Published in final edited form as:

J Mol Biol. 2007 March 16; 367(1): 224–233.

The Crystal Structure of C2a, the Catalytic Fragment of Classical Pathway C3- and C5-convertase of Human Complement

Vengadesan Krishnan¹, Yuanyuan Xu², Kevin Macon¹, John E. Volanakis³, and Sthanam V.L. Narayana^{1,*}

¹ Center for Biophysical Sciences and Engineering, School of Optometry, University of Alabama at Birmingham, Birmingham, AL 35294.

² Division of Clinical Immunology and Rheumatology, University of Alabama at Birmingham, Birmingham, AL 35294.

³ Department of Medicine, University of Alabama at Birmingham, Birmingham, AL 35294.

Summary

The multi-domain serine protease C2 provides the catalytic activity for the C3- and C5- convertases of the classical and lectin pathways of complement activation. Formation of these convertases requires the Mg²⁺-dependent binding of C2 to C4b and the subsequent cleavage of C2 by C1s or MASP2, respectively. The C-terminal fragment C2a consisting of a serine protease (SP) and a von Willebrand factor type A (vWFA) domain, remains attached to C4b, forming the C3 convertase, C4b2a. Here we present the crystal structure of Mg²⁺-bound C2a to 1.9 Å resolution in comparison to its homolog Bb, the catalytic subunit of the alternative pathway C3 convertase, C3bBb. Although the overall domain arrangement of C2a is similar to Bb, there are certain structural differences. Unexpectedly, the conformation of the metal ion-dependent adhesion site and the position of the α 7 helix of the vWFA domain indicate a ‘co-factor-bound or open’ conformation. The active site of the SP domain is in zymogen-like inactive conformation. Based on these structural features we suggest a model for the initial steps of C3 convertase assembly.

Keywords

human complement system; complement serine protease; C2; Factor B; classical pathway; C3/C5 Convertase; glycoprotein

Introduction

Complement is an intricate system of about 35 interactive proteins that recognize and eliminate invading microorganisms and altered cells of the host. It is an important component of host defense, participating in both innate and adaptive immunity¹. Inappropriate activation and/or inefficient regulation of complement can lead to inflammatory or immunologic diseases. Effective pharmacological control of such complex system requires a detailed understanding

*Corresponding author: Sthanam V. L. Narayana, Associate Professor, Center for Biophysical Sciences and Engineering, School of Optometry, University of Alabama at Birmingham, 1025 18th Street South, Birmingham, AL 35294-0005, Phone: 205 934 0119, Fax: 205 975 0538, Email: Narayana@uab.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

of the topology and structure of binding and catalytic sites of its constituent proteins, particularly of the pivotal complement enzymes, C3 and C5 convertases.

Depending on the nature of the invading pathogen and the availability of specific antibodies, complement activation is initiated through one of three pathways termed classical, lectin, and alternative (Fig. 1a). End result of complement activation is the formation of the convertases, which produce biologically-active protein fragments by cleaving C3 and C5. The classical and lectin pathway formation of the C3 convertase proceeds through three steps: First, cleavage of C4 by C1s or MASP2 results in the covalent attachment of its major fragment, C4b, to the surface of the activator, next, C2-Mg²⁺ binds to C4b, and last, C2 is cleaved by C1s or MASP2 giving rise to the C4b2a C3 convertase. A similar reaction sequence leads to assembly of the alternative pathway C3 convertase, C3bBb. Attachment of C3b to either C3 convertase changes its specificity from C3 to C5 (Fig 1a).

C2 is a glycosylated modular protein consisting of three domains (Fig. 1b). The N-terminal domain comprising three complement control protein (CCP) modules is termed C2b and is cleaved off during formation of the C3 convertase. The middle domain is a typical von Willebrand factor type A (vWFA) domain containing a metal ion-dependent adhesion site (MIDAS), similar to that present in the I domains of integrins². The C-terminal domain is an atypical chymotrypsin-like serine protease (SP) displaying trypsin-like specificity³. The main difference from typical SPs is that transition from the zymogen to the catalytically active form is not associated with the formation of a new N-terminus. Instead, the SP domain of C2 remains linked to the vWFA domain and expresses catalytic activity against C3 only in the context of the C4b2a complex. The structure of factor B is similar to C2 and the two proteins display 39% amino acid residue identity^{4, 5} (Fig. 1b).

In previous studies we explored the catalytic apparatus of the alternative pathway convertases, by solving the crystal structure of the serine protease domain of factor B⁶ and of Bb^{C428-C435}⁷. The latter has an engineered disulfide bond aimed at locking the vWFA domain in the ligand-bound “open” conformation by analogy to the I domain of integrins^{8; 9; 10}. Here we present the structure of Mg²⁺-bound C2a to 1.9 Å resolution. Surprisingly, the structure of the vWFA domain of C2a has an open conformation being very similar to Bb^{C428-C435}.

Results and Discussion

Overall Structure of C2a

Attempts to produce crystals of intact recombinant C2 expressed in insect cells by the hanging drop vapor diffusion method yielded diffraction quality single crystals of C2a. The identity of this fragment was indicated by the unit cell volume of the crystals and their solvent content and by SDS-PAGE of dissolved single crystals (Fig. 1c); it was confirmed by N-terminal amino acid sequencing by Edman degradation which yielded the sequence KIQIQ, which is the N-terminal of C2a⁴. A trypsin-like protease contaminant not detectable by SDS-PAGE could be responsible for the cleavage of C2 or less likely cleavage might be the result of autocatalytic activity of highly concentrated C2. C2 treated with diisopropylfluoro-phosphate produced no crystals using similar crystallization conditions.

