp-A1 complex. Dilution series of the proteases were generated. The protease:protein (w/w) ratios of the reactions are shown above the 15% SDS-PAGE gels.

6.6 Discussion

Various biophysical techniques were used to characterise the interaction between properdin and CIRp-A1. For all binding experiments, the pseudo-monomeric properdin constructs FPc and FPc Δ 3 (Pedersen et al., 2019) were used, as purified in **Section 3.7.6**. The calculated masses from SEC-MALS of FPc-CIRp-A1 and FPc Δ 3-CIRp-A1 complexes suggest that properdin and CIRp-A1 bind in 1:1 ratio (**Figure 6.9**). The 1:1 binding model was subsequently used for curve fitting in binding studies. Although K_D could not be determined with confidence from SPR experiments, it was consistent with it being in the 100s nM (**Figure 6.11**). MST experiments suggested a K_D between properdin and CIRp-A1 of 112 ± 9.3 nM (**Figure 6.18B**). The interaction between labelled properdin and CIRp-A1 could be detected by changes in the MST T-Jump, which measures rapid changes in fluorescence when the IR laser is switched on. As the T-Jump is related to the local environment of the fluorophore, this could suggest that CIRp-A1 binds close to the fluorophore on labelled properdin (Jerabek-Willemsen et al., 2011). The T-Jump signal also relates to changes in the flexibility of the labelled protein, which could mean that CIRp-A1 induces a conformational change of labelled properdin in close proximity to the fluorophore (Jerabek-Willemsen et al., 2011). On the other hand, no binding was observed when CIRp-A1 was labelled (**Figure 6.19**), which suggested that the binding site on CIRp-A1 could be masked by the amine coupled fluorescent label.

MST experiments highlighted the possible involvement of lysine residues in the interaction between properdin and CIRp-A1, or at least the close proximity of the interaction site to lysine residues. Therefore, biophysical techniques that involve amine coupling could affect the interaction between properdin and CIRp-A1. SPR also utilises amine coupling of one of the binding partners to the surface. If lysine residues are involved in the interaction between properdin and CIRp-A1, the interaction site might be masked following amine coupled immobilisation on the chip surface. This could result in lower responses from SPR experiments. ITC does not require protein labelling. However, ITC requires larger sample quantity compared to MST and SPR. Proteins are used in the μ M range for ITC and in the nM range for MST and SPR. ITC experiments to study the interaction between properdin and CIRp-A1 were attempted, but the proteins did not seem stable under the experimental conditions (**Section 6.4.3**).

The K_D values obtained from MST are higher than expected from complement inhibition assays. Alternative pathway WIESLAB and haemolysis assays suggested a higher affinity between properdin and CIRp-A1 by an order of magnitude; in those assays, 50% inhibition was observed at 10-15 nM (**Figure 4.18** and **Figure 4.20**). There are a few differences between MST and the inhibition assays. The incubation time was quite different between them. For MST, properdin and CIRp-A1 were mixed and incubated at room temperature for 5 min; for the inhibition assays, incubation took place at 37 °C for 60 min. The incubation before MST measurements was kept short as there was some indication that CIRp-A1 stuck to the tubes during incubation (data not shown). As it is important to incubate MST samples for long enough to reach equilibrium (Scheuermann et al., 2016), the short incubation time might have affected binding. The interaction between properdin and CIRp-A1 occur in the fluid phase during MST measurements. In contrast, both WIESLAB and haemolysis assays rely on complement activation on the surface. The interaction between properdin and CIRp-A1 might occur both in the solid and fluid phases under the assay conditions. Additionally, since serum is used in inhibition assays, other complement components could affect the interaction between properdin and CIRp-A1, such as C3b and FB.

To understand the molecular basis of the interaction between properdin and CIRp-A1, the binding sites on the two proteins were characterised. For CIRp-A1, mutants were designed based on the structures of CIRp-A1 and homologs (**Chapter 5**), and the sequence comparison of these homologs. In AP WIESLAB assays, a deletion mutant of CIRp-A1, which had three residues deleted within the $\beta 7$ - $\beta 8$ loop of the barrel, showed reduced complement inhibitory activity (**Figure 6.5**). The activity was not completely abolished, indicating that this loop only forms a part of the binding site on CIRp-A1.

To characterise where CIRp-A1 binds on properdin, TSR domains were expressed and purified from CHO cells. Pulldown assays using these proteins and immobilised CIRp-A1 suggested that CIRp-A1 binds to TSR6 (**Figure 6.7**). As TSR6 had been suggested to be involved in forming vertices of properdin oligomers (Alcorlo et al., 2013; Higgins et al., 1995; Pedersen et al., personal communication), CIRp-A1 might inhibit the oligomerisation of properdin, thereby affecting the activity of properdin. However, SEC-MALS analysis of serum properdin and CIRp-A1 showed that serum properdin was still

oligomeric in the presence of CIRp-A1, and CIRp-A1 was able to bind all oligomeric forms of properdin (**Figure 6.8**).

TSR6 domain of properdin had been shown to be functionally relevant, as mutants of TSR6 were unable to bind C3b (Fredrikson et al., 1996; Higgins et al., 1995). Therefore, the effect of CIRp-A1 on the interaction between properdin and C3b was investigated via pulldown assays using biotin-C3b and streptavidin beads. Properdin was unable to bind C3b in the presence of CIRp-A1, and also, CIRp-A1 displaced properdin that was already bound to C3b (**Figure 6.21**). Structural studies that were published during this thesis had implicated TSR5 in binding to C3b within the C3 convertase (Pedersen et al., 2017). By binding to TSR6, CIRp-A1 could physically block the interaction between TSR5 and C3b. The crystal structure of properdin showed that TSR6 contains a large loop which interacts with TSR5 (Pedersen et al., personal communication). Therefore, an alternative mode of inhibition is that CIRp-A1 binds to TSR6, and then alters the conformation of TSR5 such that it can no longer bind C3b. Salp20, which is a member of the properdin-binding Isac-like protein (ILP) family of tick complement inhibitors, was also shown to displace properdin from pre-formed C3 convertases (Tyson et al., 2008). As there is little sequence homology between CIRp-A1 and Salp20, it would be interesting to compare their mechanisms of inhibition.

Structural studies of properdin in complex with CIRp-A1 would help understand the mode of properdin inhibition by CIRp-A1, as well as determine the exact interaction sites on properdin and CIRp-A1. For crystallisation trials, properdin-CIRp-A1 complexes were purified using HEK293F stable cell lines expressing FPc or FPc Δ 3 (Pedersen et al., 2019). Despite setting up crystallisation trials at various concentrations and conditions, crystals were not observed for FPc-CIRp-A1 complex (**Section 6.5.2**). Small needles were observed for FPc Δ 3-CIRp-A1, but could not be replicated. This suggests that other crystallisation parameters could be modified, for example, temperature or using additives.

Since FPc and FPc Δ 3 could be crystallised on their own (Pedersen et al., 2017, 2019), CIRp-A1 could disrupt crystal contacts and hinder crystallisation of the complexes. Additionally, properdin is a flexible molecule. CIRp-A1 binds to TSR6 (**Section 6.3.2**), which is within the relatively stable vertex of properdin. This means that even after CIRp-A1 binding, there could still be flexibility from domains outside of the vertex, such as

TSR2 and TSR3. Since FPc could not be proteolytically cleaved (**Figure 6.25**), other properdin constructs would need to be designed by cloning. A small amount of FPc Δ 2,3 protein was acquired from the lab of Gregers Andersen, which was shown to still bind CIRp-A1 (**Figure 6.9C**). In the future, the FPc Δ 2,3 construct will be expressed in HEK293F, and crystallisation trials of FPc Δ 2,3-CIRp-A1 will be carried out. FPc Δ 2,3 is expected to be less flexible and therefore, may enable crystallisation.

CHAPTER 7: THESIS SUMMARY

In this thesis, I described structural and functional characterisations of properdin and the CIRp-A1 family of properdin-binding complement inhibitors from the tick *Rhiphicephalus pulchellus*. CIRp-A1 specifically inhibited the alternative pathway in WIESLAB assays, and the analyses of the supernatants from the alternative pathway WIESLAB assay suggested that CIRp-A1 significantly reduced C3a formation via the AP. The results reported in this thesis are similar to the inhibitory activities of other properdin-binding tick proteins (Couvreur et al., 2008; Tyson et al., 2007; Valenzuela et al., 2000). Reduced C3a formation suggests that CIRp-A1 acts at the point of C3 cleavage.

I also characterised homologs of CIRp-A1, which were identified by searching for similar sequences in other tick species. Alternative pathway haemolysis assays using human and other animal sera showed that CIRp-A1 and homologs have different complement inhibitory activity profiles in different animals. However, there was a lack of correlation between sequence identity and activity, and the inhibitory activities of the homologs were not obvious by simply analysing their sequences. Therefore, to better understand the host specificity of CIRp-A1 and homologs, I solved the crystal structures of CIRp-A1, CIRp-A2, CIRp-A4 and CIRp-A6. As a part of this, I established a refold purification protocol for CIRp-A1 and homologs, and the refolded materials were used for crystallisation trials. All homologs displayed the characteristic lipocalin fold. Overlay of the structures of the inhibitors showed overall structural homology and fold conservation, with the loop between the 7th and 8th β -strands of the β -barrel being shorter in some of the homologs.

This loop region was compared between inactive homologs that did not show complement inhibitory activity in any of the animals tested in AP haemolysis assays and active homologs that did. The inactive homologs CIRp-A2 and CIRp-A4 have a slightly shorter loop (by three amino acid residues) compared to the active homologs CIRp-A1 and CIRp-A6. Due to the lack of sequence identity within and around this loop region amongst the active homologs, the length of the loop, and not the sequence, was hypothesised to be important for activity. To test this hypothesis, CIRp-A1 mutants were generated where the loop insert residues were either substituted with alanine residues or deleted. The alanine mutant was minimally affected, with a 2-fold increase in IC₅₀

compared to wild-type CIRp-A1, whereas the deletion mutant had a 10-fold increase in IC_{50} compared to wild-type CIRp-A1. This suggested that the loop length is important for activity. However, adding the three extra residues into CIRp-A5, which is non-inhibitory in human serum despite sharing 82% sequence identity with CIRp-A1, did not change its activity, indicating that there is no simple correlation between loop length and activity. Additionally, CIRp-A5 did show complement inhibitory activity in monkey serum, despite having a shorter loop. These results suggest that this loop region might form the binding site for properdin on CIRp-A1, but there are clearly other regions involved. More extensive mutagenesis studies would be required to map the exact binding site on CIRp-A1. Given sufficient time, it would have been interesting to generate CIRp-A1 mutants in which CIRp-A1 residues are swapped with corresponding residues from other active homologs, and investigate the impact on host specificity.

To determine the binding site for CIRp-A1 on properdin, I performed pulldown assays using recombinantly expressed TSR domains of properdin, which suggested that CIRp-A1 binds to TSR6 domain. This domain had been suggested to be involved in forming vertices of properdin oligomers (Alcorlo et al., 2013; Higgins et al., 1995; Pedersen et al., personal communication). SEC-MALS analysis of serum properdin and CIRp-A1 showed that CIRp-A1 does not make properdin oligomers fall apart. TSR6 domain had also been shown to be important for ligand binding of properdin, as mutants of TSR6 were unable to bind C3b (Fredrikson et al., 1996; Higgins et al., 1995). I performed pulldown assays using biotinylated C3b and streptavidin beads to investigate the effect of CIRp-A1 on C3b binding by properdin. CIRp-A1 inhibited the interaction between properdin and C3b, and it displaced properdin that was already bound to C3b. Structural studies that were published during this thesis showed TSR5 interacting with C3b within the AP C3 convertase (Pedersen et al., 2017) and TSR6 containing a large loop which interacts with TSR5 (Pedersen et al., personal communication). These results lead to two possible mechanisms of properdin inhibition by CIRp-A1. By binding to TSR6, CIRp-A1 could sterically hinder the interaction between TSR5 and C3b, or change the conformation of TSR5 such that it can no longer interact with C3b. CIRp-A1 displacing properdin from C3b could leave C3b and C3bBb susceptible to inactivation and dissociation by complement regulators. It should be noted that the results from this thesis do not indicate whether CIRp-A1 would be able to displace properdin from a pre-formed C3 convertase complex. Since properdin binds more tightly to C3bB and C3bBb than to C3b on its own

(Farries et al., 1988a), the effect of CIRp-A1 on properdin binding to these complexes should be tested using pulldown assays.

