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ScienceDirect
Molecular Immunology 45 (2008) 1752–1760
Molecular Immunology
www.elsevier.com/locate/molimm

Revisiting the mechanism of the autoactivation of the complement protease C1r in the C1 complex: Structure of the active catalytic region of C1r

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Received 15 August 2007; received in revised form 25 September 2007; accepted 27 September 2007

Abstract

C1r is a modular serine protease which is the autoactivating component of the C1 complex of the classical pathway of the complement system. We have determined the first crystal structure of the entire active catalytic region of human C1r. This fragment contains the C-terminal serine protease (SP) domain and the preceding two complement control protein (CCP) modules. The activated CCP1–CCP2–SP fragment makes up a dimer in a head-to-tail fashion similarly to the previously characterized zymogen. The present structure shows an increased number of stabilizing interactions. Moreover, in the crystal lattice there is an enzyme–product relationship between the C1r molecules of neighboring dimers. This enzyme–product complex exhibits the crucial S1–P1 salt bridge between Asp631 and Arg446 residues, and intermolecular interaction between the CCP2 module and the SP domain. Based on these novel structural information we propose a new split-and-reassembly model for the autoactivation of the C1r. This model is consistent with experimental results that have not been explained adequately by previous models. It allows autoactivation of C1r without large-scale, directed movement of C1q arms. The model is concordant with the stability of the C1 complex during activation of the next complement components.

Keywords: Complement system; Mechanism of C1r autoactivation; C1r catalytic domain; Modular serine protease; Complement control protein

1. Introduction

Modular serine proteases consist of several distinct domains of various protein families of different size and fold (Lorand and Mann, 1993). The noncatalytic modules provide both high specificity and elasticity in the catalytic properties and are responsible for the regulation of the enzyme function regarding the localization, timing, or recognition of interacting partners. In such a context, the enzyme function can be interpreted only in the detailed knowledge of the structure and function of the individual regulatory domains even if the reaction catalyzed by the catalytic domain is well known. A representative example is the complement system, one of the proteolytic cascades found in the blood of vertebrates. It mediates several effector functions of the innate and adaptive immunity such as eliminating invading pathogens and altered host cells as well as triggering inflammatory reactions. The uncontrolled activation of the complement system, however, can result in self-tissue damage and/or pathologic inflammation. The main components of the complement system are modular serine proteases, which activate each other in a strictly ordered manner. There are three distinct pathways through which the complement system can be activated: the classical, the lectin and the alternative pathways. The first component of the classical pathway (C1) is a 790 kDa Ca^{2+}-dependent heteropentamer complex, which consists of a recognition subunit C1q and a tetramer of C1r and C1s serine proteases (Schumaker et al., 1987; Arlaud et al.,

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0161-5890/$ – see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.molimm.2007.09.031

2001). The C1q protein has an overall shape of a bunch of six tulips with six C-terminal heterotrimeric globular heads connected to six collagen-like stems that merge into a stalk at the N-terminus. According to the current models of the C1 complex, the C1s–C1r–C1r–C1s tetramer is wrapped around the collagen-like arms of the C1q. The C1r and C1s serine proteases are present in zymogenic state until the C1 complex meets an activator structure. The binding of the globular C1q heads to the activator surface results in the activation of the zymogen proteases. The first enzymatic event in the classical pathway is the autoactivation of C1r, which involves the cleavage of the Arg446–Ile447 bond in the protease domain. Active C1r then cleaves and activates zymogen C1s that in turn cleaves C4 and C2, the subsequent components of the cascade. Activation and activity of C1 is controlled by the serpin C1-inhibitor, which inhibits both serine proteases (Beinrohr et al., 2007). C1r and C1s, together with the MBL-associated serine proteases (MASPs) of the lectin pathway, form a family of serine proteases with identical domain organization (Gál et al., 1989; Gál et al., 2007). The N-terminal CUB1-EGF-CUB2 region is followed by a tandem repeat of complement control protein (CCP) modules and a chymotrypsin-like serine protease (SP) domain. The CUB1-EGF-CUB2 region mediates the Ca^{2+}-dependent C1r–C1s as well as the $C1q–C1r_2C1s_2$ association. The C-terminal CCP1–CCP2–SP catalytic region of C1r shows the enzymatic properties characteristic of the full-length molecule and forms a homodimer providing the core of the C1s–C1r–C1r–C1s tetramer. Previously it was shown on synthetic substrates that the isolated SP domain itself is an active serine protease with trypsin-like specificity. However, on protein substrates its activity is highly restricted to a unique dual function of self-activating ability and the capability of cleaving zymogen C1s (Kardos et al., 2001).