A diffraction data set to 2.3 Å resolution was collected when C2a crystals, obtained from recombinant C2 purified using buffers containing 5 mM EDTA, were exposed to an in-house X-ray source. We collected diffraction data to 1.9 Å resolution using SERCAT 19-BM beamline after soaking similarly obtained C2a crystals in 5 mM MgCl₂ overnight. The two crystal structures refined using above 2.3 Å and 1.9 Å resolution diffraction data respectively are almost identical (rms deviation 0.80 Å), except that the latter has clear density for the bound Mg²⁺ ion, whereas the former has weak density at the same position consistent with a water molecule.

The crystallographic data and the refinement statistics for the two structures are summarized in Tables 1 and 2, respectively. The data indicate that Mg^{2+} binding does not induce a conformational change in C2a. Therefore, the requirement for Mg^{2+} for formation of the C4bC2 complex may indicate the requirement for a residue(s) of C4b to complete the coordination of the metal ion. We will refer to the 1.9 Å, Mg^{2+} -bound crystal structure throughout this manuscript.

The three-dimensional structure of the C2a molecule is shown in Figure 2a. The overall dimensions of the molecule are $90 \times 50 \times 50$ Å; it consists of two compact domains, namely vWFA and SP, and has the shape of a distorted dumbbell, a close match to its appearance by electron microscopy¹¹. The two domains are joined by a 13 residues linker (433–446; amino acid residue numbering throughout from the N-terminus of the mature secreted protein). The linker contains a Cys residue at position 443, which is disulfide bonded to Cys⁵⁶¹ of the SP domain (Fig. 2a).

The vWFA domain contains 209 residues (224–432) including 13 residues of the C2b–C2a linker at the N-terminus. The domain proper comprises seven α helices, six β strands, and three 3_{10} helices and adopts the dinucleotide binding or ‘Rossman’ fold with a central β sheet surrounded by α helices (Fig. 2a & 3a). A shallow crevice present at the bottom of the domain contains the MIDAS motif, while the top of the domain is linked to the SP domain. The latter comprises 286 residues (447–732) and adopts a typical chymotrypsin fold with two anti-parallel β -barrels, each composed of six β -strands surrounded by α and 3_{10} helical segments. The catalytic center is situated topologically at the opposite pole of the ‘distorted dumbbell’ from the MIDAS motif of the vWFA domain (Fig. 2a).

C2a has six potential N-linked glycosylation sites, two in the vWFA domain (Asn²⁷⁰ and 313) and four in the SP domain (Asn⁴⁴⁷, 451, 601, and 631). The presence of bulky electron densities at these sites except for Asn⁴⁵¹, which is exposed on a flexible loop, gives clear indication that these residues are glycosylated. Asn⁴⁴⁷ is buried at the domain interface cleft and as a result the associated density is clearer and reveals more sugar residues than seen at the other sites. Well defined density is seen for at least two complete N-acetylglucosamine residues at this site. The other four sites, Asn²⁷⁰, 313, 601, and 631 show the presence of at least one sugar residue. An extended shapeless bulky electron density into the solvent, linked to all of these single sugar residues suggests that additional sugar moieties are present. Bb has only two glycosylation sites both in the vWFA domain; however, no strong density was seen at either site in the crystal structure of Bb^{C428–C435}, although it was expressed in insect cells similarly to C2a⁷.

Structure of the vWFA Domain

Fig. 3a shows the overall similarities between vWFA domains of C2 and factor B. By comparison to structurally homologous integrin α -chain I domains, the vWFA domain of C2a adopt a conformation similar to the ‘ligand bound open’ form^{8–10}. It has been shown that ligand binding induces changes mainly in two features of I domains. The most impressive change is a 10 Å shift of the C-terminal $\alpha 7$ helix away from the MIDAS motif. This large movement is associated with changes in the conformational angles of residues that contribute to MIDAS. Superposition of the vWFA domain of C2a on the I domains of αM and $\alpha 2$ in ligand-bound ‘open’ conformations (1IDO and 1DZI) yielded rms deviations of 1.3 Å and 1.5 Å for 138 and 124 common C α atoms, respectively. A similar comparison of C2a with the $\alpha 2$ I domain in ligand-free ‘closed’ conformation (1AOX) yielded rms deviation of 1.6 Å for 120 common C α atoms. The main difference between C2a and $\alpha 2$ in “open” conformation is in the position of the $\alpha 7$ helix which is 3 Å closer to the MIDAS motif in C2a. However, the $\alpha 7$ helix of C2a is much closer to that of the “open” than that in the “closed” conformation of $\alpha 2$ (Fig. 3b). More importantly, the linker between C2a and C2b domains, i.e. the N-terminus of the crystallized C2a fragment is observed occupying the exact space in which the shifted $\alpha 7$ helix

of Bb^{C428–C435} was positioned (Fig. 3c). This observation is significant, since the co-factor induced conformational changes emanating from MIDAS site, by shifting the $\alpha 7$ helix can push out and expose the scissile bond in the interdomain linker between C2a and C2b, and Bb and Ba respectively, facilitating the proteolytic cleavage of C2b and Ba domains by C1s and factor D respectively. This co-factor mediated conformational changes playing a role in N-terminal domain cleavage might be specially true for factor B activation, since factor D can cleave factor B only when the later is bound to the cofactor C3b, mediated by a Mg^{2+} ion. The placement and direction of C2a N-terminus, directed towards the face in which the S1 site is located on SP domain, possibly suggest the orientation and location of C2b in relation to the catalytic site of C2. As shown in Fig. 3c, the position of the $\alpha 7$ helix of C2a is determined to a great extent by the N-terminal region of the fragment, which precludes further upward movement of the helix. Moreover, the torsional angles (Table 3) and positions (Fig. 4a) of residues of the MIDAS motif of C2a are similar to those of the “open” conformation of $\alpha 2$.

