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CONFORMATIONAL COMPLEXITY OF COMPLEMENT COMPONENT C3

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1. INTRODUCTION

The complement system is an important component of the humoral immune response in vertebrates. The complement system consists of 30–40 soluble plasma proteins and cell-surface receptors that form a complex set of regulatory pathways (reviewed by Walport[1]). It enables the host to recognize invading microorganisms. Activation of the complement system may result in a range of effector functions: bacterial lysis, initiation of inflammatory responses, phagocytosis, and stimulation of B-cell response (reviewed by Carroll[2]). Regulation of this defense system in blood plasma is of critical importance for the homeostasis of the host, as indicated by a wide range of (auto-)immune disorders that are associated with uncontrolled complement response.

The molecule C3 plays a central role in the complement activation pathways (reviewed by Sahu and Lambris[3]). Three pathways of complement activation exist: (i) the antibody-mediated classical pathway, (ii) the lectin-mediated pathway and (iii) the alternative pathway. These pathways are characterized by protein–protein complex formation and proteolytic activation of the large multi-domain complement proteins. The first two pathways involve antibodies or lectins that bind to the surface of microorganisms. The third pathway, the alternative pathway, reacts intrinsically onto any, host or foreign, surface. All three pathways converge in the proteolytic activation of C3, which generates the biologically active fragments C3a and C3b. The small fragment C3a functions as an anaphylatoxin, initiating inflammatory responses. The large fragment, C3b, plays a central role in localization and amplification of complement response

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and initiation of the terminal phase of complement activation. Localization is achieved by covalent attachment through a highly reactive thioester moiety in C3b. Surface-bound C3b serves multiple purposes. It acts as an opsonin providing a strong signal for phagocytosis by macrophages, and it provides a molecular platform for formation of the convertases of the complement system. The convertases induce amplification of the response and initiate the terminal complement phase. The level of complement response is primarily determined by the lifetime of the convertase complexes, which are inherently instable with half-life times of 90 seconds[4]. One complement protein has been identified that enhances the lifetime of the convertases, i.e. properdin, whereas various "decay-accelarating and cofactor activity" regulators are known that induce dissociation of the convertases and proteolytic degradation of C3b by factor I. The cleavage products iC3b, C3dg, and C3d have important signaling roles. iC3b acts as an opsonin-facilitating phagocytosis of foreign material by leukocytes. Next to that, iC3b, C3dg, and C3d amplify B-cell responses and thereby provide a key interface between innate and adaptive immunity.

Structural data are instrumental in understanding the molecular mechanisms of complex formation and activation of the large multi-domain complement proteins. Resolving these structural details, however, is technically challenging. The proteins involved are typically large, multi-domain, and flexible molecules, and they associate into large and, in some cases inherently, unstable complexes. Moreover, due to size and post-translation modifications, these proteins are often hard to produce by recombinant protein expression techniques. Nonetheless, in the last decade significant advances have been made in the structure determination of complement proteins and protein domains (see Table 1). Most recently, we published the structures of native C3 and its major proteolytic fragment C3c[5]. These structures provide insight into the domain organization, structure, and dynamics of the central C3 protein of the complement system. Here, we present an overview of these new structural insights and discuss the implications for complement convertase formation, decay acceleration, and cofactor activity, and the signaling roles of the proteolytic fragments iC3b, C3dg, and C3d.

2. STRUCTURAL ORGANIZATION OF C3

The protein molecule C3 is the most abundant complement protein in human blood plasma with levels of 1–1.5 g/l in healthy individuals. It is synthesized as a single poly-peptide chain of 1641 amino-acid residues and secreted into the blood as a glycosylated two-chain protein. Residues 1–645 form the β-chain and residues 650–1641 form the α-chain with N-linked glycans on positions Asn-63 and on Asn-917 of the β- and α-chain, respectively[42,43]. Residues Arg-646–Arg-649 are proteolytically removed during post-translational processing. A remarkable aspect of its post-translational modifications is the formation of a thioester