During the recent years the 3D structures of several fragments of C1r, C1s and C1q have been solved and low resolution models for the C1 complex have been proposed (Bersch et al., 1998; Budayova-Spano et al., 2002a; Budayova-Spano et al., 2002b; Gaboriaud et al., 2000, 2003; Gregory et al., 2003). Despite all these achievements, some basic questions remain unanswered. The structure of zymogen catalytic CCP1–CCP2–SP fragment of C1r shows a dimer, which is stabilized by intermolecular CCP1–SP interactions (Budayova-Spano et al., 2002a; Lacroix et al., 2001). In this structure, the active site of one monomer and the cleavage site of the other are separated by a distance of 92 Å which prevents the physical contact that is necessary for autoactivation. On the other hand, we have shown earlier that the presence of the CCP2 module stabilizes the structure of the catalytic domain and greatly enhances the rate of cleavage of protein substrates (*i.e.* autoactivation and C1s cleavage) in solution phase (Kardos et al., 2001). It indicates that the CCP2 module mediates important protein–protein interactions during proteolysis. Here we report the first functional dimeric structure of the active C1r CCP1–CCP2–SP fragment. In the crystal lattice an enzyme–product complex is formed. The complex shows the S1–P1 salt bridge (characteristic to trypsin-like enzymes) and the CCP2–SP intermolecular interactions. Based on this novel structural information and careful consideration of former experimental data we propose an improved model for the autoactivation of C1r in the C1 complex.

2. Materials and methods

2.1. Protein expression, renaturation and purification

CCP1–CCP2–SP fragment of human C1r (corresponding to amino acids 292–688) was cloned from cDNA containing four extra amino acids (Ser-Thr-Gln-Ala) at their N-terminus and expressed in BL21(DE3)pLysS *E. coli* strain. After purification, inclusion bodies were solubilized in GdnHCl. The protein was renatured in GdnHCl using a glutathione redox system and further purified by ion exchange and gel filtration chromatography. The protocol was presented in full details previously (Kardos et al., 2001).

2.2. Functional characterization of the renatured C1r CCP1–CCP2–SP fragment

Recombinant, human C1s CCP2–SP fragment, which has been expressed in the same expression system as the C1r fragments, renatured similarly (Kardos et al., 2001), and purified by ion exchange and gel filtration chromatography was incubated with the C1r catalytic fragment using 1:50 enzyme–substrate molar ratio in 50 mM Hepes (pH 8), 100 mM NaCl. The activation (*i.e.* cleavage) was analyzed on a 12.5% SDS-PAGE, under reducing conditions.

2.3. Crystallization and data collection

Crystals were grown by the hanging-drop method at 15 °C. Crystals were obtained by mixing 8 μl of reservoir solution and 8 μl of protein solution. The reservoir solution contained 14% (w/v) PEG 6000, 0.2 M NaCl, 10% (v/v) glycerol and 0.1 M Tris–HCl (pH 7.4). The protein solution contained 0.2 mg/ml of the active C1r CCP1–CCP2–SP fragment in 20 mM Tris–HCl, 130 mM NaCl at pH 7.4. The low solubility and relatively high propensity for crystallization or precipitation of the protein required the use of large drops and low protein concentration. To obtain cryoprotective conditions crystals were soaked in solutions with glycerol content raised to 20%. Crystals were tested using the ID14 EH1 beam line at the European Synchrotron Radiation Facility. Data sets were collected at ID29 beam line at the European Synchrotron Radiation Facility at a wavelength of 0.979 Å at 100 K. A data set with improved resolution and mosaicity was collected at the BL41-XU in SPring-8 with the approval of the Japan Synchrotron Radiation Research Institute (JASRI) using a wavelength of 1.000 Å at 100 K. This data set was used for structure determination. Data processing and data reduction were carried out with the MOSFLM (Leslie, 1992) and SCALA (Evans, 1993) programs of the CCP4 package (CCP4, 1994). More details of the data collection statistics are shown in Table 1.