Two crystal structures of the vWFA domain of factor B have been published and both are in “open” conformation. For the recombinant vWFA domain (1Q0P), the “open” conformation in the absence of bound co-factor C3b was attributed to a fortuitous crystallographic contact that completes the octahedral coordination of Mg^{2+} at MIDAS, representing a ligand-mimetic conformation¹². For the vWFA domain of Bb^{C428–C435} (1RRK), the “open” conformation was intentional and attributed to the introduction of the disulfide bond⁷. Superposition of the vWFA domain of C2a to that of factor B (1Q0P) (Fig. 3a) resulted in rms deviation of 1.0 Å for 164 common C α atoms. The major differences between the two structures are: (1) A 13 residue fragment of the C2b-C2a linker is not present in the vWFA domain of factor B, (2) The loop between $\alpha 4$ and βD is 3 residues longer in C2a, (3) In the $\alpha 5$ - βE loop a short 3_{10} helix present at the beginning of βE in C2a, is extended by 5 residues in factor B, (4) The short loop between βE and $\alpha 6$ is longer in C2a by about 1 residue, and (5) The $\alpha 7$ helix is disordered and not seen in the factor B vWFA domain, while its position is clear in C2a. This may be due to the stabilization of the $\alpha 7$ helix of C2a by a number of close interactions with the SP domain and also with the N-glycan attached to Asn⁴⁴⁷ at the domain interface.

Superposition of C2a vWFA domain to that of Bb^{C428–C435} (1RRK) yields rms deviation of 0.9 Å for 164 common C α atoms. The main difference between the two structures is in the loops between $\alpha 4$ and $\alpha 4'$ and between $\alpha 5$ and βE , which are not seen in Bb^{C428–C435}. More importantly, the $\alpha 7$ helix is shifted further away from the MIDAS motif in Bb^{C428–C435}, as it is locked by a disulfide bridge to stay in that position. The corresponding position of the $\alpha 7$ helix of C2a is 3 Å nearer to the MIDAS motif, because of the position of its N-terminus (Fig. 3a & 3b). It is relevant that Bb^{C428–C435} is missing the portion of the Bb-Ba linker corresponding to the N-terminus of C2a, because the cDNA sequence encoding this region had been deleted. Despite the difference in the position of the $\alpha 7$ helix, the torsional angles (Table 3) and positions (Fig. 4b) of MIDAS motif residues are similar in the Bb^{C428–C435} and C2a structures.

The MIDAS Motif

Buffer solutions used for C2 purification contained 5 mM EDTA and the 2.3 Å resolution structure of the resulting C2a crystals showed weak density at the MIDAS motif equivalent to water molecules with identical distribution and distance from the coordinating residues as the Mg^{2+} seen in the 1.9 Å resolution crystal structure. The difference electron density map from the latter structure revealed clear density ($> 5\sigma$) for the bound Mg^{2+} ion with octahedral coordination geometry. The average bond length is 2.1 Å, as expected for Mg^{2+} ion.

Residues Asp²⁴⁰, Ser²⁴², Ser²⁴⁴, Thr³¹⁷ and Asp³⁵⁶ constitute the MIDAS motif of C2a (Fig. 4a & 4b). The side chain hydroxyl oxygen atoms of Ser²⁴², Ser²⁴⁴, and Thr³¹⁷ and three water molecules complete the six coordinating ligands of Mg^{2+} , whereas Asp²⁴⁰ and Asp³⁵⁶

coordinate with Mg^{2+} through two of the three water molecules observed. The second carboxylate oxygen of these Asp²⁴⁰ and Asp³⁵⁶ residues makes hydrogen bonds to Ser²⁴² and Ser²⁴⁴, respectively. Asp²⁴⁰, Ser²⁴², and Ser²⁴⁴ represent the conserved sequence motif DXSXS on the β A- α 1 loop, one of the three loops that surround the MIDAS motif. Mutation of Asp²⁴⁰ or Ser²⁴⁴ to Leu or Ala resulted in almost complete loss of C2 activity¹³. Two other loops α 3- α 4 and β D- α 5 contribute Thr³¹⁷ and Asp³⁵⁶ residues, respectively. These MIDAS features of the C2a vWFA domain are identical to those observed in the Bb^{C428-C435} structure⁷ and the factor B vWFA domain structure¹². Asp³⁵⁶ of C2a lies within a loop containing a TDG³⁵⁵⁻³⁵⁷ sequence; the Gly of the corresponding sequence of integrins undergoes a dramatic flip on switching from the “closed” to “open” conformation¹². As shown in Fig. 4b, the TDG³⁵⁵⁻³⁵⁷ loop of C2a closely superposes on the corresponding loop of Bb^{C428-C435} and also on that observed in “ligand-bound open” conformation of the I domain of the α 2 chain (Fig. 4a). These similarities are reflected in the (ϕ , ψ) torsion angles of the TDG³⁵⁵⁻³⁵⁷ residues of C2a and the corresponding residues of the other proteins (Table 3).

Structure of the SP domain

The SP domain of C2a (Fig. 5a) adopts a structural fold identical to that of the isolated SP domain of factor B⁶ (1IDLE) and of Bb^{C428-C435} 7 (1RRK). When the above two structures are superimposed on the SP domain of C2a, the observed rms deviation is 1.2 Å for 192 common C α atoms and 1.2 Å for 235 common C α atoms, respectively. Comparison with the typical SP structures of trypsin (1TRN) and chymotrypsin (2CGA) yields rms deviations of 1.3 Å and 1.2 Å for 157 and 151 common C α residues, respectively. A unique loop segment Cys⁵⁶⁴–Asn⁵⁸⁹ is inserted in the middle of the SP domain of C2, adopting a helix-loop-helix conformation with a disulfide bond (Cys⁵⁶⁴–Cys⁵⁸⁰) linking the N-terminal ends of the two helices. This feature is also observed in Bb^{C428-C435} 7, but is absent from typical serine proteases.