Table 1. Solved Structures in the Complement System

	Protein	Domain/fragment	Method	Ref.
Central comple-ment compo-nents	C1r	EGF-like	NMR	6
		CCP1-CCP2-SP	X-ray	7
	C1s	CCP2-SP	X-ray	8
		CUB1-EGF	X-ray	9
	C1q	Globular head	X-ray	10
	C3	C3a	X-ray	11
		C3d	X-ray	12
		C3	X-ray	5
		C3c	X-ray	5
	C4	C4d	X-ray	13
	C5	C5a	NMR	14
		C345C	NMR	15
	C8	C8	X-ray	16
	Factor B	SP	X-ray	17
		vWA	X-ray	18
		factor Bb	X-ray	19
	Factor D	Factor D	X-ray	20
	MASP2	CUB1-EGF-CUB2	X-ray	21
		CCP1-CCP2-SP	X-ray	22
	Map19	Map19	X-ray	23
Regu-lators	Factor H	CCP16	NMR	24
		CCP5	NMR	25
		CCP15-CCP16	NMR	26
	DAF (CD55)	CCP3-CCP4	X-ray	27
		CCP2-CCP3	NMR	28
		DAF (CCP1,2,3,4)	X-ray	29
	MCP (CD46)	CCP1-CCP2	X-ray	30
	CR1 (CD35)	CCP15-CCP16	NMR	31
		CCP16-CCP17	NMR	31
	CR2 (CD21)	CCP1-CCP2	X-ray	32
	CR3 (CD11b/CD18)	I-domain	X-ray	33
	CR4 (CD11c/CD18)	I-domain	X-ray	34
	CD59	CD59 (soluble form)	NMR	35
In complex	C3d-CR2	C3d CR2-CCP1,2	X-ray	36
Foreign	VCP	CCP3-CCP4	NMR	37
		VCP	X-ray	38
		CCP2-CCP3	NMR	39
	BbCRASP-1	BbCRASP-1	X-ray	40
	CHIPS	CHIPS	NMR	41

bond in the protein. The side chains of residues Cys-988 and Gln-991 form a C^{α}–C^{β}–S^{γ}–(C^{δ}=O^{ε})–C^{γ}–C^{β}–C^{α} thioester linkage[44]. The length of the polypeptide chain and the extensive post-translational modifications (twofold glycosylation, formation of thirteen disulfide bridges[11,45], removal of the tetra arginine (646–

649), and formation of a thioester moiety) indicate a complicate folding process of this intricate protein molecule (Figure 1).

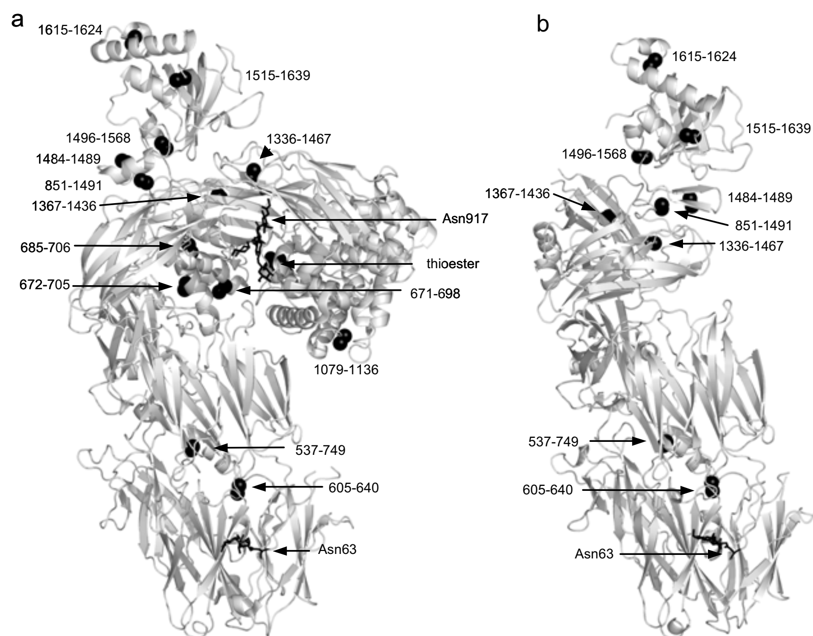

Figure 1. Structures of C3 and C3c. (a,b) Ribbon representation of C3 and C3c, respectively. Disulfide bonds and the thioester are shown as black spheres. Glycan moieties are shown as black sticks.