Alternative pathway WIESLAB and haemolysis assays using human serum suggested IC_{50} values of 13 nM and 16 nM, respectively. MST experiments suggested a lower affinity between properdin and CIRp-A1, with K_D of 112 nM. The interaction between properdin and CIRp-A1 is likely more physiologically relevant in inhibition assays since serum is used and the assays rely on complement activation on the surface. Properdin binds more favourably to surface-bound AP C3 convertase than fluid-phase AP C3 convertase (Farries et al., 1988b), and therefore, properdin inhibition on the surface is likely more relevant. On the other hand, the interaction between properdin and CIRp-A1 occur in the fluid phase in MST experiments.

Despite the structural and functional data generated in this thesis, the lack of correlation between sequence and activity of CIRp-A1 homologs makes it difficult to speculate on the molecular mechanism of properdin inhibition by these proteins. More detailed mutagenesis and interaction studies would be required to determine the exact binding mechanism between properdin and CIRp-A1, as outlined above. Ultimately, the structure of properdin-CIRp-A1 complex will be required to fully understand how CIRp-A1 inhibits properdin function and ligand binding. Crystallisation was attempted with properdin-CIRp-A1 complexes purified using stable HEK293F cell lines of pseudo-monomeric FPc and FPc Δ 3 (Pedersen et al., 2019). In the future, FPc Δ 2,3 construct obtained from the authors of the aforementioned study will be used to purify FPc Δ 2,3-CIRp-A1 complex for crystallisation trials.

Complement dysregulation has been linked to various immune-mediated and inflammatory disorders, and there is great interest in developing novel complement-targeting therapeutics. Properdin is an attractive target for therapeutic inhibition since it is alternative pathway-specific (discussed in **Chapter 1**). CIRp-A1 itself might be immunogenic and therefore, unsuitable for therapeutic use, but there is the potential to develop CIRp-A1 mimics, such as small peptides, which still bind properdin. Further insights into the mechanism of inhibition by CIRp-A1, including the structure of properdin-CIRp-A1 complex, would facilitate the design and development of properdin-targeting small molecules.

References

- Adam, B., Charlotiaux, B., Beaufays, J., Vanhamme, L., Godfroid, E., Brasseur, R., and Lins, L. (2008). Distantly related lipocalins share two conserved clusters of hydrophobic residues: use in homology modeling. *BMC Structural Biology* 8, 1.
- Adams, P.D., Afonine, P.V., Bunkóczi, G., Chen, V.B., Davis, I.W., Echols, N., Headd, J.J., Hung, L.-W., Kapral, G.J., Grosse-Kunstleve, R.W., et al. (2010). *PHENIX*: a comprehensive Python-based system for macromolecular structure solution. *Acta Crystallographica Section D Biological Crystallography* 66, 213–221.
- Afonine, P.V., Grosse-Kunstleve, R.W., Echols, N., Headd, J.J., Moriarty, N.W., Mustyakimov, M., Terwilliger, T.C., Urzhumtsev, A., Zwart, P.H., and Adams, P.D. (2012). Towards automated crystallographic structure refinement with *phenix.refine*. *Acta Crystallographica Section D Biological Crystallography* 68, 352–367.
- Agarwal, S., Ferreira, V.P., Cortes, C., Pangburn, M.K., Rice, P.A., and Ram, S. (2010). An Evaluation of the Role of Properdin in Alternative Pathway Activation on *Neisseria meningitidis* and *Neisseria gonorrhoeae*. *The Journal of Immunology* 185, 507–516.
- Agarwal, S., Specht, C.A., Huang, H., Ostroff, G.R., Ram, S., Rice, P.A., and Levitz, S.M. (2011). Linkage Specificity and Role of Properdin in Activation of the Alternative Complement Pathway by Fungal Glycans. *MBio* 2.
- Agarwal, V., Talens, S., Grandits, A.M., and Blom, A.M. (2015). A Novel Interaction between Complement Inhibitor C4b-binding Protein and Plasminogen That Enhances Plasminogen Activation. *Journal of Biological Chemistry* 290, 18333–18342.
- Alcorlo, M., Tortajada, A., Rodríguez de Córdoba, S., and Llorca, O. (2013). Structural basis for the stabilization of the complement alternative pathway C3 convertase by properdin. *Proc. Natl. Acad. Sci. U.S.A.* 110, 13504–13509.
- Ali, Y.M., Hayat, A., Saeed, B.M., Haleem, K.S., Alshamrani, S., Kenawy, H.I., Ferreira, V.P., Saggu, G., Buchberger, A., Lachmann, P.J., et al. (2014). Low-dose recombinant properdin provides substantial protection against *Streptococcus pneumoniae* and *Neisseria meningitidis* infection. *Proceedings of the National Academy of Sciences* 111, 5301–5306.
- Amara, U., Flierl, M.A., Rittirsch, D., Klos, A., Chen, H., Acker, B., Bruckner, U.B., Nilsson, B., Gebhard, F., Lambris, J.D., et al. (2010). Molecular Intercommunication between the Complement and Coagulation Systems. *The Journal of Immunology* 185, 5628–5636.
- Andersson, D., Carlsson, U., and Freskgård, P.-O. (2001). Contribution of tryptophan residues to the CD spectrum of the extracellular domain of human tissue factor: Application in folding studies and prediction of secondary structure. *European Journal of Biochemistry* 268, 1118–1128.
- Andersson, J., Ekdahl, K.N., Larsson, R., Nilsson, U.R., and Nilsson, B. (2002). C3 adsorbed to a polymer surface can form an initiating alternative pathway convertase. *J. Immunol.* 168, 5786–5791.

- Audemard-Verger, A., Descoux, E., Ponard, D., Deroux, A., Fantin, B., Fieschi, C., John, M., Bouldouyre, A., Karkowski, L., Moulis, G., et al. (2016). Infections Revealing Complement Deficiency in Adults: A French Nationwide Study Enrolling 41 Patients. *Medicine* 95, e3548.
- Aygören-Pürsün, E., Soteres, D., Moldovan, D., Christensen, J., Van Leerberghe, A., Hao, J., Schranz, J., Jacobson, K.W., and Martinez-Saguer, I. (2017). Preventing Hereditary Angioedema Attacks in Children Using Cinryze®: Interim Efficacy and Safety Phase 3 Findings. *International Archives of Allergy and Immunology* 173, 114–119.
- Bajic, G., Degn, S.E., Thiel, S., and Andersen, G.R. (2015). Complement activation, regulation, and molecular basis for complement-related diseases. *The EMBO Journal* 34, 2735–2757.
- Banerjee, S., Coussens, N.P., Gallat, F.-X., Sathyanarayanan, N., Srikanth, J., Yagi, K.J., Gray, J.S.S., Tobe, S.S., Stay, B., Chavas, L.M.G., et al. (2016). Structure of a heterogeneous, glycosylated, lipid-bound, *in vivo* -grown protein crystal at atomic resolution from the viviparous cockroach *Diploptera punctata*. *IUCrJ* 3, 282–293.
- Barbour, T.D., Pickering, M.C., and Terence Cook, H. (2013). Dense Deposit Disease and C3 Glomerulopathy. *Seminars in Nephrology* 33, 493–507.
- Beaufays, J., Adam, B., Decrem, Y., Prévôt, P.-P., Santini, S., Brasseur, R., Brossard, M., Lins, L., Vanhamme, L., and Godfroid, E. (2008). *Ixodes ricinus* Tick Lipocalins: Identification, Cloning, Phylogenetic Analysis and Biochemical Characterization. *PLoS ONE* 3, e3941.
- Berends, E.T.M., Gorham, R.D., Ruyken, M., Soppe, J.A., Orhan, H., Aerts, P.C., de Haas, C.J.C., Gros, P., and Rooijackers, S.H.M. (2015). Molecular insights into the surface-specific arrangement of complement C5 convertase enzymes. *BMC Biology* 13.
- Bhattacharya, S., Ploplis, V.A., and Castellino, F.J. (2012). Bacterial Plasminogen Receptors Utilize Host Plasminogen System for Effective Invasion and Dissemination. *Journal of Biomedicine and Biotechnology* 2012, 1–19.
- Blackmore, T.K., Hellwage, J., Sadlon, T.A., Higgs, N., Zipfel, P.F., Ward, H.M., and Gordon, D.L. (1998). Identification of the second heparin-binding domain in human complement factor H. *J. Immunol.* 160, 3342–3348.
- Blatt, A.Z., Pathan, S., and Ferreira, V.P. (2016a). Properdin: a tightly regulated critical inflammatory modulator. *Immunological Reviews* 274, 172–190.
- Blatt, A.Z., Saggi, G., Kulkarni, K.V., Cortes, C., Thurman, J.M., Ricklin, D., Lambris, J.D., Valenzuela, J.G., and Ferreira, V.P. (2016b). Properdin-Mediated C5a Production Enhances Stable Binding of Platelets to Granulocytes in Human Whole Blood. *The Journal of Immunology* 196, 4671–4680.
- Blaum, B.S., Hannan, J.P., Herbert, A.P., Kavanagh, D., Uhrin, D., and Stehle, T. (2014). Structural basis for sialic acid-mediated self-recognition by complement factor H. *Nature Chemical Biology* 11, 77–82.

- Blom, A.M., Hallström, T., and Riesbeck, K. (2009). Complement evasion strategies of pathogens—Acquisition of inhibitors and beyond. *Molecular Immunology* 46, 2808–2817.
- van den Bogaard, R., Fijen, C.A., Schipper, M.G., de Galan, L., Kuijper, E.J., and Mannens, M.M. (2000). Molecular characterisation of 10 Dutch properdin type I deficient families: mutation analysis and X-inactivation studies. *European Journal of Human Genetics* 8, 513–518.
- Bonifati, D., and Kishore, U. (2007). Role of complement in neurodegeneration and neuroinflammation. *Molecular Immunology* 44, 999–1010.
- Braconier, J.H., Sjöholm, A.G., and Söderström, C. (1983). Fulminant meningococcal infections in a family with inherited deficiency of properdin. *Scand. J. Infect. Dis.* 15, 339–345.
- Brodsky, R.A. (2014). Paroxysmal nocturnal hemoglobinuria. *Blood* 124, 2804–2811.
- Camous, L., Roumenina, L., Bigot, S., Brachemi, S., Fremeaux-Bacchi, V., Lesavre, P., and Halbwachs-Mecarelli, L. (2011). Complement alternative pathway acts as a positive feedback amplification of neutrophil activation. *Blood* 117, 1340–1349.
- Cavener, D.R. (1987). Comparison of the consensus sequence flanking translational start sites in *Drosophila* and vertebrates. *Nucleic Acids Research* 15, 1353–1361.
- Chen, C.-B., and Wallis, R. (2004). Two mechanisms for mannose-binding protein modulation of the activity of its associated serine proteases. *J. Biol. Chem.* 279, 26058–26065.
- Chen, J.Y., Cortes, C., and Ferreira, V.P. (2018). Properdin: A multifaceted molecule involved in inflammation and diseases. *Molecular Immunology* 102, 58–72.
- Chen, V.B., Arendall, W.B., Headd, J.J., Keedy, D.A., Immormino, R.M., Kapral, G.J., Murray, L.W., Richardson, J.S., and Richardson, D.C. (2010). *MolProbity*: all-atom structure validation for macromolecular crystallography. *Acta Crystallographica Section D Biological Crystallography* 66, 12–21.
- Claesson, R., Kanasi, E., Johansson, A., and Kalfas, S. (2010). A new cleavage site for elastase within the complement component 3: A NEW CLEAVAGE SITE WITHIN THE C3 MOLECULE. *APMIS* 118, 765–768.
- Clark, S.J., Schmidt, C.Q., White, A.M., Hakobyan, S., Morgan, B.P., and Bishop, P.N. (2014). Identification of Factor H-like Protein 1 as the Predominant Complement Regulator in Bruch's Membrane: Implications for Age-Related Macular Degeneration. *The Journal of Immunology* 193, 4962–4970.
- Cortes, C., Ferreira, V.P., and Pangburn, M.K. (2011). Native Properdin Binds to *Chlamydia pneumoniae* and Promotes Complement Activation. *Infection and Immunity* 79, 724–731.
- Cortes, C., Ohtola, J.A., Saggu, G., and Ferreira, V.P. (2013). Local release of properdin in the cellular microenvironment: role in pattern recognition and amplification of the alternative pathway of complement. *Frontiers in Immunology* 3.