Perona and Craik¹⁴ described eight conserved loops that determine the geometry of the S1 pocket and the subsite specificity. Of these, loops 1, A, and D are shorter in C2a than in Bb, while loops B and C are of equal length, and loops 3 and E are longer in C2a. The most prominent difference between the SP domains of C2a and Bb is observed in the length, composition, and flexibility of loop 2 (Fig. 5b). The backbone of the loop is clear in C2a while no electron density was seen for the corresponding loop of the factor B and Bb^{C428-C435} SP domains^{7; 15}. However, clear density for most of the backbone of the loop was seen in the structure of the Bb^{C428-C435}-BCX583 inhibitor complex^{7; 15}.

The N-terminal end of the C2a SP domain is connected to the vWFA domain through a 13 residues linker, whereas the corresponding linker is 14 residues long in Bb. In all other serine proteases, transition from the zymogen to the catalytically active form is associated with the generation of a new N-terminus. The free NH₃⁺ ion at the terminus forms an ion pair with the carboxylate side chain of Asp¹⁹⁴ (chymotrypsinogen numbering) that is adjacent to the catalytic Ser¹⁹⁵. Formation of this bond is thought to cause rigidity of the S1 pocket suitable for substrate binding and to force the 192–194 segment to adopt an active ‘oxyanion hole’ β -turn conformation, one of the prerequisite structural correlates of catalytic efficiency. In the SP domain of C2a although the overall backbone conformation of the Ser⁶⁵³⁽¹⁸⁹⁾–Glu⁶⁵⁸⁽¹⁹⁴⁾ (numbers in parentheses are for chymotrypsinogen) segment is similar to the corresponding segments of activated serine proteases, its conformation is zymogen-like due to the inward orientation of the carbonyl oxygen of Lys⁶⁵⁶⁽¹⁹²⁾ and its interactions (3.2 Å separation) with the hydroxyl group of Ser⁶⁵⁹⁽¹⁹⁵⁾. As a result, the hydroxyl of Ser⁶⁵⁹⁽¹⁹⁵⁾ is not free to perform nucleophilic attack on the scissile bond of the substrate and the Lys⁶⁵⁶⁽¹⁹²⁾–Glu⁶⁵⁸⁽¹⁹⁴⁾ segment adopts a 3_{10} helix conformation (Fig. 5b), similar to that observed in the factor B and Bb^{C428-C435} SP domains. The corresponding separation between

the main chain carbonyl of Arg⁶⁷¹⁽¹⁹²⁾ to the hydroxyl oxygen of Ser⁶⁷⁴⁽¹⁹⁵⁾ in factor B is 2.89 Å. In contrast to other typical serine proteases, where the Lys⁶⁵⁶⁽¹⁹²⁾–Glu⁶⁵⁸⁽¹⁹⁴⁾ segment displays interactions with the buried N-terminus and with loop D, in C2a it exhibits considerably more contacts with the much extended L2 loop, while such interactions are limited in the Bb^{C428–C435} structure. Interestingly, Arg⁶⁹⁴ from the L2 loop of C2a forms a salt bridge with Glu⁶⁵⁸⁽¹⁹⁴⁾, such that the Arg⁶⁹⁴ side chain occupies the space in which the charged N-terminus Ile¹⁶ occupies in typical active SPs. The Glu⁶⁵⁸⁽¹⁹⁴⁾–Arg⁶⁹⁴ salt bridge seems to be responsible for the stabilization of the L2 loop in C2a, while similar interactions were possible in the factor B SP domain only when the S1 pocket was occupied by the BCX583 inhibitor that is a mimetic of the substrate P1 Arg side chain⁷. However, even in that structure the oxyanion hole displayed a 3_{10} helix, zymogen-like conformation. The failure of the Glu⁶⁵⁸⁽¹⁹⁴⁾–Arg⁶⁹⁴ salt bridge in C2a and the corresponding Asp⁶⁷³⁽¹⁹⁴⁾–Arg⁷⁰⁵ bridge in the Bb^{C428–C435}–BCX583 inhibitor complex to induce the active conformation of the oxyanion hole is puzzling. It suggests that additional structural features present in typical SPs, but absent from C2 and factor B, are necessary for induction of a functional oxyanion hole. Despite the inactive conformation of the oxyanion hole and the hydrophobic interaction between the catalytic Ser⁶⁵⁹⁽¹⁹⁵⁾ and Arg⁶⁷¹⁽¹⁹²⁾, the catalytic triad residues Asp⁵⁴¹⁽¹⁰²⁾, His⁴⁸⁷⁽⁵⁷⁾, and Ser⁶⁵⁹⁽¹⁹⁵⁾ of C2a display conventional active site disposition.