We recently published the crystal structure of native C3 and its major proteolytic fragment C3c[5]. Both proteins were purified from human blood plasma: native C3 was purified from fresh plasma[46]; whereas C3c was purified from outdated plasma stored at 4°C. First we determined the crystal structure of C3c, which is a proteolytic fragment representing the core structure of C3 and is conceivably less flexible and hence possibly easier to crystallize. Indeed, in our hands C3c crystallizes more readily than C3. C3c crystals typically diffracted to approx. 3-Å resolution and best diffraction data was collected up to 2.4-Å resolution. Since the protein was isolated from a natural source and no significant structural models were a priori available, we obtained phase information through the classical method of multiple-isomorphous replacement using heavy-atom compounds. Overall, the process involved many crystals of varying diffraction quality (mostly 3–3.5 Å resolution) in three different space groups. An interpretable electron-density map was obtained after multi-crystal averaging, combining data from multiple crystal forms after extensive optimization of the operators and masks for mapping corresponding structural fragments in the various crystal

forms. This experimental electron-density map was used to build an atomic model of C3c. The structure of C3c consisting of ten domains was instrumental in solving the structure of native C3. Diffraction data of C3 was collected from a single crystal diffracting to 3.3-Å resolution. The structure was solved by maximum-likelihood molecular replacement placing known structures of domains (derived from the structures of C3c and C3d[12]) into the unit cell. After successfully placing 10 domains, the model was completed by model building and refinement. The resulting final model of C3 consisted of a total of 13 domains. The thioester is intact in the crystals, as indicated by the electron density at the thioester linkage, and is occluded in the structure of C3 as expected for the native conformation of C3.

The structure of C3 is characterized by an intricate arrangement of 13 domains (see Figure 1; domain names and secondary structure labeling as defined in [5]). The core of the structure is formed by eight homologous domains from both the β- and α-chains with an additional five domains appearing as inserts and a C-terminal extension. We named the core domains macroglobulin (MG) domains 1 through 8 (referring to the α2-macroglobulin protein family). The MG domains display a fibronection type-III like fold that is part of the superfamily of immunoglobulin folds. Domains MG1–MG5 are formed by residues of the β-chain, whereas domains MG7 and MG8 are formed by the α-chain. Surprisingly, domain MG6 is formed by residues of both the β-chain (res. 535–577) and the α-chain (res. 746–806). This reflects that C3 is a single gene product that should not be considered a gene fusion of a β- and α-part. C3 is rich in disulfide linkages. One disulfide bridge covalently links the β- and α-chains in domain MG6 (537-794), one disulfide bridge is inter-domain (from MG7 to the anchor region; 851–1491), and the eleven remaining are intra-domain disulfide bonds. C3 has two N-linked glycosylation sites — on Asn-63 and Asn-917. At both positions electron density confirms the presence of glycan moieties. The glycan on Asn-917 has been implicated in folding and correctly predicted to be concealed[47]. The base of the glycan, i.e., the first two N-acetylglucosamines is well protected in C3 by the CUB, TED, and MG8 domains. This glycan is close to the factor I cleavage sites (it is 13 Å away from cleavage site, 1298–1299); proteolysis at these sites induce conversion of C3b into iC3b that is accompanied by conformational changes exposing the glycan at Asn-917 for conglutinin binding[48]. A central feature of the C3 molecule is the reactive thioester moiety. In native C3 the thioester is protected from the surrounding solvent by close packing of the TED domain, which harbours the thioester, against domain MG8. This arrangement appears to be stabilized by the overall domain—domain arrangement in native C3, where the ANA domain plays a decisive role by holding MG8 in place with respect to domain MG3 of the β-ring. Residues Phe-1047 from the TED domain and Met-1378, Tyr-1425, and Tyr-1460 from the MG8 domain form a shield around the thioester, limiting access of small amino and hydroxyl nucleophiles. High reactivity toward hydroxyl nucleophiles requires a transformation of the thioester (Cys-988–Gln-991) to a free thiolate anion (Cys-

988) and an acylimidazole (Gln-991–His-1104)[49-51]. Comparison of the native C3 and C3d[12] structures shows significant structural differences that can be correlated with this transformation from thioester to thiolate anion and acylimidazole intermediates. The fact that the structures of C3d[12,52] and C4d[13] correlates best with the thiolate/acylimidazole form indicates that this is the most stable conformation. Consequently, the domain–domain interactions observed in native C3 function to maintain the thioester state of the protein. Activation of C3 induces large conformational changes. Comparison of the structures of C3 and C3c shows that the arrangements of domains in the β-chain are structurally relatively stable and that the domains of the α-chain (as exemplified by MG7 and MG8) undergo large rearrangements.