- Couvreux, B., Beaufays, J., Charon, C., Lahaye, K., Gensale, F., Denis, V., Charloteaux, B., Decrem, Y., Prévôt, P.-P., Brossard, M., et al. (2008). Variability and Action Mechanism of a Family of Anticomplement Proteins in *Ixodes ricinus*. *PLoS ONE* 3, e1400.
- Cowtan, K. (2006). The *Buccaneer* software for automated model building. 1. Tracing protein chains. *Acta Crystallographica Section D Biological Crystallography* 62, 1002–1011.
- Crehan, H., Hardy, J., and Pocock, J. (2012). Microglia, Alzheimer's Disease, and Complement. *International Journal of Alzheimer's Disease* 2012, 1–10.
- Daha, M.R., and van Kooten, C. (2000). Is there a role for locally produced complement in renal disease? *Nephrology Dialysis Transplantation* 15, 1506–1509.
- Daix, V., Schroeder, H., Praet, N., Georgin, J.-P., Chiappino, I., Gillet, L., de Fays, K., Decrem, Y., Lebouille, G., Godfroid, E., et al. (2007). *Ixodes* ticks belonging to the *Ixodes ricinus* complex encode a family of anticomplement proteins. *Insect Molecular Biology* 16, 155–166.
- Damian, L. (2013). Isothermal Titration Calorimetry for Studying Protein–Ligand Interactions. In *Protein-Ligand Interactions*, M.A. Williams, and T. Daviter, eds. (Totowa, NJ: Humana Press), pp. 103–118.
- Das, S., Banerjee, G., DePonte, K., Marcantonio, N., Kantor, F.S., and Fikrig, E. (2001). Salp25D, an *Ixodes scapularis* Antioxidant, Is 1 of 14 Immunodominant Antigens in Engorged Tick Salivary Glands. *The Journal of Infectious Diseases* 184, 1056–1064.
- Dauter, Z. (2003). Twinned crystals and anomalous phasing. *Acta Crystallographica Section D Biological Crystallography* 59, 2004–2016.
- Davis, A.E., Mejia, P., and Lu, F. (2008). Biological activities of C1 inhibitor. *Molecular Immunology* 45, 4057–4063.
- Denk, S., Neher, M.D., Messerer, D.A.C., Wiegner, R., Nilsson, B., Rittirsch, D., Nilsson-Ekdahl, K., Weckbach, S., Ignatius, A., Kalbitz, M., et al. (2017). Complement C5a Functions as a Master Switch for the pH Balance in Neutrophils Exerting Fundamental Immunometabolic Effects. *The Journal of Immunology* 198, 4846–4854.
- Dimitrova, P., Ivanovska, N., Schwaeble, W., Gyurkovska, V., and Stover, C. (2010). The role of properdin in murine zymosan-induced arthritis. *Molecular Immunology* 47, 1458–1466.
- Dimitrova, P., Ivanovska, N., Belenska, L., Milanova, V., Schwaeble, W., and Stover, C. (2012). Abrogated RANKL expression in properdin-deficient mice is associated with better outcome from collagen-antibody-induced arthritis. *Arthritis Research & Therapy* 14, R173.
- Eisenberg, D., Schwarz, E., Komaromy, M., and Wall, R. (1984). Analysis of membrane and surface protein sequences with the hydrophobic moment plot. *Journal of Molecular Biology* 179, 125–142.

- Emsley, P., Lohkamp, B., Scott, W.G., and Cowtan, K. (2010). Features and development of *Coot*. *Acta Crystallographica Section D Biological Crystallography* 66, 486–501.
- Ermert, D., and Blom, A.M. (2016). C4b-binding protein: The good, the bad and the deadly. Novel functions of an old friend. *Immunology Letters* 169, 82–92.
- Evans, P. (2006). Scaling and assessment of data quality. *Acta Crystallographica Section D Biological Crystallography* 62, 72–82.
- Evans, P.R. (2011). An introduction to data reduction: space-group determination, scaling and intensity statistics. *Acta Crystallographica Section D Biological Crystallography* 67, 282–292.
- Evans, P.R., and Murshudov, G.N. (2013). How good are my data and what is the resolution? *Acta Crystallographica Section D Biological Crystallography* 69, 1204–1214.
- Farkas, I., Baranyi, L., Ishikawa, Y., Okada, N., Bohata, C., Budai, D., Fukuda, A., Imai, M., and Okada, H. (2002). CD59 blocks not only the insertion of C9 into MAC but inhibits ion channel formation by homologous C5b-8 as well as C5b-9. *The Journal of Physiology* 539, 537–545.
- Farries, T.C., Lachmann, P.J., and Harrison, R.A. (1988a). Analysis of the interactions between properdin, the third component of complement (C3), and its physiological activation products. *Biochem. J.* 252, 47–54.
- Farries, T.C., Lachmann, P.J., and Harrison, R.A. (1988b). Analysis of the interaction between properdin and factor B, components of the alternative-pathway C3 convertase of complement. *Biochem. J.* 253, 667–675.
- Fearon, D.T., and Austen, K.F. (1975). Properdin: binding to C3b and stabilization of the C3b-dependent C3 convertase. *J. Exp. Med.* 142, 856–863.
- Ferreira, V.P., Herbert, A.P., Hocking, H.G., Barlow, P.N., and Pangburn, M.K. (2006). Critical Role of the C-Terminal Domains of Factor H in Regulating Complement Activation at Cell Surfaces. *The Journal of Immunology* 177, 6308–6316.
- Ferreira, V.P., Cortes, C., and Pangburn, M.K. (2010). Native polymeric forms of properdin selectively bind to targets and promote activation of the alternative pathway of complement. *Immunobiology* 215, 932–940.
- Feussner, A., Kalina, U., Hofmann, P., Machnig, T., and Henkel, G. (2014). Biochemical comparison of four commercially available C1 esterase inhibitor concentrates for treatment of hereditary angioedema: Biochemical Analyses of C1-INH Products. *Transfusion* 54, 2566–2573.
- Fijen, C.A., van den Bogaard, R., Schipper, M., Mannens, M., Schlesinger, M., Nordin, F.G., Dankert, J., Daha, M.R., Sjöholm, A.G., Truedsson, L., et al. (1999). Properdin deficiency: molecular basis and disease association. *Mol. Immunol.* 36, 863–867.
- Fisher, R.G., and Sweet, R.M. (1980). Treatment of diffraction data from crystals twinned by merohedry. *Acta Crystallographica Section A* 36, 755–760.

- Francischetti, I.M.B., My Pham, V., Mans, B.J., Andersen, J.F., Mather, T.N., Lane, R.S., and Ribeiro, J.M.C. (2005). The transcriptome of the salivary glands of the female western black-legged tick *Ixodes pacificus* (Acari: Ixodidae). *Insect Biochemistry and Molecular Biology* 35, 1142–1161.
- Fraser, D.A., Pisalyaput, K., and Tenner, A.J. (2010). C1q enhances microglial clearance of apoptotic neurons and neuronal blebs, and modulates subsequent inflammatory cytokine production. *Journal of Neurochemistry* 112, 733–743.
- Fredrikson, G.N., Westberg, J., Kuijper, E.J., Tijssen, C.C., Sjöholm, A.G., Uhlén, M., and Truedsson, L. (1996). Molecular characterization of properdin deficiency type III: dysfunction produced by a single point mutation in exon 9 of the structural gene causing a tyrosine to aspartic acid interchange. *J. Immunol.* 157, 3666–3671.
- Fredrikson, G.N., Gullstrand, B., Westberg, J., Sjöholm, A.G., Uhlén, M., and Truedsson, L. (1998). Expression of properdin in complete and incomplete deficiency: normal in vitro synthesis by monocytes in two cases with properdin deficiency type II due to distinct mutations. *J. Clin. Immunol.* 18, 272–282.
- Fredslund, F., Laursen, N.S., Roversi, P., Jenner, L., Oliveira, C.L.P., Pedersen, J.S., Nunn, M.A., Lea, S.M., Discipio, R., Sottrup-Jensen, L., et al. (2008). Structure of and influence of a tick complement inhibitor on human complement component 5. *Nature Immunology* 9, 753–760.
- Fritsche, L.G., Lauer, N., Hartmann, A., Stippa, S., Keilhauer, C.N., Oppermann, M., Pandey, M.K., Köhl, J., Zipfel, P.F., Weber, B.H.F., et al. (2010). An imbalance of human complement regulatory proteins CFHR1, CFHR3 and factor H influences risk for age-related macular degeneration (AMD). *Human Molecular Genetics* 19, 4694–4704.
- Fujita, T., Inoue, T., Ogawa, K., Iida, K., and Tamura, N. (1987). The mechanism of action of decay-accelerating factor (DAF). DAF inhibits the assembly of C3 convertases by dissociating C2a and Bb. *J. Exp. Med.* 166, 1221–1228.
- Furmanek, A., and Hofsteenge, J. (2000). Protein C-mannosylation: facts and questions. *Acta Biochim. Pol.* 47, 781–789.
- Gaarkeuken, H., Siezenga, M.A., Zuidwijk, K., van Kooten, C., Rabelink, T.J., Daha, M.R., and Berger, S.P. (2008). Complement activation by tubular cells is mediated by properdin binding. *American Journal of Physiology-Renal Physiology* 295, F1397–F1403.
- Gaboriaud, C., Frachet, P., Thielens, N.M., and Arlaud, G.J. (2012). The Human C1q Globular Domain: Structure and Recognition of Non-Immune Self Ligands. *Frontiers in Immunology* 2.
- Gao, Z., Ma, Z., Qu, B., Jiao, D., and Zhang, S. (2017). Identification and characterization of properdin in amphioxus: Implications for a functional alternative complement pathway in the basal chordate. *Fish & Shellfish Immunology* 65, 1–8.
- Garcia, B.L., Ramyar, K.X., Tzekou, A., Ricklin, D., McWhorter, W.J., Lambris, J.D., and Geisbrecht, B.V. (2010). Molecular Basis for Complement Recognition and Inhibition Determined by Crystallographic Studies of the Staphylococcal Complement Inhibitor (SCIN) Bound to C3c and C3b. *Journal of Molecular Biology* 402, 17–29.

- Goicoechea de Jorge, E., Harris, C.L., Esparza-Gordillo, J., Carreras, L., Arranz, E.A., Garrido, C.A., López-Trascasa, M., Sánchez-Corral, P., Morgan, B.P., and Rodríguez de Córdoba, S. (2007). Gain-of-function mutations in complement factor B are associated with atypical hemolytic uremic syndrome. *Proc. Natl. Acad. Sci. U.S.A.* *104*, 240–245.
- Goicoechea de Jorge, E., Caesar, J.J.E., Malik, T.H., Patel, M., Colledge, M., Johnson, S., Hakobyan, S., Morgan, B.P., Harris, C.L., Pickering, M.C., et al. (2013). Dimerization of complement factor H-related proteins modulates complement activation in vivo. *Proceedings of the National Academy of Sciences* *110*, 4685–4690.
- Greenfield, N.J. (2007). Using circular dichroism spectra to estimate protein secondary structure. *Nature Protocols* *1*, 2876–2890.
- Gulla, K.C., Gupta, K., Krarup, A., Gal, P., Schwaeble, W.J., Sim, R.B., O'Connor, C.D., and Hajela, K. (2010). Activation of mannan-binding lectin-associated serine proteases leads to generation of a fibrin clot: MASPs generate a fibrin clot. *Immunology* *129*, 482–495.
- Gullipalli, D., Zhang, F., Sato, S., Ueda, Y., Kimura, Y., Golla, M., Miwa, T., Wang, J., and Song, W.-C. (2018). Antibody Inhibition of Properdin Prevents Complement-Mediated Intravascular and Extravascular Hemolysis. *The Journal of Immunology* *201*, 1021–1029.
- Gupta-Bansal, R., Parent, J.B., and Brunden, K.R. (2000). Inhibition of complement alternative pathway function with anti-properdin monoclonal antibodies. *Molecular Immunology* *37*, 191–201.
- Hammel, M., Sfyroera, G., Ricklin, D., Magotti, P., Lambris, J.D., and Geisbrecht, B.V. (2007a). A structural basis for complement inhibition by *Staphylococcus aureus*. *Nature Immunology* *8*, 430–437.
- Hammel, M., Sfyroera, G., Pyrpassopoulos, S., Ricklin, D., Ramyar, K.X., Pop, M., Jin, Z., Lambris, J.D., and Geisbrecht, B.V. (2007b). Characterization of Ehp, a Secreted Complement Inhibitory Protein from *Staphylococcus aureus*. *Journal of Biological Chemistry* *282*, 30051–30061.
- Harboe, M., Ulvund, G., Vien, L., Fung, M., and Mollnes, T.E. (2004). The quantitative role of alternative pathway amplification in classical pathway induced terminal complement activation. *Clin. Exp. Immunol.* *138*, 439–446.
- Harboe, M., Thorgersen, E.B., and Mollnes, T.E. (2011). Advances in assay of complement function and activation. *Advanced Drug Delivery Reviews* *63*, 976–987.
- Harboe, M., Garred, P., Lindstad, J.K., Pharo, A., Müller, F., Stahl, G.L., Lambris, J.D., and Mollnes, T.E. (2012). The Role of Properdin in Zymosan- and *Escherichia coli* - Induced Complement Activation. *The Journal of Immunology* *189*, 2606–2613.
- Harboe, M., Johnson, C., Nymo, S., Ekholt, K., Schjalm, C., Lindstad, J.K., Pharo, A., Hellerud, B.C., Nilsson Ekdahl, K., Mollnes, T.E., et al. (2017). Properdin binding to complement activating surfaces depends on initial C3b deposition. *Proceedings of the National Academy of Sciences* *201612385*.