The S1 pocket of C2a is composed of three polypeptide segments: Ser⁶⁵³⁽¹⁸⁹⁾–Ser⁶⁵⁹⁽¹⁹⁵⁾, Ser⁶⁷⁸⁽²¹⁴⁾–Asn^{683(218a)}, and Arg⁷⁰⁶⁽²²⁵⁾–His⁶⁹⁷⁽²²⁸⁾. Notable differences in C2a from typical SPs with trypsin-like specificity are the substitutions of Ser⁶⁵³⁽¹⁸⁹⁾ and Asp⁷⁰⁷⁽²²⁶⁾ for the highly conserved Asp¹⁸⁹ and Gly²²⁶, respectively (Fig. 5b). A loss of crucial negative charge in the S1 pocket affecting substrate specificity was anticipated in factor B due to the presence of an Asn⁶⁵³⁽¹⁸⁹⁾ instead of Asp¹⁸⁹, however, a switch back to Asp from Asn at position 653 (189) by site directed mutagenesis did not improve the catalytic efficiency and had no effect on specificity¹⁵. In chymotrypsin, specificity for bulky aromatics is largely determined by Ser¹⁸⁹ and the hydrophobicity of the walls of S1. Despite the presence of Pro⁶⁵⁴⁽¹⁹⁰⁾, which imparts hydrophobicity to the S1 pocket, and Ser⁶⁵³⁽¹⁸⁹⁾, C2 exhibits trypsin-like specificity for positively charged Arg residues. As we showed earlier for factor B¹⁵ the requirement for negative charge in the S1 pocket of C2a despite the loss of Asp at position 653(189) can apparently be compensated by the Asp residue present at position 707(226), which can effectively hold the P1-Arg and position the scissile bond for cleavage. Asn⁶⁵³⁽¹⁸⁹⁾ present in the S1 site of factor B was shown to point away from the substrate binding pocket. In contrast, Ser⁶⁵³⁽¹⁸⁹⁾ in C2a points towards the binding pocket and thus contributes to the electro-negativity of the pocket (Fig. 5b).

Domain Interface

The buried surface area at the interface between the vWFA and SP domains was calculated to be 1890 Å² compared to 1280 Å² in Bb^{C428–C435}. For this analysis the linker residues Leu⁴³³–Thr⁴³⁹ in the vWFA domain and Gly⁴⁴⁶–Asp⁴⁴⁰ in the SP domain and sugar molecules were included. The interface is constituted of a total of 39 residues (excepting sugars) from both domains, 12 of which are charged. The residues of the two domains make 30 contacts measuring less than 4 Å, including 2 salt bridges and 14 hydrogen bonds. Specifically, the $\alpha 7$ helix of the vWFA domain, which is situated at the domain interface, makes 4 hydrogen bonds with the 448–452 loop segment and two hydrogen bonds with the sugar molecule at Asn⁴⁴⁷ of the SP domain. Of note are interactions between two His residues (His⁴²⁶ and His⁴³¹) at the C-terminal part of the $\alpha 7$ helix with the SP domain. Specifically, His⁴²⁶ participates in hydrophobic interactions with Phe⁶⁶⁹ and Met⁴⁴⁸, while the polar side chain of His⁴³¹ and the main chain carbonyl of Glu⁴³⁰ form strong hydrogen bonds with the N-acetylglucosamine residue of Asn⁴⁴⁷. Probably, these are the interactions that hold the $\alpha 7$ helix in its present shifted position. In the isolated SP domain structure of factor B this segment has no density⁶, and

similarly the $\alpha 7$ helix is not seen in the individual vWFA domain structure¹², which suggests that the above mentioned interactions are important in the domain interface.

Implications for the formation of the C3 convertase

The present data dictate several changes in the current paradigm of the mechanism of classical/lectin pathway convertase assembly. It is currently held that initial binding of C2 to C4b is mediated by two low-affinity sites one on C2b, formed by all three CCP modules¹⁶, and the other on the vWFA domain, formed by the MIDAS motif¹³. It is further held that cleavage by C1s or MASP2 causes a transient conformational change in the latter site, leading to increased binding avidity for C4b, sequestration of Mg^{2+} and expression of proteolytic activity against C3³. Concepts about the nature of the conformational change of the vWFA domain have been influenced by studies on the homologous domain of the α chain of integrins, variously termed I or A domain, which mediates ligand-binding. Mutational and biophysical studies have demonstrated that the I domain can exist in one of two reversible conformations called “closed” and “open”. The open conformation is induced by the ligand, is characterized by high affinity binding, transduces a signal to neighboring domains of the protein and can be stabilized in the absence of ligand by the introduction of a disulfide bond^{8; 9; 10; 17; 18}. Previous data on factor B, the structural and functional homolog of C2 in the alternative pathway, were only partially consistent with this model.

The MIDAS motif of the recombinant vWFA domain of factor B was shown to display a conformation similar to the “open” one of integrins. This was attributed to the presence of an Asp residue from another domain in the crystal which completed the coordination of the bound Mg^{2+} ¹². Computer modeling indicated the domain can also adopt a “closed” conformation. Similarly, the vWFA domain in the crystal structure of Bb^{C428–C435} was shown to adopt an “open” conformation⁷, which in this case was attributed to the introduction of a disulfide bond analogous to that locking the open conformation of the integrin I domain^{8; 9}. A major difference from the I domain was that the avidity of Bb^{C428–C435} for C3b was only slightly increased over that of Bb as opposed to a 100-fold increased affinity for ligand displayed by I domains in “open” conformation.

The present structure demonstrates clearly that the “open” conformation of the vWFA domain of C2a is not induced by the cofactor C4b or by any other chance or deliberate contact. Whether it is induced by the cleavage of C2 by C1s or MASP2 or exists in the structure of intact C2 is not clear from the present data. It is clear however, that in contrast to the I domains, the “open” conformation is not by itself sufficient for high avidity binding to C4b. Apparently transient conformational changes of other regions of the vWFA domain are necessary for binding of C2a to C4b. These changes are reversed when C2a is dissociated from C4b and obviously cannot be induced again. The contribution of the MIDAS motif to C4b binding appears to be mainly through the Mg^{2+} , which probably coordinates to one or more residues of the C4b binding site.