3. CONVERTASE FORMATION

C3 undergoes profound conformational changes[53] upon proteolytic activation generating C3a and C3b. C3b, the activated form of C3, exposes various protein-binding sites[54] among which is that for factor B. Factor B binding to C3b results in formation of the bimolecular complex C3bB, followed by cleavage of factor B by factor D and formation of the C3 convertase C3bBb. C3b is a large 1560-residue protein fragment (with MW = 176 kDa) for which its 12-domain organization may be inferred from the structure of full-length C3 and C3c. Factor B consists of 739 residues (MW 90 kDa) that form five domains: three N-terminal short complement-control-protein (CCP1-3) domains consisting of approx. 60 residues each, a Von Willebrand-factor type A (VWA) domain of 215 residues, and a C-terminal trypsin-like serine protease (SP) domain of 297 residues. Factor B shows no proteolytic activity toward its substrate C3 in solution. It requires complex formation with C3b and proteolytic activation for its activity. First, factor B associates with C3b. Factor B then becomes sensitive to proteolysis by factor D, which cleaves the Arg-234–Lys-235 peptide bond generating fragments Ba (consisting of the three N-terminal CCP domains) and Bb (consisting of the VWA and SP domains). Ba then dissociates from the complex. This yields the active (and short-lived) convertase complex of the alternative pathway C3bBb that will amplify the complement response by cleaving additional C3 into C3a and C3b. Binding sites for C3b on factor B have been reviewed previously[55]. The proposed C3b-binding sites include peptide stretches on CCP2 and CCP3[56], the MIDAS site on VWA[57-61], and a possible binding site on the SP domain[62]. The resulting complexes, C3bB and C3bBb, are labile. When dissociated, factor B may re-associate with C3b, yielding a new complex; whereas fragment Bb cannot re-associate. This would suggest that dissociated Bb has undergone conformational changes, making it unsuitable for re-association. However, conformational changes in C3b induced by binding of factor B, and not by fragment Bb, cannot be excluded at this moment. In the

following section we discuss the putative binding sites for factor B onto C3b in the formation of the C3 convertase.

Four separate sites have been identified that either present putative binding sites for factor B or are indirectly involved in binding factor B, possibly through conformational changes. One site of importance has been identified by peptide-binding and mutagenesis studies. Peptide binding studies[63] have shown that residues 727–745, which form the N-terminal region of the α′ chain (α′NT) in C3b, are involved in the interaction of C3b with factor B. Mutation studies in this region have pinpointed four acidic residues, i.e., Asp-730, Glu-731, Glu-736, and Glu-737, to be involved in the convertase formation[64]. Another study[65] showed that cleavage fragment C3o of C3 was able to form a C3 convertase complex with factor B. In contrast, fragment C3c, which resembles C3o, cannot form a convertase complex. C3o and C3c differ by the presence of residues 933–942 in C3o. Thus, the binding data suggest that this residue stretch (forming strand β5 and its flanking loops[5] in the CUB domain in C3) is involved in factor B binding. One possible binding site for factor B located on the β-chain of C3 has been suggested based on sequence homology. Complement C2 receptor inhibitor trispanning (CRIT) binds C2[66]. The C2 binding site was shown to reside in a short segment of the extracellular domain of CRIT. This segment has high sequence homology with a segment of C4. It was concluded that this segment in C4 may be directly involved in binding C2[66,67]. The analogous site in C3 is formed by residues 200–220 of the β-chain. They are located on the last β-strand (βG) of MG2, the first strand (βA) of MG3, and the connecting loop. Recently, it has been shown that the C-terminal C345C domain of C3 is also involved in the binding of factor B[68,69]. In these studies various parts of the C terminus of C3 have been replaced by the corresponding parts of the snake venom homolog cobra-venom factor. The resulting chimaeras yielded enhanced lifetimes of the convertase complex, suggesting that there is at least one binding site for factor B on the C345C domain of C3. As shown in Figure 2, these data indicate four separate regions located in the top half, primarily α-chain, of the C3 molecule.

To date, structures have been published of C3, C3c, and of a disulfide-bridge engineered construct of fragment Bb[19]. Structures of C3b and B, are not available yet. Discussions on structural implications of the putative factor B binding sites must consider the possibility of conformational changes. The C345C domain is surface exposed in both C3 and C3c. Its outward position in the molecule suggests it will be exposed in C3b as well and accessible for factor B binding. The “C3o segment” formed by residues 933–942 is surface exposed in C3 and is absent in C3c due to proteolytic processing by factor I. This segment is structurally close to the C345C domain, suggesting that, possibly even after conformational changes, factor B might bind both regions simultaneously. The “CRIT segment” (formed by residues 200–220), however, is exposed to the solvent but inaccessible to proteins in both the C3 and C3c structures. Direct involvement in factor B binding here would imply large conformational changes of the β-ring in the conversion of C3 to C3b. These changes are not observed