Table 1
Data collection and refinement statistics of the crystal structure of the dimeric activated catalytic fragment of C1r

Crystal parameters	
Space group	$P2_12_12_1$
Cell constants	$a = 74.541$ Å, $b = 92.416$ Å, $c = 162.848$ Å
Data quality	
Resolution range (last resolution shell)	54.98–2.60 (2.67–2.60)
R_{meas} [a]	0.089 (0.489)
Completeness	99.7% (98.9%)
No. of observed/unique reflections	236034/35308 (15814/2533)
$I/\sigma(I)$	6.1 (1.5)
Refinement residuals	
R	0.2131
R_{free} [b]	0.2594
Model quality	
RMS bond lengths (Å)	0.015
RMS bond angles (°)	1.498
RMS general planes (Å)	0.006
Ramachandran plot: residues in core/allowed/disallowed regions	552/96/0
Model contents	
Protein residues	780
Residues in dual conformations	4
Side chains with disordered atoms	55
Glycerol molecules	7
Water molecules	160

[a] $R_{meas} = (\Sigma_h(n/(n-1))^{0.5}\Sigma_j|\langle I_h\rangle - I_{hj}|)/(\Sigma_{hj}I_{hj})$ with $\langle I_h\rangle = (\Sigma_j I_{hj})/n_j$.
[b] 5.0% of the reflections in a test set for monitoring the refinement process.

2.4. Crystal structure determination and refinement

The phase problem was solved by molecular replacement using BEAST (Read, 2001) program of the CCP4 package (CCP4, 1994). We used search models representing individual domains of the zymogen structure of the CCP1–CCP2–SP fragment of C1r (PDB accession code 1GPZ). The asymmetric unit contains a head-to-tail dimer of the C1r fragment. Manual model building was carried out using the Coot program (Emsley and Cowtan, 2004). Refinement was carried out with the REFMAC5 program (Murshudov et al., 1997) using restrained maximum-likelihood refinement and TLS refinement (Winn et al., 2001). During refinement non-crystallographic restraints were added to the regions of C1r molecules possessing similar conformation.

The final model contains 2 residues of the cloning tag and residues 292–686 of C1r monomers A and B, as well as 7 glycerol molecules and 160 water molecules. Stereochemistry of the structures was assessed with PROCHECK (Laskowski et al., 1993). Model statistics are shown in Table 1. The atomic coordinates and structure factors were deposited in the Protein Data Bank (Berman et al., 2000) with accession code 2QY0. Figs. 2 and 3D–F were created using PyMOL (DeLano, 2002). Superposition of structures was carried out using SwissPdb Viewer v3.7 (Guex and Peitsch, 1997). AREAIMOL program (Lee and Richards, 1971) of the CCP4 package was used for calculating total buried surface area and identifying corresponding residue segments. Intermolecular contacts were calculated using CONTACT program of the CCP4 package (CCP4, 1994). The hydrogen bonds were sorted out using distance limit of 3.5 Å and constraint on the donor-hydrogen bond orientation to the acceptor atom.