A C2-binding site identified on C4b is located at the N-terminal of its α' chain and consists of two acidic sequences, ⁷⁴⁴EED and ⁷⁴⁹DEDD¹⁹. Assuming an ionic interaction between this site and the corresponding C2 site, it is perhaps relevant that the region around the MIDAS motif is strongly electronegative, while the main positively-charged region is on part of the loop 2 of the SP domain (Fig. 2b). Electron micrographs of the C4b2a complex¹¹ show C2a attached on C4b through only one of its two domains. Current evidence indicates that this single site is on the vWFA domain and includes the MIDAS motif¹³. Therefore, binding of the electropositive patch of the SP domain to the N-terminal electronegative region of the C4b α' chain seems unlikely, which suggests that perhaps the CCP modules of C2b bind at that site.

The C2a structure also indicates that the active center of the SP domain, like that of Bb is in inactive, zymogen-like conformation, a finding consistent with the lack of proteolytic activity. The proposed transduction of an activating signal from the C4b-bound vWFA to the SP domain seems highly unlikely, because of the presence of the Cys⁴⁴³–Cys⁵⁶¹ bond, which would stop transmission of a signal. If that were so, two not mutually exclusive hypotheses can explain expression of C3-cleaving activity only by C4b-bound C2a. The necessary conformational change of the active center is induced by a direct interaction of the SP domain with the cofactor or with the substrate. As mentioned above the former interaction could be through the electropositive loop 2 and it seems unlikely. The second possibility, which we favor has also been proposed by us to explain expression of proteolytic activity by C3b-bound Bb⁷.

In conclusion, we have presented the crystal structure of the catalytic fragment of C2, C2a. The structure shows the vWFA domain in “open” conformation and the catalytic center in inactive zymogen-like conformation. On the basis of the structure we propose a new model for the assembly of the C3 convertase of the classical and lectin pathway.

Methods

Expression and Purification

Full-length C2 cDNA⁵ was cloned into the baculovirus expression vector pACgp67A (BD Biosciences Pharmingen, San Diego, CA) and co-transfected with BD Baculogold Bright linearized baculovirus DNA into sf9 insect cells. A C241A mutation had been introduced into the cDNA to avoid formation of undesired disulfide bonds and protein aggregation. We showed previously that the C241A mutation results in slight increase of C2 hemolytic activity¹³. High titer stocks were produced and used to infect High Five insect cells (Invitrogen, Carlsbad, CA). Media from the infection were harvested at day five and C2 purified by successive chromatography on S Sepharose, Heparin HT, and Mono S using a FLCP system (AKTA Purifier; GE Healthcare, Piscataway, NJ).

Crystallization

Crystallization was carried out by the vapor-diffusion method using purified C2 at 20 mg/ml and the hanging drop technique at 22°C. Initially nine conditions were obtained using various crystallization screen kits with high throughput nano technique. Two of them were reproducible, and gave long tree like crystals not suitable for X-rays diffraction. Additives screening was carried out to improve the crystal quality and the following condition was found most suitable. A droplet containing 2 µl C2 at 20mg/ml mixed with 2 µl well solution (20% w/v polyethylene glycol 10000, 0.1 M HEPES, pH 7.5: Hampton crystal screen 2, #38) and 0.4µl of 0.3 M glycyl-glycyl-glycine (Hampton additive screen #35) was equilibrated against the reservoir well solution. Long thick rod-like crystals grew in 3–7 days.

Data Collection

C2a native data were collected on a Rigaku R-axis IV imaging-plate detector using CuK α radiation from an in-house Rigaku rotating-anode X-ray generator operating at 100 mA and 50 kV. Ethylene glycol (20%) was used as cryoprotectant and crystals were flash-frozen in a nitrogen stream at 100K mounted in litho loops. The cell dimensions and V_m calculations showed that space group is P2₁ and that one molecule is present in the asymmetric unit. The data were processed and scaled using program D*trek²⁰. High-resolution 2.0 Å diffraction for Magnesium soaked C2a crystal was collected on a 3 X 3 mosaic CCD detector at the SBC-CAT 19 BM beamline of the Advance Photon Source at the Argonne National Laboratory. Ethylene glycol (20%) was used as cryoprotectant before freezing the crystal in liquid Nitrogen. The data was processed and scaled using programs DENZO and SCALEPACK²¹. The crystallographic data statistics are presented in Table 1.

Structure determination, Model Building, and Refinement

Initial phases for the C2a data were obtained by MR (molecule replacement) method using the Bb^{C428–C435} structure (1RRK) as a search model with the CNS²² program. A clear solution was obtained in the cross rotation function and subsequent translation function. The location of the SP domain was first obtained and fixed while the location of vWFA domain was searched. The positions of the two domains in the asymmetric unit were subjected to rigid body refinement and the resultant orientation between the two domains was very similar to the Bb^{C428–C435} structure. Examination of the electron density map revealed clear density for the core of both SP and vWFA domains. Chain tracing and model building were carried out with the program COOT²³. Several rounds of model building and final refinement with Refmac24 and the addition of sugars and water molecules at the final stage gave a final R_{cryst} of 0.21.7 and an R_{free} of 0.28. The C2a native structure was used in refinement of Mg ion soaked data. The refinement statistics of C2a and C2a-Mg²⁺ structure are listed in Table 2. The final structure was checked using PROCHECK²⁵.

Acknowledgements

The project is funded by NIH grant AI1064815 to S.V.L.N. High-resolution diffraction data for this study were collected at Argonne National Laboratory, Structural Biology Center beamline 19-BM. Coordinates of C2a are deposited in PDB with an accession code XXX.