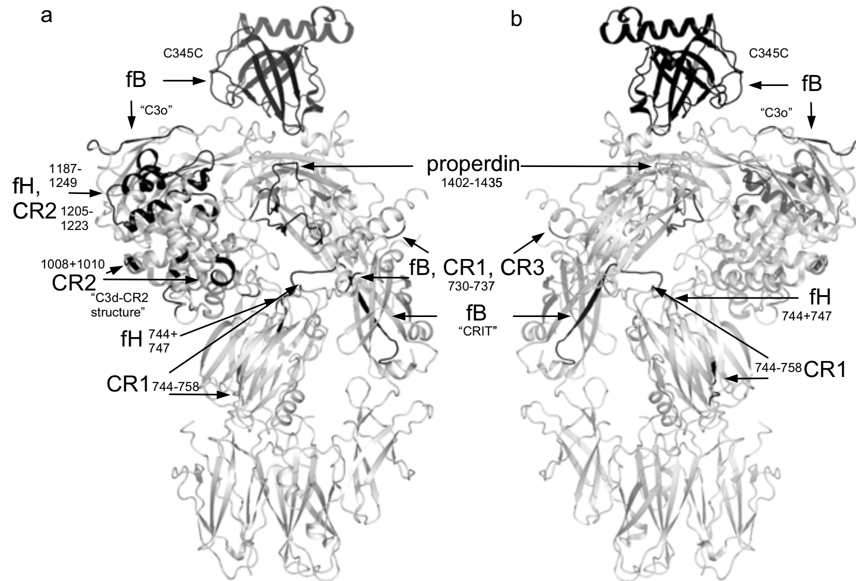

Figure 1. Possible binding sites mapped on the structure of C3. (a,b) Possible binding sites for factor B, receptors CR1, CR2, CR3, and soluble regulators factor H and properdin are indicated in black cartoon drawing, by arrows and residue numbering or "keywords" of possible binding sites. (b) C3 rotated 180° with respect to the view shown in (a).

when comparing the structures of C3 and C3c. Interestingly, the α′NT that carries the four acidic residues important for factor B binding is buried in C3 but exposed in C3c. In C3 the acidic residues occupy a cone formed by the ANA, MG3, and MG8 domains, where they are loosely structured as judged by the quality of the electron density. At Glu-737 the chain exits this cone and makes a 90º turn at 737–738 and runs through the β-ring passing residues Glu-202–Pro-206 (these latter residues are part of the CRIT segment and hence implied in factor B binding[66,67]). At ^{745}FPES^{748} the chain kinks and starts MG6$^{\alpha}$ of the intertwined MG6$^{\beta}$/MG6$^{\alpha}$ domain. In C3c the α′NT resides fully on the opposite, MG6 side of the molecule. In the structure of C3c residues Asp-730–Arg-742 interact with MG7. The kink 745–748 is reoriented and the chain continues into MG6. Thus, the α′NT has slid through the ring formed by the β-chain in the conversion of C3 into C3c. Consequently, residues 727–768 (as studied by antibody binding[70]) form one continuous solvent exposed region in C3c, whereas this region is on two separate sides of the molecule and partially shielded in C3. The large structural relocation of α′NT from C3 to C3c poses the question of where the α′NT region resides in C3b. In other words, on which side of C3b does factor B bind, assuming that α′NT is directly involved in factor B binding? The C3 convertase can be formed either with C3b or C3(H_2O) that still has the

ANA domain covalently attached. This observation argues for factor B binding to α′NT on the ANA side of the molecule. However, this argument is not conclusive when the possible flexibility of C3 is taken into account. The α′NT is poorly structured and its preceding loop 720–729 is not structured in C3. Thus, it is possible that ANA may reorient in the activated C3(H_2O), yielding enough leverage for the α′NT to slip under the bridge. This is supported by deletion mutants of C3 lacking residues 759–765, 759–762, or 762–765 of MG6 that result in threefold or more increased concentrations of C3(H_2O) in COS cell supernatants[71]. On fragment Bb three positively charged regions have been proposed to bind the acidic residues of the α′NT[19]. These regions are located on the SP domain (residues 701–708) and two patches at the VWA–SP domain intersection. These data, taken together, do not yet provide a conclusive model of factor B or fragment Bb binding to C3b. Since the C3bB and C3bBb complexes are labile, a more achievable goal is to obtain a structure of C3b that will provide the arrangements of the multiple binding sites for factor B.