- Harris, C.L. (2018). Expanding horizons in complement drug discovery: challenges and emerging strategies. *Seminars in Immunopathology* 40, 125–140.
- Harrison, R.A. (2017). The properdin pathway: an “alternative activation pathway” or a “critical amplification loop” for C3 and C5 activation? *Seminars in Immunopathology*.
- Hartmann, S. (2000). Properdin, the Positive Regulator of Complement, Is Highly C-Mannosylated. *Journal of Biological Chemistry* 275, 28569–28574.
- Hartmann, S., and Hofsteenge, J. (2000). Properdin, the Positive Regulator of Complement, Is Highly C-Mannosylated. *Journal of Biological Chemistry* 275, 28569–28574.
- Hebecker, M., and Józsi, M. (2012). Factor H-related Protein 4 Activates Complement by Serving as a Platform for the Assembly of Alternative Pathway C3 Convertase via Its Interaction with C3b Protein. *Journal of Biological Chemistry* 287, 19528–19536.
- Heeger, P.S., Lalli, P.N., Lin, F., Valujskikh, A., Liu, J., Muqim, N., Xu, Y., and Medof, M.E. (2005). Decay-accelerating factor modulates induction of T cell immunity. *The Journal of Experimental Medicine* 201, 1523–1530.
- Helminen, M., Seitsonen, S., Jarva, H., Meri, S., and Järvelä, I.E. (2012). A Novel Mutation W388X Underlying Properdin Deficiency in a Finnish Family: A Novel Mutation Underlying Properdin Deficiency. *Scandinavian Journal of Immunology* 75, 445–448.
- Helmy, K.Y., Katschke, K.J., Gorgani, N.N., Kljavin, N.M., Elliott, J.M., Diehl, L., Scales, S.J., Ghilardi, N., and van Lookeren Campagne, M. (2006). CR1g: A Macrophage Complement Receptor Required for Phagocytosis of Circulating Pathogens. *Cell* 124, 915–927.
- Henderson, A.L., Lindorfer, M.A., Kennedy, A.D., Foley, P.L., and Taylor, R.P. (2002). Concerted clearance of immune complexes bound to the human erythrocyte complement receptor: development of a heterologous mouse model. *Journal of Immunological Methods* 270, 183–197.
- Hepburn, N.J., Williams, A.S., Nunn, M.A., Chamberlain-Banoub, J.C., Hamer, J., Morgan, B.P., and Harris, C.L. (2007). *In Vivo* Characterization and Therapeutic Efficacy of a C5-specific Inhibitor from the Soft Tick *Ornithodoros moubata*. *Journal of Biological Chemistry* 282, 8292–8299.
- Heurich, M., Martinez-Barricarte, R., Francis, N.J., Roberts, D.L., Rodriguez de Cordoba, S., Morgan, B.P., and Harris, C.L. (2011). Common polymorphisms in C3, factor B, and factor H collaborate to determine systemic complement activity and disease risk. *Proceedings of the National Academy of Sciences* 108, 8761–8766.
- Heutinck, K.M., ten Berge, I.J.M., Hack, C.E., Hamann, J., and Rowshani, A.T. (2010). Serine proteases of the human immune system in health and disease. *Molecular Immunology* 47, 1943–1955.

- Higgins, J.M., Wiedemann, H., Timpl, R., and Reid, K.B. (1995). Characterization of mutant forms of recombinant human properdin lacking single thrombospondin type I repeats. Identification of modules important for function. *J. Immunol.* *155*, 5777–5785.
- Hill, A., Weston-Davies, W., Nunn, M., Robak, T., Windyga, J., Hellmann, A., Kulasekararaj, A.G., Piekarska, A., Szmigielska-Kaplon, A., Griffin, M., et al. (2017). Coversin, a Novel C5 Complement Inhibitor, Is Safe and Effective in the Treatment of PNH: Results of a Phase II Clinical Trial. *Blood* *130*, 4747–4747.
- Hillmen, P., Young, N.S., Schubert, J., Brodsky, R.A., Socié, G., Muus, P., Röth, A., Szer, J., Elebute, M.O., Nakamura, R., et al. (2006). The Complement Inhibitor Eculizumab in Paroxysmal Nocturnal Hemoglobinuria. *New England Journal of Medicine* *355*, 1233–1243.
- Hofstra, J.J., Budde, I.K., van Twuyver, E., Choi, G., Levi, M., Leebeek, F.W.G., de Monchy, J.G.R., Ypma, P.F., Keizer, R.J., Huitema, A.D.R., et al. (2012). Treatment of hereditary angioedema with nanofiltered C1-esterase inhibitor concentrate (Cetor®): Multi-center phase II and III studies to assess pharmacokinetics, clinical efficacy and safety. *Clinical Immunology* *142*, 280–290.
- Holers, V.M. (2008). The spectrum of complement alternative pathway-mediated diseases. *Immunological Reviews* *223*, 300–316.
- Holers, V.M., and Banda, N.K. (2018). Complement in the Initiation and Evolution of Rheumatoid Arthritis. *Frontiers in Immunology* *9*.
- Holm, L. (2019). Benchmarking Fold Detection by DaliLite v.5. *Bioinformatics*.
- Hong, Y.Q., and Ghebrehiwet, B. (1992). Effect of *Pseudomonas aeruginosa* elastase and alkaline protease on serum complement and isolated components C1q and C3. *Clin. Immunol. Immunopathol.* *62*, 133–138.
- Hourcade, D.E. (2006). The Role of Properdin in the Assembly of the Alternative Pathway C3 Convertases of Complement. *Journal of Biological Chemistry* *281*, 2128–2132.
- Hourcade, D.E., Mitchell, L.M., and Oglesby, T.J. (1999). Mutations of the type A domain of complement factor B that promote high-affinity C3b-binding. *J. Immunol.* *162*, 2906–2911.
- Hourcade, D.E., Akk, A.M., Mitchell, L.M., Zhou, H., Hauhart, R., and Pham, C.T.N. (2016). Anti-complement activity of the *Ixodes scapularis* salivary protein Salp20. *Molecular Immunology* *69*, 62–69.
- Hovingh, E.S., van den Broek, B., and Jongerius, I. (2016). Hijacking Complement Regulatory Proteins for Bacterial Immune Evasion. *Frontiers in Microbiology* *7*.
- Huang, Y., Qiao, F., Abagyan, R., Hazard, S., and Tomlinson, S. (2006). Defining the CD59-C9 Binding Interaction. *Journal of Biological Chemistry* *281*, 27398–27404.
- Huber-Lang, M., Sarma, J.V., Zetoune, F.S., Rittirsch, D., Neff, T.A., McGuire, S.R., Lambris, J.D., Warner, R.L., Flierl, M.A., Hoesel, L.M., et al. (2006). Generation of C5a

in the absence of C3: a new complement activation pathway. *Nature Medicine* 12, 682–687.

Hughes-Jones, N.C., and Gardner, B. (1979). Reaction between the isolated globular subunits of the complement component C1q and IgG-complexes. *Molecular Immunology* 16, 697–701.

Janssen, B.J.C., Huizinga, E.G., Raaijmakers, H.C.A., Roos, A., Daha, M.R., Nilsson-Ekdahl, K., Nilsson, B., and Gros, P. (2005). Structures of complement component C3 provide insights into the function and evolution of immunity. *Nature* 437, 505–511.

Janssen, B.J.C., Christodoulidou, A., McCarthy, A., Lambris, J.D., and Gros, P. (2006). Structure of C3b reveals conformational changes that underlie complement activity. *Nature* 444, 213–216.

Jaroszewski, L., Rychlewski, L., Li, Z., Li, W., and Godzik, A. (2005). FFAS03: a server for profile--profile sequence alignments. *Nucleic Acids Res.* 33, W284–288.

Jerabek-Willemsen, M., Wienken, C.J., Braun, D., Baaske, P., and Duhr, S. (2011). Molecular interaction studies using microscale thermophoresis. *Assay Drug Dev Technol* 9, 342–353.

Jerabek-Willemsen, M., André, T., Wanner, R., Roth, H.M., Duhr, S., Baaske, P., and Breitsprecher, D. (2014). MicroScale Thermophoresis: Interaction analysis and beyond. *Journal of Molecular Structure* 1077, 101–113.

Jokiranta, T.S., Cheng, Z.-Z., Seeberger, H., Jözsi, M., Heinen, S., Noris, M., Remuzzi, G., Ormsby, R., Gordon, D.L., Meri, S., et al. (2005). Binding of Complement Factor H to Endothelial Cells Is Mediated by the Carboxy-Terminal Glycosaminoglycan Binding Site. *The American Journal of Pathology* 167, 1173–1181.

Jönsen, A., Nilsson, S.C., Ahlqvist, E., Svenungsson, E., Gunnarsson, I., Eriksson, K.G., Bengtsson, A., Zickert, A., Eloranta, M.-L., Truedsson, L., et al. (2011). Mutations in genes encoding complement inhibitors CD46 and CFH affect the age at nephritis onset in patients with systemic lupus erythematosus. *Arthritis Research & Therapy* 13, R206.

Jore, M.M., Johnson, S., Sheppard, D., Barber, N.M., Li, Y.I., Nunn, M.A., Elmlund, H., and Lea, S.M. (2016). Structural basis for therapeutic inhibition of complement C5. *Nature Structural & Molecular Biology*.

Józsi, M., Tortajada, A., Uzonyi, B., Goicoechea de Jorge, E., and Rodríguez de Córdoba, S. (2015). Factor H-related proteins determine complement-activating surfaces. *Trends in Immunology* 36, 374–384.

Junker, Baatrup, Svehag, Wang, Holmström, Sturfelt, and Sjöholm (1998). Binding of Properdin to Solid-Phase Immune Complexes: Critical Role of the Classical Activation Pathway of Complement. *Scandinavian Journal of Immunology* 47, 481–486.

Jurrus, E., Engel, D., Star, K., Monson, K., Brandi, J., Felberg, L.E., Brookes, D.H., Wilson, L., Chen, J., Liles, K., et al. (2018). Improvements to the APBS biomolecular solvation software suite: Improvements to the APBS Software Suite. *Protein Science* 27, 112–128.

- Kabsch, W. (2010). *XDS*. *Acta Crystallographica Section D Biological Crystallography* 66, 125–132.
- Kajander, T., Lehtinen, M.J., Hyvarinen, S., Bhattacharjee, A., Leung, E., Isenman, D.E., Meri, S., Goldman, A., and Jokiranta, T.S. (2011). Dual interaction of factor H with C3d and glycosaminoglycans in host-nonhost discrimination by complement. *Proceedings of the National Academy of Sciences* 108, 2897–2902.
- Kantardjieff, K.A., and Rupp, B. (2003). Matthews coefficient probabilities: Improved estimates for unit cell contents of proteins, DNA, and protein-nucleic acid complex crystals. *Protein Science* 12, 1865–1871.
- Kardos, J., Gál, P., Szilágyi, L., Thielens, N.M., Szilágyi, K., Lőrincz, Z., Kulcsár, P., Gráf, L., Arlaud, G.J., and Závodszky, P. (2001). The Role of the Individual Domains in the Structure and Function of the Catalytic Region of a Modular Serine Protease, C1r. *The Journal of Immunology* 167, 5202–5208.
- Katschke, K.J., Stawicki, S., Yin, J., Steffek, M., Xi, H., Sturgeon, L., Hass, P.E., Loyet, K.M., DeForge, L., Wu, Y., et al. (2009). Structural and Functional Analysis of a C3b-specific Antibody That Selectively Inhibits the Alternative Pathway of Complement. *Journal of Biological Chemistry* 284, 10473–10479.
- Kaufmann, S.H.E. (2008). Immunology's foundation: the 100-year anniversary of the Nobel Prize to Paul Ehrlich and Elie Metchnikoff. *Nature Immunology* 9, 705–712.
- Keller, P.M., Waxman, L., Arnold, B.A., Schultz, L.D., Condra, C., and Connolly, T.M. (1993). Cloning of the cDNA and expression of moubatin, an inhibitor of platelet aggregation. *J. Biol. Chem.* 268, 5450–5456.
- Kemper, C., Mitchell, L.M., Zhang, L., and Hourcade, D.E. (2008). The complement protein properdin binds apoptotic T cells and promotes complement activation and phagocytosis. *Proc. Natl. Acad. Sci. U.S.A.* 105, 9023–9028.
- Kerr, F.K., O'Brien, G., Quinsey, N.S., Whisstock, J.C., Boyd, S., de la Banda, M.G., Kaiserman, D., Matthews, A.Y., Bird, P.I., and Pike, R.N. (2005). Elucidation of the Substrate Specificity of the C1s Protease of the Classical Complement Pathway. *Journal of Biological Chemistry* 280, 39510–39514.
- Kimura, Y., Miwa, T., Zhou, L., and Song, W.-C. (2008). Activator-specific requirement of properdin in the initiation and amplification of the alternative pathway complement. *Blood* 111, 732–740.
- Kimura, Y., Zhou, L., Miwa, T., and Song, W.-C. (2010). Genetic and therapeutic targeting of properdin in mice prevents complement-mediated tissue injury. *Journal of Clinical Investigation* 120, 3545–3554.
- Kinoshita, T., Takata, Y., Kozono, H., Takeda, J., Hong, K.S., and Inoue, K. (1988). C5 convertase of the alternative complement pathway: covalent linkage between two C3b molecules within the trimolecular complex enzyme. *J. Immunol.* 141, 3895–3901.
- Kolev, M., Dimeloe, S., Le Friec, G., Navarini, A., Arbore, G., Povoleri, G.A., Fischer, M., Belle, R., Loeliger, J., Develioglou, L., et al. (2015). Complement Regulates Nutrient

Influx and Metabolic Reprogramming during Th1 Cell Responses. *Immunity* 42, 1033–1047.