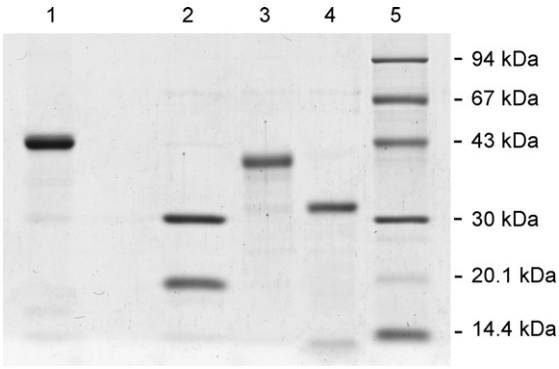

Fig. 1. SDS-PAGE analysis of the recombinant, renatured C1r catalytic domain. Autoactivation of C1r results in two chains linked by a disulfide bridge. (1) 10 μg renatured C1r CCP1–CCP2–SP fragment under non-reducing and (2) under reducing conditions. An empty lane was applied between the two samples to separate reducing and non-reducing conditions. To check the activity of C1r CCP1–CCP2–SP on its natural substrate, C1s, a proteolysis experiment was carried out on the recombinant CCP2–SP fragment of C1s. (3) 5 μg intact C1s CCP2–SP fragment, (4) C1s CCP2–SP fragment cleaved by C1r CCP1–CCP2–SP using 1:50 enzyme–substrate ratio (see Section 2). (5) molecular weight marker. The samples were analyzed on a 12.5% SDS-PAGE. The gel shows that the renatured C1r fragment has been fully activated (*i.e.* cleaved) during the renaturation process. It exerts its biological activity by cleaving the C1s molecule.

3. Results

3.1. Proteolytic activity of the recombinant C1r CCP1–CCP2–SP fragment

As it was described previously, the CCP1–CCP2–SP fragment of C1r was transformed to and expressed in

Table 2
CCP1/SP intermolecular contacts within the C1r core dimer

	Zymogen C1r[a]	Activated C1r
Total buried surface area (Å^2)	2202	2547
Number of hydrogen bonds[b]	11	15
Number of contacts (within 4.2 Å dist.)[c]	207	271
CCP1 segments with interacting residues	305–306	305–306
	313–320	313–320
	335–342	335–343
	348–349	348–349
SP segments with interacting residues	463, 465, 469	465
	499–503	497–503
	509–519	508–519
	548–549	548
	588	588

[a] PDB code 1GPZ.
[b] Donor–acceptor geometry was checked using Coot program (distance limit: 3.5 Å).
[c] Calculated using CONTACT program of the CCP4 package (CCP4, 1994).

BL21(DE3)pLysS *E. coli* strain (Kardos et al., 2001). The solubilized inclusion body protein was renatured in 2 M GdnHCl, 50 mM Tris, pH 8.3 in the presence of 1 mM oxidized and 3 mM reduced glutathione. The recombinant protein was further purified using Q-sepharose Fast Flow ion-exchange chromatography and Superose-12 gel filtration. Full autoactivation of the C1r catalytic fragment took place during the renaturation procedure resulting in two chains linked by a disulfide bridge as it is shown in Fig. 1, lane 1, 2. The esterolytic activity of the renatured C1r fragment was verified on Z-Gly-Arg-S-Bzl, a sensitive thioester substrate of C1r, and was found to be identical to the value published earlier (Kardos et al., 2001). Proteolytic activity of the recombinant C1r on its natural substrate, C1s, was checked through the cleavage of the recombinant CCP2-SP fragment of C1s. The reaction was followed by SDS-PAGE and showed that after 1 h incubation at an 1:50 enzyme–substrate molar ratio the renatured C1r catalytic fragment fully cleaved the substrate C1s fragment (Fig. 1, lane 3, 4).