The labile C3bB and C3bBb complexes are stabilized by properdin. Properdin consists of 410 residues that form six or seven trombospondin (TSR) domains[72] and exists as either dimers, trimers, or tetramers[73]. It increases the affinity of factor B for C3b, prevents cleavage of C3b to iC3b by factor I, and enhances the convertase lifetime to approx. 30 minutes[74]. A binding site for properdin on C3b has been identified on residues 1402–1435[75]. This site is situated on MG8, partly on an insertion between strands βC and βC′, which changes conformation in the conversion of C3 to C3c from β–α–β to β–α–α. This site is hidden in C3 and exposed in C3c. Properdin binding indicates that this site is probably exposed in the C3bB and C3bBb complexes. In addition, the properdin–C3bBb interaction has been described to be dependent mainly on ionic strength[76]. Properdin is predominantly positively charged, especially the domains TSR-3, 5, and 6[72] (TSR-4, 5, and 6 have been shown to be involved in C3b binding[72,77]). C3 and C3c are mainly negatively charged[5], and factor Bb has dispersed positive- and negative-charged patches[19]. Possibly some of the positive patches of Bb are involved in electrostatic interaction with C3b, resulting in a mainly negatively charged C3bBb complex in good agreement with ionic strength-dependent binding of the positively charged properdin. A new and totally different mode of convertase stabilization has recently been described for a complement inhibitor from *S. aureus*, called SCIN[78]. This 85-residue protein not only stabilizes but also inhibits the C3bBb convertases, indicating that these two proteins probably interact in distinct ways to stabilize the convertase complex.

4. DECAY ACCELERATION

When complement is not properly regulated it may cause severe damage to host tissues. To prevent self tissue from complement-mediated destruction, host cells express various regulators that downregulate convertase activity. These

regulators form a family of related proteins, called "regulators of complement activation" (RCA), which consist almost entirely of CCP domains arranged in a beads-on-a-string fashion. They act on convertases by accelerating the dissociation of the complexes, termed "decay-acceleration activity,"[79] and function as markers to discriminate between self and non-self. Factor H, decay-accelerating factor (DAF, CD55), and complement receptor 1 (CR1, CD35) are three important members of this family that are composed of 20, 4, and 30 CCP domains respectively. Whereas DAF and CR1 are cell-surface proteins, factor H is a soluble regulator and thus must possess additional binding sites to distinguish self from foreign. The tick-borne pathogen *Borrelia burgdorferi* utilizes this regulator from the infected host; it hijacks factor H by binding it to its Bb-CRASP surface protein, thereby avoiding complement activation on its surface[80]. In addition, other pathogens protect themselves from complement activation by expressing CCP containing proteins that have similarly decay-accelerating and cofactor activities[81]. Thus, strings of CCP domains provide an important framework for regulating convertase activity. Although the regulators differ enormously in number of CCP domains, it has been shown that typically three CCP domains suffice to achieve decay-accelerating activity: e.g., CCP2-4 of DAF[82], CCP1-3 of CR1[83], and CCP1-4 of factor H[84]. Various structural data have been reported of CCP domains of these proteins: CCP2-3[28], CCP3-4[27], and more recently all four CCP's[29] of DAF have been solved; CCP15-17 of CR1[31]; and, CCP15-16[26] and CCP5[25] of factor H (see Table 1). Many questions, however, remain unanswered with respect to multiple binding sites and conformational flexibility of the CCP containing molecules. Understanding the molecular mechanisms that underlie the decay-accelerating activities is expected to have a significant impact in developing inhibitors of complement for therapeutic purposes[85].

Multiple studies[63,64,70,86,87] indicate that residues 727–767 of C3b form an important interaction site for CR1 and factor H. Moreover, factor B, factor H, and CR1 compete for interaction with C3b or C3(H_2O). As for factor B, mutagenesis studies have identified various acidic residues important for binding. Factor H binding depends on Glu-744 and Glu-747 located in domain MG6. For CR1 binding Asp-730, Glu-731, Glu-736, Glu-737, Glu-747, Glu-754, and Glu-755 of C3b are important. The latter residues are part of the α'NT region (729–745) and MG6 domain (535–577 and 746–806). These data possibly indicate that both factor H and CR1 have a primary binding site on MG6 (Figure 2). Two possible modes of action exist for decay-accelerating activity of factor H and CR1. If the factor B binding site lies on the MG6 side on C3b, then factor H and CR1 might at least in part act through steric hindrance. If, in contrast, the factor B binding site is on the ANA side of C3b, then factor H and CR1 may affect the α'NT by binding to MG6 and altering the conformation of α'NT by pulling these residues completely or in part through the β-ring. The effect of factor H, however, must be reversible, since C3b can bind factor B after factor H is removed from a C3b–H complex[88]. Furthermore, factor H has at least one addi-