Konar, M., and Granoff, D.M. (2017). Eculizumab treatment and impaired opsonophagocytic killing of meningococci by whole blood from immunized adults. *Blood* 130, 891–899.

Kouser, L., Abdul-Aziz, M., Tsolaki, A.G., Singhal, D., Schwaebler, W.J., Urban, B.C., Khan, H.A., Sim, R.B., and Kishore, U. (2016). A recombinant two-module form of human properdin is an inhibitor of the complement alternative pathway. *Molecular Immunology* 73, 76–87.

Kozak, M. (1991). An analysis of vertebrate mRNA sequences: intimations of translational control. *J. Cell Biol.* 115, 887–903.

Kraiczy, P., and Stevenson, B. (2013). Complement regulator-acquiring surface proteins of *Borrelia burgdorferi*: Structure, function and regulation of gene expression. *Ticks and Tick-Borne Diseases* 4, 26–34.

Krurup, A., Wallis, R., Presanis, J.S., Gál, P., and Sim, R.B. (2007). Simultaneous Activation of Complement and Coagulation by MBL-Associated Serine Protease 2. *PLoS ONE* 2, e623.

Krych-Goldberg, M., and Atkinson, J.P. (2001). Structure-function relationships of complement receptor type 1. *Immunol. Rev.* 180, 112–122.

Kühn, S., and Zipfel, P.F. (1996). Mapping of the domains required for decay acceleration activity of the human factor H-like protein 1 and factor H. *European Journal of Immunology* 26, 2383–2387.

Laarman, A.J., Ruyken, M., Malone, C.L., van Strijp, J.A.G., Horswill, A.R., and Rooijakkers, S.H.M. (2011). *Staphylococcus aureus* Metalloprotease Aureolysin Cleaves Complement C3 To Mediate Immune Evasion. *The Journal of Immunology* 186, 6445–6453.

Laarman, A.J., Bardoel, B.W., Ruyken, M., Fernie, J., Milder, F.J., van Strijp, J.A.G., and Rooijakkers, S.H.M. (2012). *Pseudomonas aeruginosa* Alkaline Protease Blocks Complement Activation via the Classical and Lectin Pathways. *The Journal of Immunology* 188, 386–393.

Lachmann, P.J. (2018). Looking back on the alternative complement pathway. *Immunobiology*.

Lambris, J.D., Ricklin, D., and Geisbrecht, B.V. (2008). Complement evasion by human pathogens. *Nature Reviews Microbiology* 6, 132–142.

Langford-Smith, A., Day, A.J., Bishop, P.N., and Clark, S.J. (2015). Complementing the Sugar Code: Role of GAGs and Sialic Acid in Complement Regulation. *Frontiers in Immunology* 6.

Langley, R., Wines, B., Willoughby, N., Basu, I., Proft, T., and Fraser, J.D. (2005). The *Staphylococcal* Superantigen-Like Protein 7 Binds IgA and Complement C5 and Inhibits

IgA-FcαRI Binding and Serum Killing of Bacteria. *The Journal of Immunology* 174, 2926–2933.

Laursen, N.S., Gordon, N., Hermans, S., Lorenz, N., Jackson, N., Wines, B., Spillner, E., Christensen, J.B., Jensen, M., Fredslund, F., et al. (2010). Structural basis for inhibition of complement C5 by the SSL7 protein from *Staphylococcus aureus*. *Proceedings of the National Academy of Sciences* 107, 3681–3686.

Law, S.K., Lichtenberg, N.A., and Levine, R.P. (1980). Covalent binding and hemolytic activity of complement proteins. *Proceedings of the National Academy of Sciences* 77, 7194–7198.

Lawrie, C.H., Randolph, S.E., and Nuttall, P.A. (1999). Ixodes Ticks: Serum Species Sensitivity of Anticomplement Activity. *Experimental Parasitology* 93, 207–214.

Lee, L.Y.L., Höök, M., Haviland, D., Wetsel, R.A., Yonter, E.O., Syribeys, P., Vernachio, J., and Brown, E.L. (2004). Inhibition of Complement Activation by a Secreted *Staphylococcus aureus* Protein. *The Journal of Infectious Diseases* 190, 571–579.

Legendre, C.M., Licht, C., Muus, P., Greenbaum, L.A., Babu, S., Bedrosian, C., Bingham, C., Cohen, D.J., Delmas, Y., Douglas, K., et al. (2013). Terminal Complement Inhibitor Eculizumab in Atypical Hemolytic–Uremic Syndrome. *New England Journal of Medicine* 368, 2169–2181.

Leshner, A.M., Zhou, L., Kimura, Y., Sato, S., Gullipalli, D., Herbert, A.P., Barlow, P.N., Eberhardt, H.U., Skerka, C., Zipfel, P.F., et al. (2013). Combination of Factor H Mutation and Properdin Deficiency Causes Severe C3 Glomerulonephritis. *Journal of the American Society of Nephrology* 24, 53–65.

Li, R. (2015). Transient transfection of CHO cells using linear polyethylenimine is a simple and effective means of producing rainbow trout recombinant IFN-γ protein. *Cytotechnology* 67, 987–993.

Li, K., Fazekasova, H., Wang, N., Sagoo, P., Peng, Q., Khamri, W., Gomes, C., Sacks, S.H., Lombardi, G., and Zhou, W. (2011). Expression of complement components, receptors and regulators by human dendritic cells. *Molecular Immunology* 48, 1121–1127.

Linton, S.M., and Morgan, B.P. (1999). Properdin deficiency and meningococcal disease—identifying those most at risk. *Clin. Exp. Immunol.* 118, 189–191.

Liu, Y., Endo, Y., Iwaki, D., Nakata, M., Matsushita, M., Wada, I., Inoue, K., Munakata, M., and Fujita, T. (2005). Human M-Ficolin Is a Secretory Protein That Activates the Lectin Complement Pathway. *The Journal of Immunology* 175, 3150–3156.

Longo, P.A., Kavran, J.M., Kim, M.-S., and Leahy, D.J. (2013). Transient Mammalian Cell Transfection with Polyethylenimine (PEI). In *Methods in Enzymology*, (Elsevier), pp. 227–240.

Lubbers, R., van Essen, M.F., van Kooten, C., and Trouw, L.A. (2017). Production of complement components by cells of the immune system: Production of complement components. *Clinical & Experimental Immunology* 188, 183–194.

- Luzzatto, L. (2006). Paroxysmal nocturnal hemoglobinuria: an acquired X-linked genetic disease with somatic-cell mosaicism. *Current Opinion in Genetics & Development* 16, 317–322.
- Ma, Y.J., Skjoedt, M.-O., and Garred, P. (2013). Collectin-11/MASP Complex Formation Triggers Activation of the Lectin Complement Pathway - The Fifth Lectin Pathway Initiation Complex. *Journal of Innate Immunity* 5, 242–250.
- Makou, E., Herbert, A.P., and Barlow, P.N. (2013). Functional Anatomy of Complement Factor H. *Biochemistry* 52, 3949–3962.
- Mans, B.J., and Ribeiro, J.M.C. (2008). A novel clade of cysteinyl leukotriene scavengers in soft ticks. *Insect Biochemistry and Molecular Biology* 38, 862–870.
- Mans, B.J., Ribeiro, J.M.C., and Andersen, J.F. (2008). Structure, Function, and Evolution of Biogenic Amine-binding Proteins in Soft Ticks. *Journal of Biological Chemistry* 283, 18721–18733.
- Manuelian, T., Hellwage, J., Meri, S., Caprioli, J., Noris, M., Heinen, S., Jozsi, M., Neumann, H.P.H., Remuzzi, G., and Zipfel, P.F. (2003). Mutations in factor H reduce binding affinity to C3b and heparin and surface attachment to endothelial cells in hemolytic uremic syndrome. *Journal of Clinical Investigation* 111, 1181–1190.
- Masaki, T., Matsumoto, M., Nakanishi, I., Yasuda, R., and Seya, T. (1992). Factor I-dependent inactivation of human complement C4b of the classical pathway by C3b/C4b receptor (CR1, CD35) and membrane cofactor protein (MCP, CD46). *J. Biochem.* 111, 573–578.
- Mastellos, D.C., Yancopoulou, D., Kokkinos, P., Huber-Lang, M., Hajishengallis, G., Biglarnia, A.R., Lupu, F., Nilsson, B., Risitano, A.M., Ricklin, D., et al. (2015). Compstatin: a C3-targeted complement inhibitor reaching its prime for bedside intervention. *European Journal of Clinical Investigation* 45, 423–440.
- Matsushita, M., and Fujita, T. (1992). Activation of the classical complement pathway by mannose-binding protein in association with a novel C1s-like serine protease. *J. Exp. Med.* 176, 1497–1502.
- Matsushita, M., Thiel, S., Jensenius, J.C., Terai, I., and Fujita, T. (2000). Proteolytic activities of two types of mannose-binding lectin-associated serine protease. *J. Immunol.* 165, 2637–2642.
- Matsushita, M., Kuraya, M., Hamasaki, N., Tsujimura, M., Shiraki, H., and Fujita, T. (2002). Activation of the Lectin Complement Pathway by H-Ficolin (Hakata Antigen). *The Journal of Immunology* 168, 3502–3506.
- Matthews, B.W. (1968). Solvent content of protein crystals. *J. Mol. Biol.* 33, 491–497.
- McCoy, A.J., Grosse-Kunstleve, R.W., Adams, P.D., Winn, M.D., Storoni, L.C., and Read, R.J. (2007). Phaser crystallographic software. *J Appl Crystallogr* 40, 658–674.
- McGeer, P.L., Lee, M., and McGeer, E.G. (2017). A review of human diseases caused or exacerbated by aberrant complement activation. *Neurobiology of Aging* 52, 12–22.