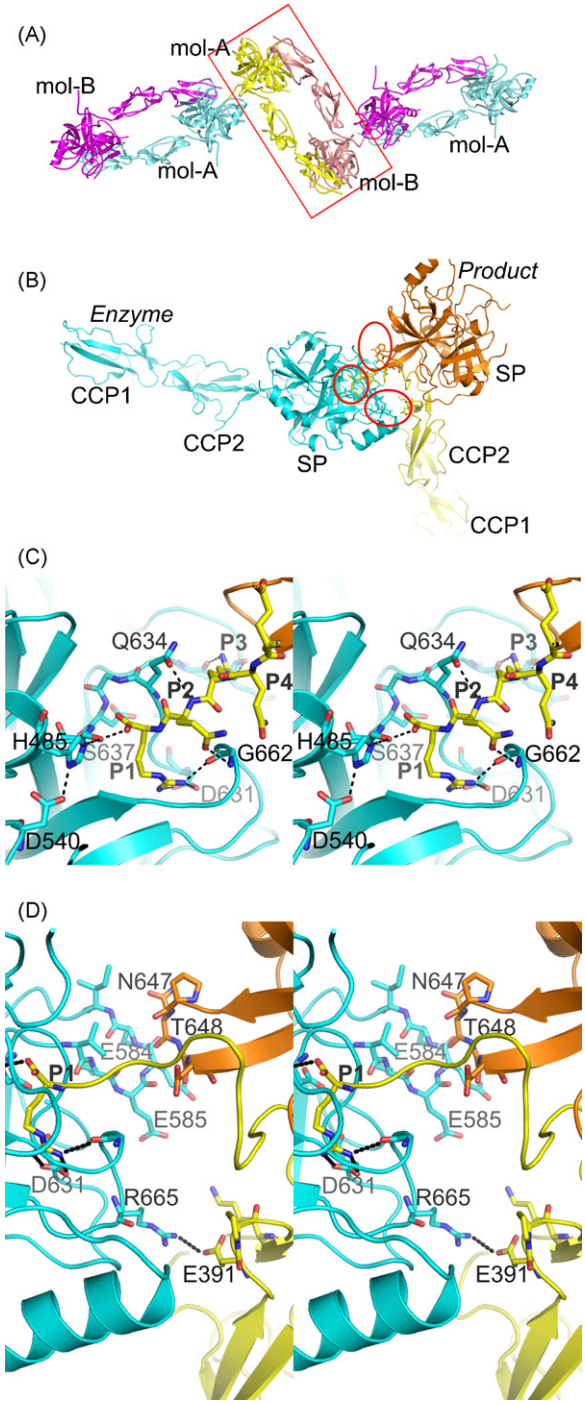

Fig. 2. Enzyme/product-like contacts in the crystal structure of the activated CCP1–CCP2–SP fragment of C1r. (A) The asymmetric unit contains one head-to-tail dimer of molecule A and molecule B (framed). Three dimers are shown.. Both molecules of the middle dimer are in the role of the "product" here (yellow and rose molecules), with neighbors acting as "enzymes" of these. All molecules are "enzymes" and "products" at the same time. It is possible to find neighbors for opposite roles (not shown). (B) Cartoon representation of the molecule in the role of the enzyme (cyan) and in the role of the product of the enzyme reaction (the N-terminal and C terminal segments of the cleaved chain are yellow and orange, respectively). Cleavage site is between residues Arg446 (P1) and Ile447 (P1′). The contact sites of the enzyme for the product activation peptide, SP domain and CCP2 module are shown with red ellipses, with stick representation of the contacting residues. (C) Blow up of the binding site of the activation peptide of the product (stereo view). Hydrogen bonds of the P1–P4 residues of the product and the enzyme are shown as black dashed lines. (D) Blow up of further molecular contacts of the enzyme and product (stereo view). These are: enzyme SP domain (cyan)/product SP domain (residues 646–650, orange), and enzyme SP domain/product CCP2 module (yellow, loop between residues 391 and 395). The S1–P1 hydrogen bonds are also shown as reference.

3.2. Overall structure of activated C1r CCP1–CCP2–SP dimer

The CCP1–CCP2–SP fragment was successfully crystallized as described in detail in Section 2. We determined the crystal structure of this C1r fragment to 2.6 Å resolution. The structure is well defined by electron density, except for some surface side chains, and short segments of a flexible loop of CCP2 module and loop B of the SP domain. The asymmetric unit contains a dimer of C1r with non-crystallographic twofold rotation symmetry. The dimer structure is formed by head-to-tail interaction of the monomers (*i.e.* SP domain of one monomer contacting the CCP1 module of the other, Fig. 2A), and it resembles to that of the zymogen form of the CCP1–CCP2–SP fragment (PDB code 1GPZ, Budayova-Spano et al., 2002a). However, here the dimer is somewhat twisted along its longest dimension. The conformation at the module barriers (*i.e.* CCP1–CCP2 and CCP2–SP junctions) is constrained by the head-to-tail contacts within the dimer, suggesting the rigidity of the C1r/C1r dimer core of $C1r_2s_2$ tetramer.

The comparison of the contacting residues to that of the zymogen structure reveals significantly higher number of contacts and hydrogen bonds as well as 15% greater buried surface area of the CCP1/SP dimer interface in the activated structure (Table 2). This means more favorable interactions of similar contact regions in the activated structure.