tional interaction site on C3b, i.e., helices α9–α11 and neighboring loops in the TED domain (1187–1249)[89,90]. For DAF the binding site on C3b is unknown, though it has been shown that DAF can bind to C3b without Bb present[91]. Important sites for interaction with factor H, DAF, and CR1 on fragment Bb have been identified on the VWA domain[60]. Mutations around helix α1 and neighboring loops were shown to be important for resistance to decay acceleration by all three regulators. A second site important for interaction with DAF and CR1, but not factor H, was shown to reside on helices α4 and 5. The interactions of the regulators with C3b, and its homolog C4b of the classical pathway, have been found to be dominated by electrostatic interactions, as demonstrated by the salt dependency[76,92]. Furthermore, mutation studies on the regulators have indicated that positive charges are favorable in binding to C3b (and C4b)[28,31,83,93-95]. This is in good agreement with the predicted overall negative charge of the C3bBb complex, as discussed above. Although the recently solved DAF structure indicates that there is also a negatively charged region on CCP3 and 4 that might be important for the interaction with the C3bBb convertase[29]. Possibly the positively charged region on MG1 and MG5 of C3b or one of the positive patches in factor Bb is involved in this interaction. As of yet it is not clear what the mode of action for these regulators is; however, as factor Bb cannot bind C3b after its dissociation from the convertase, it is possible that the regulators induce a conformational change in factor Bb that is unfavorable for binding to C3b and thus accelerate the decay.

5. COFACTOR ACTIVITY

Next to decay-accelerating activity, factor H and CR1 serve, as does membrane-cofactor protein (MCP), as cofactors in the cleavage of C3b by factor I[96-99], i.e., these proteins exhibit so-called "cofactor activity." Like factor H and CR1, MCP is a member of the RCA family of proteins. It consists of four N-terminal CCP domains, an O-glycosilated serine, threonine and proline-rich region, a transmembrane region, and a C-terminal intracellular domain[100]. The structure of the CCP1-2 pair has been solved[30]. CCP3–4 are sufficient for binding to C3b; however, CCP2 is required for cofactor activity[101]. The cofactor-mediated factor I proteolytic inactivation of C3b involves three cleavages in the CUB domain of C3b. The first cleavage, between residues Arg-1281–Ser-1282 in loop β6–β7 (i.e., the loop connecting strands β6 and β7) of the CUB^f part of CUB, generates $iC3b_1$. The second cleavage occurs between Arg-1298–Ser-1299, situated in strand β8 of CUB^f and generates fragment C3f (2 kDa)[102] and $iC3b_2$. The third cleavage, between Arg-932–Ser-933 in loop β3′–β4 of the CUB^g part of CUB, results in the formation of C3dg (40 kDa)[103] and C3c (135 kDa). This indicates variable binding modes of the protease, factor I, with respect to the CUB domain that carries the three scissile bonds. Residues of the TED domain that are important for factor H binding (1187–1249)[89] lie adjacent to CUB (Figure 2), suggest-

ing that factor H binding to this site may be involved directly in orienting factor I with respect to cleavage sites in CUB. Moreover, factor H, CR1, and MCP are possibly involved in unraveling of the CUB domain, enabling factor I to bind and cleave CUB three times. So far no interaction sites on C3b for MCP are known. Mutations in C3(H_2O), however, that affect factor H and CR1 binding have no effect on MCP binding, indicating a different mode of interaction for MCP[87]. Taken together, the available data suggest a model in which the compact arrangement of the TED domain in C3 is relaxed and opened up in C3b (with TED attached to the target surface), while the CUB domain is gradually unfolded in iC3b due to cleavages, until the bond between C3dg and C3c is finally severed.