- Medicus, R.G., Esser, A.F., Fernandez, H.N., and Müller-Eberhard, H.J. (1980). Native and activated properdin: interconvertibility and identity of amino- and carboxy-terminal sequences. *J. Immunol.* *124*, 602–606.
- Mendes-Sousa, A.F., Vale, V.F. do, Silva, N.C.S., Guimaraes-Costa, A.B., Pereira, M.H., Sant’Anna, M.R.V., Oliveira, F., Kamhawi, S., Ribeiro, J.M.C., Andersen, J.F., et al. (2017). The Sand Fly Salivary Protein Lufaxin Inhibits the Early Steps of the Alternative Pathway of Complement by Direct Binding to the Proconvertase C3b-B. *Frontiers in Immunology* *8*.
- Meri, S., and Pangburn, M.K. (1990). Discrimination between activators and nonactivators of the alternative pathway of complement: regulation via a sialic acid/polyanion binding site on factor H. *Proceedings of the National Academy of Sciences* *87*, 3982–3986.
- Merle, N.S., Noe, R., Halbwachs-Mecarelli, L., Fremeaux-Bacchi, V., and Roumenina, L.T. (2015). Complement System Part II: Role in Immunity. *Frontiers in Immunology* *6*.
- Miao, J., Leshner, A.M., Miwa, T., Sato, S., Gullipalli, D., and Song, W.-C. (2014). Tissue-specific deletion of Crry from mouse proximal tubular epithelial cells increases susceptibility to renal ischemia–reperfusion injury. *Kidney International* *86*, 726–737.
- Miao, W.M., Seng, W.L., Duquette, M., Lawler, P., Laus, C., and Lawler, J. (2001). Thrombospondin-1 type 1 repeat recombinant proteins inhibit tumor growth through transforming growth factor-beta-dependent and -independent mechanisms. *Cancer Res.* *61*, 7830–7839.
- Michels, M.A.H.M., Volokhina, E.B., van de Kar, N.C.A.J., and van den Heuvel, L.P.W.J. (2019). The role of properdin in complement-mediated renal diseases: a new player in complement-inhibiting therapy? *Pediatric Nephrology* *34*, 1349–1367.
- Minta, J.O., Man, D., and Movat, H.Z. (1977). Kinetic studies on the fragmentation of the third component of complement (C3) by trypsin. *J. Immunol.* *118*, 2192–2198.
- Mitchell, P., Liew, G., Gopinath, B., and Wong, T.Y. (2018). Age-related macular degeneration. *The Lancet* *392*, 1147–1159.
- Miwa, T., Sato, S., Gullipalli, D., Nangaku, M., and Song, W.-C. (2013). Blocking Properdin, the Alternative Pathway, and Anaphylatoxin Receptors Ameliorates Renal Ischemia-Reperfusion Injury in Decay-Accelerating Factor and CD59 Double-Knockout Mice. *The Journal of Immunology* *190*, 3552–3559.
- Monaco, S., Gordon, E., Bowler, M.W., Delagenière, S., Guijarro, M., Spruce, D., Svensson, O., McSweeney, S.M., McCarthy, A.A., Leonard, G., et al. (2013). Automatic processing of macromolecular crystallography X-ray diffraction data at the ESRF. *Journal of Applied Crystallography* *46*, 804–810.
- Morgan, B.P. (2000). *Complement Methods and Protocols* (New Jersey: Humana Press).
- Morgan, B.P., and Harris, C.L. (2015). Complement, a target for therapy in inflammatory and degenerative diseases. *Nature Reviews Drug Discovery* *14*, 857–877.

- Morgan, B.P., Walters, D., Serna, M., and Bubeck, D. (2016). Terminal complexes of the complement system: new structural insights and their relevance to function. *Immunological Reviews* 274, 141–151.
- Morgan, H.P., Schmidt, C.Q., Guariento, M., Blaum, B.S., Gillespie, D., Herbert, A.P., Kavanagh, D., Mertens, H.D.T., Svergun, D.I., Johansson, C.M., et al. (2011). Structural basis for engagement by complement factor H of C3b on a self surface. *Nature Structural & Molecular Biology* 18, 463–470.
- Mueller, A.M., Breitsprecher, D., Duhr, S., Baaske, P., Schubert, T., and Längst, G. (2017). MicroScale Thermophoresis: A Rapid and Precise Method to Quantify Protein–Nucleic Acid Interactions in Solution. In *Functional Genomics*, M. Kaufmann, C. Klinger, and A. Savelsbergh, eds. (New York, NY: Springer New York), pp. 151–164.
- Müller-Eberhard, H.J. (1985). The Killer Molecule of Complement. *Journal of Investigative Dermatology* 85, S47–S52.
- Müller-Eberhard, H.J., Polley, M.J., and Calcott, M.A. (1967). Formation and functional significance of a molecular complex derived from the second and the fourth component of human complement. *J. Exp. Med.* 125, 359–380.
- Murshudov, G.N., Skubák, P., Lebedev, A.A., Pannu, N.S., Steiner, R.A., Nicholls, R.A., Winn, M.D., Long, F., and Vagin, A.A. (2011). *REFMAC 5* for the refinement of macromolecular crystal structures. *Acta Crystallographica Section D Biological Crystallography* 67, 355–367.
- Nash, J.T., Taylor, P.R., Botto, M., Norsworthy, P.J., Davies, K.A., and Walport, M.J. (2001). Immune complex processing in C1q-deficient mice. *Clinical and Experimental Immunology* 123, 196–202.
- Nataf, S., Davoust, N., Ames, R.S., and Barnum, S.R. (1999). Human T cells express the C5a receptor and are chemoattracted to C5a. *J. Immunol.* 162, 4018–4023.
- Nauta, A.J., Bottazzi, B., Mantovani, A., Salvatori, G., Kishore, U., Schwaeble, W.J., Gingras, A.R., Tzima, S., Vivanco, F., Egido, J., et al. (2003). Biochemical and functional characterization of the interaction between pentraxin 3 and C1q. *European Journal of Immunology* 33, 465–473.
- Nelson, R.A. (1958). An alternative mechanism for the properdin system. *J. Exp. Med.* 108, 515–535.
- Newcomer, M.E. (1993). Structure of the epididymal retinoic acid binding protein at 2.1 Å resolution. *Structure* 1, 7–18.
- Nguyen, H., Park, J., Kang, S., and Kim, M. (2015). Surface Plasmon Resonance: A Versatile Technique for Biosensor Applications. *Sensors* 15, 10481–10510.
- Nielsen, H.E., and Koch, C. (1987). Congenital properdin deficiency and meningococcal infection. *Clin. Immunol. Immunopathol.* 44, 134–139.
- Nilsson, B., and Nilsson Ekdahl, K. (2012). The tick-over theory revisited: Is C3 a contact-activated protein? *Immunobiology* 217, 1106–1110.

- Nilsson Ekdahl, K., Nilsson, B., Pekna, M., and Nilsson, U.R. (1992). Generation of iC3 at the interface between blood and gas. *Scand. J. Immunol.* 35, 85–91.
- Nolan, K.F., and Reid, K.B. (1993). Properdin. *Meth. Enzymol.* 223, 35–46.
- Nolan, K.F., Kaluz, S., Higgins, J.M., Goundis, D., and Reid, K.B. (1992). Characterization of the human properdin gene. *Biochem. J.* 287 (Pt 1), 291–297.
- Nunn, M.A., Sharma, A., Paesen, G.C., Adamson, S., Lissina, O., Willis, A.C., and Nuttall, P.A. (2005). Complement Inhibitor of C5 Activation from the Soft Tick *Ornithodoros moubata*. *The Journal of Immunology* 174, 2084–2091.
- O’Flynn, J., Dixon, K.O., Faber Krol, M.C., Daha, M.R., and van Kooten, C. (2014). Myeloperoxidase Directs Properdin-Mediated Complement Activation. *Journal of Innate Immunity* 6, 417–425.
- Olsen, J.G., and Kragelund, B.B. (2014). Who climbs the tryptophan ladder? On the structure and function of the WSXWS motif in cytokine receptors and thrombospondin repeats. *Cytokine & Growth Factor Reviews* 25, 337–341.
- Padilla, J.E., and Yeates, T.O. (2003). A statistic for local intensity differences: robustness to anisotropy and pseudo-centering and utility for detecting twinning. *Acta Crystallographica Section D Biological Crystallography* 59, 1124–1130.
- Paesen, G.C., Adams, P.L., Harlos, K., Nuttall, P.A., and Stuart, D.I. (1999). Tick histamine-binding proteins: isolation, cloning, and three-dimensional structure. *Mol. Cell* 3, 661–671.
- Païdassi, H., Tacnet-Delorme, P., Lunardi, T., Arlaud, G.J., Thielens, N.M., and Frchet, P. (2008). The lectin-like activity of human C1q and its implication in DNA and apoptotic cell recognition. *FEBS Letters* 582, 3111–3116.
- Païdassi, H., Tacnet-Delorme, P., Verneret, M., Gaboriaud, C., Houen, G., Duus, K., Ling, W.L., Arlaud, G.J., and Frchet, P. (2011). Investigations on the C1q–Calreticulin–Phosphatidylserine Interactions Yield New Insights into Apoptotic Cell Recognition. *Journal of Molecular Biology* 408, 277–290.
- Paixão-Cavalcante, D., Torreira, E., Lindorfer, M.A., Rodriguez de Cordoba, S., Morgan, B.P., Taylor, R.P., Llorca, O., and Harris, C.L. (2014). A Humanized Antibody That Regulates the Alternative Pathway Convertase: Potential for Therapy of Renal Disease Associated with Nephritic Factors. *The Journal of Immunology* 192, 4844–4851.
- Pangburn, M.K. (1989). Analysis of the natural polymeric forms of human properdin and their functions in complement activation. *J. Immunol.* 142, 202–207.
- Pangburn, M.K., and Müller-Eberhard, H.J. (1986). The C3 convertase of the alternative pathway of human complement. Enzymic properties of the bimolecular proteinase. *Biochem. J.* 235, 723–730.
- Park, D.H., Connor, K.M., and Lambris, J.D. (2019). The Challenges and Promise of Complement Therapeutics for Ocular Diseases. *Frontiers in Immunology* 10.

- Pauly, D., Nagel, B.M., Reinders, J., Killian, T., Wulf, M., Ackermann, S., Ehrenstein, B., Zipfel, P.F., Skerka, C., and Weber, B.H.F. (2014). A Novel Antibody against Human Properdin Inhibits the Alternative Complement System and Specifically Detects Properdin from Blood Samples. *PLoS ONE* 9, e96371.
- Pausa, M., Pellis, V., Cinco, M., Giulianini, P.G., Presani, G., Perticarari, S., Murgia, R., and Tedesco, F. (2003). Serum-Resistant Strains of *Borrelia burgdorferi* Evade Complement-Mediated Killing by Expressing a CD59-Like Complement Inhibitory Molecule. *The Journal of Immunology* 170, 3214–3222.
- Pedersen, D.V., Roumenina, L., Jensen, R.K., Gadeberg, T.A., Marinozzi, C., Picard, C., Rybkine, T., Thiel, S., Sørensen, U.B., Stover, C., et al. (2017). Functional and structural insight into properdin control of complement alternative pathway amplification. *The EMBO Journal* e201696173.
- Pedersen, D.V., Revel, M., Gadeberg, T.A.F., and Andersen, G.R. (2019). Crystallization and X-ray analysis of monodisperse human properdin. *Acta Crystallographica Section F Structural Biology Communications* 75.
- Perdikoulis, M.V., Kishore, U., and Reid, K.B. (2001). Expression and characterisation of the thrombospondin type I repeats of human properdin. *Biochim. Biophys. Acta* 1548, 265–277.
- Pettigrew, H.D., Teuber, S.S., and Gershwin, M.E. (2009). Clinical Significance of Complement Deficiencies. *Annals of the New York Academy of Sciences* 1173, 108–123.
- Phieler, J., Garcia-Martin, R., Lambris, J.D., and Chavakis, T. (2013). The role of the complement system in metabolic organs and metabolic diseases. *Seminars in Immunology* 25, 47–53.
- Pillemer, L., Blum, L., Lepow, I.H., Ross, O.A., Todd, E.W., and Wardlaw, A.C. (1954). The properdin system and immunity. I. Demonstration and isolation of a new serum protein, properdin, and its role in immune phenomena. *Science* 120, 279–285.
- Platonov, A. (2003). Long term effects of vaccination of patients deficient in a late complement component with a tetravalent meningococcal polysaccharide vaccine. *Vaccine* 21, 4437–4447.
- Podack, E.R., and Müller-Eberhard, H.J. (1979). Isolation of human S-protein, an inhibitor of the membrane attack complex of complement. *J. Biol. Chem.* 254, 9808–9814.
- Potempa, M., and Potempa, J. (2012). Protease-dependent mechanisms of complement evasion by bacterial pathogens. *Biological Chemistry* 393.
- Prodinger, W.M., Hellwege, J., Spruth, M., Dierich, M.P., and Zipfel, P.F. (1998). The C-terminus of factor H: monoclonal antibodies inhibit heparin binding and identify epitopes common to factor H and factor H-related proteins. *Biochemical Journal* 331, 41–47.
- Raby, A.-C., Holst, B., Davies, J., Colmont, C., Laumonnier, Y., Coles, B., Shah, S., Hall, J., Topley, N., Köhl, J., et al. (2011). TLR activation enhances C5a-induced pro-inflammatory responses by negatively modulating the second C5a receptor, C5L2. *European Journal of Immunology* 41, 2741–2752.