References

1. Volanakis, JE. Overview of the Complement System. In: Volanakis, JE.; Frank, MM., editors. The Human Complement System. Marcel Dekker, Inc; New York: 1998.
2. Kamata T, Liddington RC, Takada Y. Interaction between collagen and the alpha(2) I-domain of integrin alpha(2)beta(1). Critical role of conserved residues in the metal ion-dependent adhesion site (MIDAS) region. *Journal of Biological Chemistry* 1999;274:32108–11. [PubMed: 10542245]
3. Arlaud GJ, Volanakis JE, Thielens NM, Narayana SV, Rossi V, Xu Y. The atypical serine proteases of the complement system. *Adv Immunol* 1998;69:249–307. [PubMed: 9646846]
4. Bentley DR. Primary structure of human complement component C2. Homology to two unrelated protein families. *Biochem J* 1986;239:339–45. [PubMed: 2949737]
5. Horiuchi T, Macon KJ, Kidd VJ, Volanakis JE. cDNA cloning and expression of human complement component C2. *J Immunol* 1989;142:2105–11. [PubMed: 2493504]
6. Jing H, Xu Y, Carson M, Moore D, Macon KJ, Volanakis JE, Narayana SV. New structural motifs on the chymotrypsin fold and their potential roles in complement factor B. *Embo J* 2000;19:164–73. [PubMed: 10637221]
7. Ponnuraj K, Xu Y, Macon K, Moore D, Volanakis JE, Narayana SV. Structural analysis of engineered Bb fragment of complement factor B: insights into the activation mechanism of the alternative pathway C3-convertase. *Mol Cell* 2004;14:17–28. [PubMed: 15068800]
8. Lee JO, Bankston LA, Arnaout MA, Liddington RC. Two conformations of the integrin A-domain (I-domain): a pathway for activation? *Structure* 1995;15:1333–40. [PubMed: 8747460]
9. Shimaoka M, Lu C, Salas A, Xiao T, Takagi J, Springer TA. Stabilizing the integrin alpha M inserted domain in alternative conformations with a range of engineered disulfide bonds. *Proc Natl Acad Sci U S A* 2002;99:16737–41. [PubMed: 12466503]
10. Shimaoka M, Xiao T, Liu JH, Yang Y, Dong Y, Jun CD, McCormack A, Zhang R, Joachimiak A, Takagi J, Wang JH, Springer TA. Structures of the alpha L I domain and its complex with ICAM-1 reveal a shape-shifting pathway for integrin regulation. *Cell* 2003;112:99–111. [PubMed: 12526797]
11. Smith CA, Vogel CW, Muller-Eberhard HJ. MHC Class III products: an electron microscopic study of the C3 convertases of human complement. *J Exp Med* 1984;159:324–9. [PubMed: 6559206]
12. Bhattacharya AA, Lupher ML Jr, Staunton DE, Liddington RC. Crystal structure of the A domain from complement factor B reveals an integrin-like open conformation. *Structure* 2004;12:371–8. [PubMed: 15016353]

13. Horiuchi T, Macon KJ, Engler JA, Volanakis JE. Site-directed mutagenesis of the region around Cys-241 of complement component C2. Evidence for a C4b binding site. *J Immunol* 1991;147:584–9. [PubMed: 2071895]
14. Perona JJ, Craik CS. Evolutionary divergence of substrate specificity within the chymotrypsin-like serine protease fold. *J Biol Chem* 1997;272:29987–90. [PubMed: 9374470]
15. Xu Y, Circolo A, Jing H, Wang Y, Narayana SV, Volanakis JE. Mutational analysis of the primary substrate specificity pocket of complement factor B. Asp(226) is a major structural determinant for p(1)-Arg binding. *J Biol Chem* 2000;275:378–85. [PubMed: 10617628]
16. Xu Y, Volanakis JE. Contribution of the complement control protein modules of C2 in C4b binding assessed by analysis of C2/factor B chimeras. *J Immunol* 1997;158:5958–65. [PubMed: 9190950]
17. Li R, Rieu P, Griffith DL, Scott D, Arnaout MA. Two functional states of the CD11b A-domain: correlations with key features of two Mn²⁺-complexed crystal structures. *J Cell Biol* 1998;143:1523–34. [PubMed: 9852148]
18. McCleverty CJ, Liddington RC. Engineered allosteric mutants of the integrin alphaMbeta2 I domain: structural and functional studies. *Biochem J* 2003;372:121–7. [PubMed: 12611591]
19. Pan Q, Ebanks RO, Isenman DE. Two clusters of acidic amino acids near the NH2 terminus of complement component C4 alpha'-chain are important for C2 binding. *J Immunol* 2000;165:2518–27. [PubMed: 10946278]
20. Pflugrath JW. The finer things in X-ray diffraction data collection. *Acta Crystallogr D Biol Crystallogr* 1999;55:1718–25. [PubMed: 10531521]
21. Otwinowski Z, Minor W. Processing of X-ray Diffraction Data Collected in Oscillation Mode. *Methods in Enzymology* 1997;276:307–326.
22. Brunger AT, Adams PD, Clore GM, DeLano WL, Gros P, Grosse-Kunstleve RW, Jiang JS, Kuszewski J, Nilges M, Pannu NS, Read RJ, Rice LM, Simonson T, Warren GL. Crystallography & NMR system: A new software suite for macromolecular structure determination. *Acta Crystallogr D Biol Crystallogr* 1998;54:905–21. [PubMed: 9757107]
23. Emsley P, Cowtan K. Coot: model-building tools for molecular graphics. *Acta Crystallogr D Biol Crystallogr* 2004;60:2126–32. [PubMed: 15572765]
24. The CCP4 suite: programs for protein crystallography. *Acta Crystallogr D Biol Crystallogr* 1994;50:760–3. [PubMed: 15299374]
25. Morris AL, MacArthur MW, Hutchinson EG, Thornton JM. Stereochemical quality of protein structure coordinates. *Proteins* 1992;12:345–64. [PubMed: 1579569]
26. Nicholls A, Sharp KA, Honig B. Protein folding and association: insights from the interfacial and thermodynamic properties of hydrocarbons. *Proteins* 1991;11:281–96. [PubMed: 1758883]

Figure 1.

a) Cartoon representation of C3-convertase formation in classical, alternative and lectin pathways of the human complement. b). Domain organization of homologous C2 and factor B of human complement. c) The SDS-PAGE of the C2 recombinant utilized in crystallization and the C2a fragment that was crystallized. From left: Lane1-LMW standards, Lane2- C2a from dissolved crystals, Lane 3- C2 protein used for crystallization after a week, Lane 4- Fresh C2 recombinant.