6. SIGNALING ROLES OF C3B FRAGMENTS

The factor I- and factor H-, CR1- or MCP-mediated conversion of C3b to iC3b induces large structural changes[104] and alters the binding properties of the molecule[3]. Cleavage of C3b into iC3b results in loss of factor B and properdin binding; and gain of complement receptor 2 (CR2, CD21), 3 (CR3, $\alpha_M\beta_2$, CD11b/ CD18, Mac-1) and 4 (CR4, $\alpha_X\beta_2$, CD11c/CD18, p150,95); and conglutinin binding[54]. At the same time, the binding modes for factor H and CR1 change, since different residues of the α'NT and the MG6 domain become important for interaction with factor H and CR1[64,87]. Finally, proteolysis leads to two separate products — C3c released into the medium and C3dg fragment attached to the surface.

iC3b is the opsonin that facilitates phagocytosis of antigens by leukocytes. Recognition of iC3b by leukocytes is mediated by the integrin CR3[105-107]. The inserted (I) domain plays a critical role in ligand binding to CR3, similar to other integrins, e.g., CR4. In both cases, the binding site involves a metal-ion-dependent adhesion site (MIDAS) in the I domain[108]. Various structural studies[109,110] have shown that ligand binding to a MIDAS site involves an acidic residue of the ligand that completes the coordination sphere of the divalent ion bound at the MIDAS. In the integrins the ligand binding induces a large structural change of the C-terminal α-helix in the I domains, which activates the integrin[109-111]. These I domains are structurally homologous to the VWA domain of factor B[33,34]. Also in the case of factor B, the MIDAS site is important for the metal-dependent interaction with C3b[112]. This homology between I and VWA domains have prompted others to propose a similar activation mechanism for factor B in the formation of the C3bBb convertase[55,113]. Nonetheless, CR3 and CR4 bind iC3b, whereas factor B binds C3b. A possible binding site for both factor B and CR3 has been identified on the α'NT region of C3b and iC3b[64]. This would suggest that there are differences in or near this interaction site between C3b and iC3b or that both factor B and CR3 bind multiple regions in C3b

and iC3b respectively. The latter is supported by various studies that show that regions outside the I domain contribute to iC3b interaction[114,115].

Surface-bound iC3b and C3dg play critical roles in B-cell stimulation and the initiation of adaptive immune responses. Coligation of C3dg or iC3b with CR2 and the B-cell antigen receptor complex amplifies a signal transduction cascade through the CR2/CD19/CD81 co-activation complex[2]. CR2 is a member of the RCA family. It consists of 15 or 16 N-terminal CCP modules, a 24 residue transmembrane domain, and a C-terminal 34-residue intracellular part. Only CCP1 and 2 are necessary for binding to C3dg or iC3b[116]. The CR2 binding site is located on the TED domain. Various studies have provided information on the interaction and binding sites in the iC3b–CR2 and C3d–CR2 complexes[117-124]. A few years ago, the structure of C3d in complex with CCP1-2 of CR2 was solved[36]. This structure shows extensive main-chain interactions between C3d and CCP2 of CR2 and no direct interaction between CCP1 of CR2 and C3d. However, the observed structural arrangement of the complex is controversial. Very recently it was shown that, in addition, the CCP1 domain of CR2 probably makes direct contacts to C3d[125,126]. Nevertheless, the exact site of interaction of CCP1 on C3d still remains unknown. Though significant structural differences are apparent between the structures of C3d in the CR2–C3d complex and the TED domain in C3, the CR2–CCP2 binding site is very similar in the two structures — ruling out that conformational changes play a role for this subsite. Moreover, this site is completely exposed in native C3 (Figure 2). This indicates that CCP1 of CR2 discriminates between the conformational states of C3 vs. iC3b and C3dg, because its binding site is either inaccessible in C3 or has changed its conformation.

7. CONCLUDING REMARKS

The structures of native C3 and its major fragment C3c have provided a wealth of structural insights into the central protein of the complement system. The proteolytic activation steps, generating the important fragments C3b and iC3b, are thought to induce significant conformational changes in the molecule, yielding protein molecules with distinct binding properties. The structures of C3 and C3c reveal the extent of conformational changes that may be expected. These structures together with the large amount of biochemical, mutagenesis, and binding data available on C3, its fragments, and the various interacting partners provide for the first time a detailed map of the various proposed binding sites. Still, many questions remain unanswered and additional structural data and site-directed mutagenesis experiments, now made possible in a more rational way, are required to elucidate the complete complexity of the central component of the complement system.

8. ACKNOWLEDGMENTS

We are grateful to Fin Milder, Michael Hadders and Lucio Gomes (Utrecht), and John D. Lambris (Philadelphia) for critically reading the manuscript. We thank the members of the laboratory for Crystal and Structural Chemistry for support and discussions. Financial support by the Council for Chemical Sciences of the Netherlands Organization of Scientific Research (NWO/CW) is gratefully acknowledged.

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