- Ram, S., Sharma, A.K., Simpson, S.D., Gulati, S., McQuillen, D.P., Pangburn, M.K., and Rice, P.A. (1998). A Novel Sialic Acid Binding Site on Factor H Mediates Serum Resistance of Sialylated *Neisseria gonorrhoeae*. *The Journal of Experimental Medicine* 187, 743–752.
- Ribeiro, J.M.C., Alarcon-Chaidez, F., B. Francischetti, I.M., Mans, B.J., Mather, T.N., Valenzuela, J.G., and Wikel, S.K. (2006). An annotated catalog of salivary gland transcripts from *Ixodes scapularis* ticks. *Insect Biochemistry and Molecular Biology* 36, 111–129.
- Ricklin, D., and Lambris, J.D. (2013). Complement in Immune and Inflammatory Disorders: Pathophysiological Mechanisms. *The Journal of Immunology* 190, 3831–3838.
- Ricklin, D., and Lambris, J.D. (2016). New milestones ahead in complement-targeted therapy. *Seminars in Immunology* 28, 208–222.
- Ricklin, D., Mastellos, D.C., Reis, E.S., and Lambris, J.D. (2018). The renaissance of complement therapeutics. *Nature Reviews Nephrology* 14, 26–47.
- Riedl, M.A., Bygum, A., Lumry, W., Magerl, M., Bernstein, J.A., Busse, P., Craig, T., Frank, M.M., Edelman, J., Williams-Herman, D., et al. (2016). Safety and Usage of C1-Inhibitor in Hereditary Angioedema: Berinert Registry Data. *The Journal of Allergy and Clinical Immunology: In Practice* 4, 963–971.
- Rooijackers, S.H.M., Ruyken, M., Roos, A., Daha, M.R., Presanis, J.S., Sim, R.B., van Wamel, W.J.B., van Kessel, K.P.M., and van Strijp, J.A.G. (2005). Immune evasion by a staphylococcal complement inhibitor that acts on C3 convertases. *Nature Immunology* 6, 920–927.
- Rooijackers, S.H.M., Wu, J., Ruyken, M., van Domselaar, R., Planken, K.L., Tzekou, A., Ricklin, D., Lambris, J.D., Janssen, B.J.C., van Strijp, J.A.G., et al. (2009). Structural and functional implications of the alternative complement pathway C3 convertase stabilized by a staphylococcal inhibitor. *Nature Immunology* 10, 721–727.
- Rossi, V., Cseh, S., Bally, I., Thielens, N.M., Jensenius, J.C., and Arlaud, G.J. (2001). Substrate specificities of recombinant mannan-binding lectin-associated serine proteases-1 and -2. *J. Biol. Chem.* 276, 40880–40887.
- Rother, R.P., Rollins, S.A., Mojciak, C.F., Brodsky, R.A., and Bell, L. (2007). Discovery and development of the complement inhibitor eculizumab for the treatment of paroxysmal nocturnal hemoglobinuria. *Nature Biotechnology* 25, 1256–1264.
- Roumenina, L.T., Loirat, C., Dragon-Durey, M.-A., Halbwachs-Mecarelli, L., Sautes-Fridman, C., and Fremeaux-Bacchi, V. (2011). Alternative complement pathway assessment in patients with atypical HUS. *Journal of Immunological Methods* 365, 8–26.
- Roumenina, L.T., Frimat, M., Miller, E.C., Provot, F., Dragon-Durey, M.-A., Bordereau, P., Bigot, S., Hue, C., Satchell, S.C., Mathieson, P.W., et al. (2012). A prevalent C3 mutation in aHUS patients causes a direct C3 convertase gain of function. *Blood* 119, 4182–4191.

- Roversi, P., Lissina, O., Johnson, S., Ahmat, N., Paesen, G.C., Ploss, K., Boland, W., Nunn, M.A., and Lea, S.M. (2007). The Structure of OMCI, a Novel Lipocalin Inhibitor of the Complement System. *Journal of Molecular Biology* 369, 784–793.
- Roversi, P., Ryffel, B., Togbe, D., Maillet, I., Teixeira, M., Ahmat, N., Paesen, G.C., Lissina, O., Boland, W., Ploss, K., et al. (2013). Bifunctional Lipocalin Ameliorates Murine Immune Complex-induced Acute Lung Injury. *Journal of Biological Chemistry* 288, 18789–18802.
- Ruseva, M.M., Vernon, K.A., Leshner, A.M., Schwaebler, W.J., Ali, Y.M., Botto, M., Cook, T., Song, W., Stover, C.M., and Pickering, M.C. (2013). Loss of Properdin Exacerbates C3 Glomerulopathy Resulting from Factor H Deficiency. *Journal of the American Society of Nephrology* 24, 43–52.
- Sacks, S.H., and Zhou, W. (2012). The role of complement in the early immune response to transplantation. *Nature Reviews Immunology* 12, 431–442.
- Saggu, G., Cortes, C., Emch, H.N., Ramirez, G., Worth, R.G., and Ferreira, V.P. (2013). Identification of a Novel Mode of Complement Activation on Stimulated Platelets Mediated by Properdin and C3(H₂O). *The Journal of Immunology* 190, 6457–6467.
- Sahu, A., Kay, B.K., and Lambris, J.D. (1996). Inhibition of human complement by a C3-binding peptide isolated from a phage-displayed random peptide library. *J. Immunol.* 157, 884–891.
- Schatz-Jakobsen, J.A., Zhang, Y., Johnson, K., Neill, A., Sheridan, D., and Andersen, G.R. (2016). Structural Basis for Eculizumab-Mediated Inhibition of the Complement Terminal Pathway. *The Journal of Immunology* 197, 337–344.
- Schejbel, L., Rosenfeldt, V., Marquart, H., Valerius, N.H., and Garred, P. (2009). Properdin deficiency associated with recurrent otitis media and pneumonia, and identification of male carrier with Klinefelter syndrome. *Clinical Immunology* 131, 456–462.
- Scheuermann, T.H., Padrick, S.B., Gardner, K.H., and Brautigam, C.A. (2016). On the acquisition and analysis of microscale thermophoresis data. *Analytical Biochemistry* 496, 79–93.
- Schramm, E.C., Roumenina, L.T., Rybkina, T., Chauvet, S., Vieira-Martins, P., Hue, C., Maga, T., Valoti, E., Wilson, V., Jokiranta, S., et al. (2015). Mapping interactions between complement C3 and regulators using mutations in atypical hemolytic uremic syndrome. *Blood* 125, 2359–2369.
- Schroeder, H., Skelly, P.J., Zipfel, P.F., Losson, B., and Vanderplassen, A. (2009). Subversion of complement by hematophagous parasites. *Developmental & Comparative Immunology* 33, 5–13.
- Schuijt, T.J., Coumou, J., Narasimhan, S., Dai, J., DePonte, K., Wouters, D., Brouwer, M., Oei, A., Roelofs, J.J.T.H., van Dam, A.P., et al. (2011). A Tick Mannose-Binding Lectin Inhibitor Interferes with the Vertebrate Complement Cascade to Enhance Transmission of the Lyme Disease Agent. *Cell Host & Microbe* 10, 136–146.

Schwaebble, W., Dippold, W.G., Schäfer, M.K., Pohla, H., Jonas, D., Luttwig, B., Weihe, E., Huemer, H.P., Dierich, M.P., and Reid, K.B. (1993). Properdin, a positive regulator of complement activation, is expressed in human T cell lines and peripheral blood T cells. *J. Immunol.* 151, 2521–2528.

Schwaebble, W., Huemer, H.P., Most, J., Dierich, M.P., Strobel, M., Claus, C., Reid, K.B.M., and Ziegler-Heitbrock, H.W.L. (1994). Expression of properdin in human monocytes. *European Journal of Biochemistry* 219, 759–764.

Seddon, J.M., Yu, Y., Miller, E.C., Reynolds, R., Tan, P.L., Gowrisankar, S., Goldstein, J.I., Triebwasser, M., Anderson, H.E., Zerbib, J., et al. (2013). Rare variants in CFI, C3 and C9 are associated with high risk of advanced age-related macular degeneration. *Nature Genetics* 45, 1366–1370.

Seelen, M.A., Roos, A., Wieslander, J., Mollnes, T.E., Sjöholm, A.G., Wurzner, R., Loos, M., Tedesco, F., Sim, R.B., Garred, P., et al. (2005). Functional analysis of the classical, alternative, and MBL pathways of the complement system: standardization and validation of a simple ELISA. *Journal of Immunological Methods* 296, 187–198.

Seidel, S.A.I., Dijkman, P.M., Lea, W.A., van den Bogaart, G., Jerabek-Willemsen, M., Lazic, A., Joseph, J.S., Srinivasan, P., Baaske, P., Simeonov, A., et al. (2013). Microscale thermophoresis quantifies biomolecular interactions under previously challenging conditions. *Methods* 59, 301–315.

Servais, A., Noël, L.-H., Roumenina, L.T., Le Quintrec, M., Ngo, S., Dragon-Durey, M.-A., Macher, M.-A., Zuber, J., Karras, A., Provot, F., et al. (2012). Acquired and genetic complement abnormalities play a critical role in dense deposit disease and other C3 glomerulopathies. *Kidney International* 82, 454–464.

Sharma, A.K., and Pangburn, M.K. (1996). Identification of three physically and functionally distinct binding sites for C3b in human complement factor H by deletion mutagenesis. *Proceedings of the National Academy of Sciences* 93, 10996–11001.

Shaw, P.X., Zhang, L., Zhang, M., Du, H., Zhao, L., Lee, C., Grob, S., Lim, S.L., Hughes, G., Lee, J., et al. (2012). Complement factor H genotypes impact risk of age-related macular degeneration by interaction with oxidized phospholipids. *Proceedings of the National Academy of Sciences* 109, 13757–13762.

Sheridan, D., Yu, Z.-X., Zhang, Y., Patel, R., Sun, F., Lasaro, M.A., Bouchard, K., Andrien, B., Marozsan, A., Wang, Y., et al. (2018). Design and preclinical characterization of ALXN1210: A novel anti-C5 antibody with extended duration of action. *PLOS ONE* 13, e0195909.

Sievers, F., Wilm, A., Dineen, D., Gibson, T.J., Karplus, K., Li, W., Lopez, R., McWilliam, H., Remmert, M., Soding, J., et al. (2014). Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Molecular Systems Biology* 7, 539–539.

Silva, N.C.S., Vale, V.F., Franco, P.F., Gontijo, N.F., Valenzuela, J.G., Pereira, M.H., Sant'Anna, M.R.V., Rodrigues, D.S., Lima, W.S., Fux, B., et al. (2016). Saliva of

Rhipicephalus (Boophilus) microplus (Acari: Ixodidae) inhibits classical and alternative complement pathways. *Parasites & Vectors* 9.

Sim, R.B., and Tsiftoglou, S.A. (2004). Proteases of the complement system. *Biochemical Society Transactions* 32, 21–27.

Šimo, L., Kazimirova, M., Richardson, J., and Bonnet, S.I. (2017). The Essential Role of Tick Salivary Glands and Saliva in Tick Feeding and Pathogen Transmission. *Frontiers in Cellular and Infection Microbiology* 7.

Sjöholm, A.G., Söderström, C., and Nilsson, L.A. (1988). A second variant of properdin deficiency: the detection of properdin at low concentrations in affected males. *Complement* 5, 130–140.

Skerra, A. (2000). Lipocalins as a scaffold. *Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology* 1482, 337–350.

Skidgel, R.A., and Erdös, E.G. (2007). Structure and function of human plasma carboxypeptidase N, the anaphylatoxin inactivator. *International Immunopharmacology* 7, 1888–1899.

Smith, C.A., Pangburn, M.K., Vogel, C.W., and Müller-Eberhard, H.J. (1984). Molecular architecture of human properdin, a positive regulator of the alternative pathway of complement. *J. Biol. Chem.* 259, 4582–4588.

Smith, R.J.H., Alexander, J., Barlow, P.N., Botto, M., Cassavant, T.L., Cook, H.T., de Córdoba, S.R., Hageman, G.S., Jokiranta, T.S., Kimberling, W.J., et al. (2007). New Approaches to the Treatment of Dense Deposit Disease. *Journal of the American Society of Nephrology* 18, 2447–2456.

Smith-Jackson, K., and Marchbank, K.J. (2018). Targeting properdin in the treatment of atypical haemolytic uraemic syndrome: better than eculizumab? *Annals of Translational Medicine* 6, S62–S62.

Späth, P.J., Sjöholm, A.G., Fredrikson, G.N., Misiano, G., Scherz, R., Schaad, U.B., Uhring-Lambert, B., Hauptmann, G., Westberg, J., Uhlén, M., et al. (1999). Properdin deficiency in a large Swiss family: identification of a stop codon in the properdin gene, and association of meningococcal disease with lack of the IgG2 allotype marker G2m(n). *Clin. Exp. Immunol.* 118, 278–284.

Spitzer, D., Mitchell, L.M., Atkinson, J.P., and Hourcade, D.E. (2007). Properdin Can Initiate Complement Activation by Binding Specific Target Surfaces and Providing a Platform for De Novo Convertase Assembly. *The Journal of Immunology* 179, 2600–2608.

Stanley, E. (1972). The identification of twins from intensity statistics. *Journal of Applied Crystallography* 5, 191–194.

Stein, N. (2008). CHAINSAW: a program for mutating pdb files used as templates in molecular replacement. *Journal of Applied Crystallography* 41, 641–643.