Figure 2.

a). Overall ribbon representation of C2a structure. The SP domain is represented in magenta, the vWFA domain in cyan, linker in blue, and the N-terminal in red. A disulfide bond joining the linker to the SP domain and catalytic site Serine are shown in green and red respectively.

b) Electrostatic surface representation of C2a. Surface charge distribution was calculated using GRASP²⁶. Positively charged regions are represented in blue, negatively charged regions in red, and both polar and nonpolar regions are in white. The marked electropositive patch represents the regions of L2 loop of SP domain

Figure 3.

a) Overall comparison of the vWFA domain of C2a (magenta) and isolated vWFA domain of Bb (yellow). Mg ion of C2a and Bb is shown in silver and pink. The N-terminal linker of C2a is shown in red and the disordered $\alpha 7$ helix of Bb is shown in yellow dotted line. b) Stereo view comparison of $\alpha 7$ helix and its environment of C2a with other known integrin I-domain 'open' and 'closed' structures. C2 is in magenta color, ligand free $\alpha 2\beta 1$ I-domain closed form is in yellow, ligand bound $\alpha 2\beta 1$ I-domain is in cyan color and Bb^{C428-C435} mutant of Bb in green color. c) Stereo view of superposition of $\alpha 7$ helix and its environment of C2a (magenta) and Bb (yellow). The linker to SP domain of C2a and Bb is shown in blue and green. The additional N-terminal of C2a is shown in red. The sugar molecule of C2a which is located at

the domain interface near $\alpha 7$ helix is shown in white and labeled as NAG. Mg ion of C2a and Bb are shown in silver and yellow respectively.

Figure 4.

(a) Stereo view of MIDAS motif comparison for ligand bound and ligand free conformations. The metal ions are colored in the same color as main chain ribbons, and the water molecules are in blue color. The ligand free closed conformation, in the presence of Mg^{2+} ion for $\alpha 2\beta 1$ I-domain (yellow) is superposed on ligand bound open conformation of $\alpha 2\beta 1$ Id-main (cyan) and ligand free open-like conformation of C2a (magenta). A Glu residue from the ligand collagen completes the coordination of Mg in $\alpha 2\beta 1$ I-domain 'open' conformation. (b) Stereo view of detailed comparison of MIDAS structures of C2a (magenta) and Bb (yellow). The metal atom of C2a and Bb is shown as silver and yellow ball respectively. Thin solid lines indicate the metal coordination of C2a (magenta) and Bb (yellow). Water molecules are shown in blue.

Figure 5.

a). Overall comparison of the SP domain of C2a (magenta) and Bb (yellow). The dotted lines show the disordered loops. The catalytic triad of C2a (S⁶⁵⁹, H⁴⁸⁷, D⁵⁴¹) and Bb (S⁶⁷⁴, H⁵⁰¹, D⁵⁵¹) are shown in Red and white respectively. The surface loops are labeled respectively. b). Stereo diagram showing the superposition of S1 pocket of C2a (magenta) and Bb (yellow).

Table 1

C2a native crystal crystallographic data.

Parameter	C2a Native	C2a with Mg ²⁺ ion
Space group	P2 ₁	P2 ₁
A (Å)	51.35	51.40
B (Å)	84.20	83.82
C (Å)	74.43	75.24
β = (°)	92.1	92.1
Resolution (Å)	30.0–2.3 (2.38–2.3)	50.0–1.9 (1.97–1.90)
R _{sym} * (%)	4.7 (18.2)	4.0 (17.0)
Unique reflections	27904	49897
Completeness (%)	98.8 (97.3)	98.0 (91.3)
χ ²	0.95 (1.32)	0.99(0.90)
I/σI	19.5 (7.1)	31.7 (6.0)

Values in the parentheses refer to the outermost resolution shell.

Table 2

Refinement data for C2a structure.

Parameter	C2a Native	C2a Mg ²⁺
Resolution range	30–2.3	30–1.9
R factor/R _{free}	21.7/28.0	20.0/25.0
Average B value (Å ²)	30.29	24.31
Rmsd for bonds (Å)	0.027	0.017
Rmsd for angles (°)	2.329	1.627
No. of protein atoms/sugar atoms	3888/112	3874/112
No. of water molecules	115	270
Unique reflections	26457	46291
No. of metal atoms	Nil	1
Ramachandran plot (core/disallowed)	100.0/0.0	100.0/0.0
PDB code		

Table 3
Backbone torsion angles comparison of TDG motif

	T		D		G	
	ϕ	ψ	ϕ	ψ	ϕ	ψ
C2a-Mg ²⁺	-161.2	164.1	-89.7	3.5	73.4	30.1
C2a	-161.3	156.8	-80.5	-8.9	80.5	26.5
Bb ^{C428-C435} (1RRK)	-157.1	171.8	-99.2	2.6	75.1	13.8
FB vWFA (1Q0P)	-154.3	169.7	-100.0	2.9	74.4	6.7
$\alpha 2$ open (1DZI)	-160.6	167.5	-93.6	4.4	74.5	12.3
$\alpha 2$ closed (1AOX)	-140.3	154.8	-122.6	22.2	-159.2	152.3