- Stevens, B., Allen, N.J., Vazquez, L.E., Howell, G.R., Christopherson, K.S., Nouri, N., Micheva, K.D., Mehalow, A.K., Huberman, A.D., Stafford, B., et al. (2007). The Classical Complement Cascade Mediates CNS Synapse Elimination. *Cell* 131, 1164–1178.
- Stites, E., Le Quintrec, M., and Thurman, J.M. (2015). The Complement System and Antibody-Mediated Transplant Rejection. *The Journal of Immunology* 195, 5525–5531.
- Strobel, S., Zimmering, M., Papp, K., Prechl, J., and Józsi, M. (2010). Anti-factor B autoantibody in dense deposit disease. *Molecular Immunology* 47, 1476–1483.
- Su, H.R. (1996). S-Protein/Vitronectin Interaction with the C5b and the C8 of the Complement Membrane Attack Complex. *International Archives of Allergy and Immunology* 110, 314–317.
- Sun, Z., Reid, K.B.M., and Perkins, S.J. (2004). The Dimeric and Trimeric Solution Structures of the Multidomain Complement Protein Properdin by X-ray Scattering, Analytical Ultracentrifugation and Constrained Modelling. *Journal of Molecular Biology* 343, 1327–1343.
- Takata, Y., Kinoshita, T., Kozono, H., Takeda, J., Tanaka, E., Hong, K., and Inoue, K. (1987). Covalent association of C3b with C4b within C5 convertase of the classical complement pathway. *J. Exp. Med.* 165, 1494–1507.
- Tan, A.W.L., Francischetti, I.M.B., Slovak, M., Kini, R.M., and Ribeiro, J.M.C. (2015). Sexual differences in the sialomes of the zebra tick, *Rhipicephalus pulchellus*. *Journal of Proteomics* 117, 120–144.
- Tan, K., Duquette, M., Liu, J., Dong, Y., Zhang, R., Joachimiak, A., Lawler, J., and Wang, J. (2002). Crystal structure of the TSP-1 type 1 repeats: a novel layered fold and its biological implication. *J. Cell Biol.* 159, 373–382.
- Tan, P.L., Bowes Rickman, C., and Katsanis, N. (2016). AMD and the alternative complement pathway: genetics and functional implications. *Human Genomics* 10.
- Taylor, P.R., Carugati, A., Fadok, V.A., Cook, H.T., Andrews, M., Carroll, M.C., Savill, J.S., Henson, P.M., Botto, M., and Walport, M.J. (2000). A Hierarchical Role for Classical Pathway Complement Proteins in the Clearance of Apoptotic Cells in Vivo. *The Journal of Experimental Medicine* 192, 359–366.
- Tossavainen, H., Pihlajamaa, T., Huttunen, T.K., Raulo, E., Rauvala, H., Permi, P., and Kilpeläinen, I. (2006). The layered fold of the TSR domain of *P. falciparum* TRAP contains a heparin binding site. *Protein Science* 15, 1760–1768.
- Truedsson, L., Bengtsson, A.A., and Sturfelt, G. (2007). Complement deficiencies and systemic lupus erythematosus. *Autoimmunity* 40, 560–566.
- Tschopp, J., and French, L.E. (1994). Clusterin: modulation of complement function. *Clin. Exp. Immunol.* 97 Suppl 2, 11–14.

- Tyson, K., Elkins, C., Patterson, H., Fikrig, E., and de Silva, A. (2007). Biochemical and functional characterization of Salp20, an Ixodes scapularis tick salivary protein that inhibits the complement pathway. *Insect Mol. Biol.* 16, 469–479.
- Tyson, K.R., Elkins, C., and de Silva, A.M. (2008). A Novel Mechanism of Complement Inhibition Unmasked by a Tick Salivary Protein That Binds to Properdin. *The Journal of Immunology* 180, 3964–3968.
- Ueda, Y., Miwa, T., Gullipalli, D., Sato, S., Ito, D., Kim, H., Palmer, M., and Song, W.-C. (2018). Blocking Properdin Prevents Complement-Mediated Hemolytic Uremic Syndrome and Systemic Thrombophilia. *Journal of the American Society of Nephrology* 29, 1928–1937.
- Vagin, A., and Teplyakov, A. (1997). *MOLREP*: an Automated Program for Molecular Replacement. *Journal of Applied Crystallography* 30, 1022–1025.
- Vagin, A., and Teplyakov, A. (2010). Molecular replacement with *MOLREP*. *Acta Crystallographica Section D Biological Crystallography* 66, 22–25.
- Valenzuela, J.G., Charlab, R., Mather, T.N., and Ribeiro, J.M.C. (2000). Purification, Cloning, and Expression of a Novel Salivary Anticomplement Protein from the Tick, *Ixodes scapularis*. *Journal of Biological Chemistry* 275, 18717–18723.
- Varga, L., and Farkas, H. (2011). rhC1INH: a new drug for the treatment of attacks in hereditary angioedema caused by C1-inhibitor deficiency. *Expert Review of Clinical Immunology* 7, 143–153.
- Velazquez-Campoy, A., Leavitt, S.A., and Freire, E. (2015). Characterization of Protein-Protein Interactions by Isothermal Titration Calorimetry. In *Protein-Protein Interactions*, C.L. Meyerkord, and H. Fu, eds. (New York, NY: Springer New York), pp. 183–204.
- Vonrhein, C., Flensburg, C., Keller, P., Sharff, A., Smart, O., Paciorek, W., Womack, T., and Bricogne, G. (2011). Data processing and analysis with the *autoPROC* toolbox. *Acta Crystallographica Section D Biological Crystallography* 67, 293–302.
- Wagemakers, A., Coumou, J., Schuijt, T.J., Oei, A., Nijhof, A.M., van ‘t Veer, C., van der Poll, T., Bins, A.D., and Hovius, J.W.R. (2016). An *Ixodes ricinus* Tick Salivary Lectin Pathway Inhibitor Protects *Borrelia burgdorferi* sensu lato from Human Complement. *Vector-Borne and Zoonotic Diseases* 16, 223–228.
- Wagner, E., and Frank, M.M. (2010). Therapeutic potential of complement modulation. *Nature Reviews Drug Discovery* 9, 43–56.
- Wang, Y., Miwa, T., Ducka-Kokalari, B., Redai, I.G., Sato, S., Gullipalli, D., Zangrilli, J.G., Haczku, A., and Song, W.-C. (2015). Properdin Contributes to Allergic Airway Inflammation through Local C3a Generation. *The Journal of Immunology* 195, 1171–1181.
- Ward, P.A. (2009). Functions of C5a receptors. *Journal of Molecular Medicine* 87, 375–378.

- Waterhouse, A., Bertoni, M., Bienert, S., Studer, G., Tauriello, G., Gumienny, R., Heer, F.T., de Beer, T.A.P., Rempfer, C., Bordoli, L., et al. (2018). SWISS-MODEL: homology modelling of protein structures and complexes. *Nucleic Acids Research* 46, W296–W303.
- Waxman, F.J., Hebert, L.A., Cornacoff, J.B., VanAman, M.E., Smead, W.L., Kraut, E.H., Birmingham, D.J., and Taguam, J.M. (1984). Complement depletion accelerates the clearance of immune complexes from the circulation of primates. *Journal of Clinical Investigation* 74, 1329–1340.
- Weismann, D., Hartvigsen, K., Lauer, N., Bennett, K.L., Scholl, H.P.N., Issa, P.C., Cano, M., Brandstätter, H., Tsimikas, S., Skerka, C., et al. (2011). Complement factor H binds malondialdehyde epitopes and protects from oxidative stress. *Nature* 478, 76–81.
- Westberg, J., Fredrikson, G.N., Truedsson, L., Sjöholm, A.G., and Uhlén, M. (1995). Sequence-Based Analysis of Properdin Deficiency: Identification of Point Mutations in Two Phenotypic Forms of an X-Linked Immunodeficiency. *Genomics* 29, 1–8.
- Weston-Davies, W.H., Nunn, M.A., Pinto, F.O., Mackie, I.J., Richards, S.J., Machin, S.J., Prudo, R., and Hillmen, P. (2014). Clinical and Immunological Characterisation of Coversin, a Novel Small Protein Inhibitor of Complement C5 with Potential As a Therapeutic Agent in PNH and Other Complement Mediated Disorders. *Blood* 124, 4280–4280.
- Wikel, S.K., and Bergman, D. (1997). Tick-host immunology: Significant advances and challenging opportunities. *Parasitol. Today (Regul. Ed.)* 13, 383–389.
- Wilson, A.J.C. (1949). The probability distribution of X-ray intensities. *Acta Crystallographica* 2, 318–321.
- Winter, G. (2010). *xia2*: an expert system for macromolecular crystallography data reduction. *Journal of Applied Crystallography* 43, 186–190.
- Winter, G., Waterman, D.G., Parkhurst, J.M., Brewster, A.S., Gildea, R.J., Gerstel, M., Fuentes-Montero, L., Vollmar, M., Michels-Clark, T., Young, I.D., et al. (2018). *DIALS*: implementation and evaluation of a new integration package. *Acta Crystallographica Section D Structural Biology* 74, 85–97.
- Wirthmueller, U., Dewald, B., Thelen, M., Schäfer, M.K., Stover, C., Whaley, K., North, J., Eggleton, P., Reid, K.B., and Schwaeble, W.J. (1997). Properdin, a positive regulator of complement activation, is released from secondary granules of stimulated peripheral blood neutrophils. *J. Immunol.* 158, 4444–4451.
- Wu, J., Wu, Y.-Q., Ricklin, D., Janssen, B.J.C., Lambris, J.D., and Gros, P. (2009). Structure of complement fragment C3b-factor H and implications for host protection by complement regulators. *Nat. Immunol.* 10, 728–733.
- Xu, W., Berger, S.P., Trouw, L.A., de Boer, H.C., Schlagwein, N., Mutsaers, C., Daha, M.R., and van Kooten, C. (2008). Properdin Binds to Late Apoptotic and Necrotic Cells Independently of C3b and Regulates Alternative Pathway Complement Activation. *The Journal of Immunology* 180, 7613–7621.

- Yang, Y., Liu, F., Franc, V., Halim, L.A., Schellekens, H., and Heck, A.J.R. (2016). Hybrid mass spectrometry approaches in glycoprotein analysis and their usage in scoring biosimilarity. *Nature Communications* 7.
- Yeates, T.O. (1988). Simple statistics for intensity data from twinned specimens. *Acta Crystallographica Section A Foundations of Crystallography* 44, 142–144.
- Yeates, T.O. (1997). [22] Detecting and overcoming crystal twinning. In *Methods in Enzymology*, (Elsevier), pp. 344–358.
- Yu, H., Muñoz, E.M., Edens, R.E., and Linhardt, R.J. (2005). Kinetic studies on the interactions of heparin and complement proteins using surface plasmon resonance. *Biochim. Biophys. Acta* 1726, 168–176.
- Zhang, X., Kimura, Y., Fang, C., Zhou, L., Sfyroera, G., Lambris, J.D., Wetsel, R.A., Miwa, T., and Song, W.-C. (2007). Regulation of Toll-like receptor-mediated inflammatory response by complement in vivo. *Blood* 110, 228–236.
- Zhang, Y., Meyer, N.C., Wang, K., Nishimura, C., Frees, K., Jones, M., Katz, L.M., Sethi, S., and Smith, R.J.H. (2012). Causes of Alternative Pathway Dysregulation in Dense Deposit Disease. *Clinical Journal of the American Society of Nephrology* 7, 265–274.
- Zhou, W. (2012). The new face of anaphylatoxins in immune regulation. *Immunobiology* 217, 225–234.
- Zhou, H. -f., Yan, H., Stover, C.M., Fernandez, T.M., Rodriguez de Cordoba, S., Song, W.-C., Wu, X., Thompson, R.W., Schwaeble, W.J., Atkinson, J.P., et al. (2012). Antibody directs properdin-dependent activation of the complement alternative pathway in a mouse model of abdominal aortic aneurysm. *Proceedings of the National Academy of Sciences* 109, E415–E422.
- Zhou, H. -f., Yan, H., Bertram, P., Hu, Y., Springer, L.E., Thompson, R.W., Curci, J.A., Hourcade, D.E., and Pham, C.T.N. (2013). Fibrinogen-specific antibody induces abdominal aortic aneurysm in mice through complement lectin pathway activation. *Proceedings of the National Academy of Sciences* 110, E4335–E4344.
- Zipfel, P.F., and Skerka, C. (1999). FHL-1/reconectin: a human complement and immune regulator with cell-adhesive function. *Immunol. Today* 20, 135–140.
- Zipfel, P.F., and Skerka, C. (2009). Complement regulators and inhibitory proteins. *Nature Reviews Immunology*.
- Zipfel, P.F., Hallström, T., and Riesbeck, K. (2013). Human complement control and complement evasion by pathogenic microbes – Tipping the balance. *Molecular Immunology* 56, 152–160.