The distance of the active site of one molecule and the activation site of the other within the C1r dimer underlines the necessity of re-organization and flexibility of C1r molecules during autoactivation (Budayova-Spano et al., 2002a). Comparison of the conformation of the activated SP domain with that of the activated monomeric structure (PDB code 1MD8) reveals two

regions with different overall conformation that are important for assembly and rearrangement of C1r molecules. (i) Loop B (residues 490–498) is more ordered in our structure, possibly because of the stabilization of the subsequent residues, which form part of the CCP1/SP interface region in the dimer. (ii) Loop 2 (residues 662–666) is shifted and its conformation is modified because of different SP/SP intermolecular contacts (see next section).

3.3. Crystallographic contacts at the active site

The interactions of the symmetry equivalent enzyme molecules at the active site show several features characteristic to enzyme–product interactions in the chymotrypsin family. The S1 specificity site of one molecule is occupied with Arg446 of a molecule of a neighboring dimer (Fig. 2A and B). The S1–P1 salt bridge, crucial for the primary substrate binding of serine proteases with trypsin-like specificity, is formed with Asp631 (Fig. 2C). Moreover, the carboxylate group of the P1 arginine is hydrogen bonded to the OH group of the catalytic serine (Ser637) and its oxygen is within 4 Å distance from the NH groups of the oxyanion binding site (Fig. 2C). In contrast, in the monomeric activated structure (PDB code 1MD8, Budayova-Spano et al., 2002b) crystallographic contacts are formed between Asp631 of the S1 site and Arg444 (P3) of a symmetry equivalent molecule, which is physiologically not relevant.

The P2–P3 residues form several contacts with the residues of loops 1 and D (loop nomenclature by Perona and Craik, 1997) of the substrate binding site (Table 3, Fig. 2C). However, the backbone conformation and binding region of this segment differ from that observed in peptide complexes of enzymes of the chymotrypsin family. These contacts could correspond to a strung enzyme/product adduct before the release of the product, where the P1–P3 segment is extended and the remaining part of the SP domain of the molecule in the role of "product" forms lower number of contacts with the molecule in the role of "enzyme", namely with loop D.

3.4. Novel CCP2/SP intermolecular contacts

Inspection of the crystallographic contacts of the enzyme–product like pair of molecules described in the previous section revealed further contacts formed by the SP domain of the molecule in the role of the enzyme and the CCP2 module of the molecule in the role of product (Table 3, Fig. 2D). An intermolecular salt bridge is established by Arg665 and Glu391, both side chains stabilized by intramolecular hydrogen bond. A further water mediated hydrogen bond is formed by Ser627 and Glu391. Similar interactions of SP domain with both the CCP2 and SP modules of a symmetry equivalent molecule are not observed in other C1r structures. Because the protein was expressed in *E. coli*, it does not contain any attached glycans. We examined if the natural Asn497 and Asn564-bound glycans can interfere with the observed intermolecular interactions of the dimer and the enzyme–product complex. Superposing the glycosylated zymogen structure over our structure showed no overlaps with the carbohydrate residues.

4. Discussion

4.1. C1r catalytic domain forms a stable head-to-tail dimer

We determined the structure of the dimeric active catalytic region of C1r encompassing the two CCP modules and the C-terminal chymotrypsin-like serine-protease domain. This recombinant fragment is essentially identical to the γB fragment which was obtained through the limited proteolysis of plasma C1r (Arlaud et al., 1986). The CCP modules have both structural and functional roles, which are essential for the activation and activity of the C1r dimer in the C1 complex. Activated C1r, like the zymogen, forms a dimer in a head-to-tail fashion where the CCP1 module of one molecule interacts with the SP domain of the other molecule and *vice versa*. This conformation, however, does not allow intradimer autoactivation since it prevents the physical contact between the two SP domains.

Table 3
Enzyme–product-like molecular contacts

	Molecules A/A	Molecules B/B
Total buried surface area (Å^2)	1140	1200
Number of hydrogen bonds[a]	10	12
SP–SP interactions		
"Product" activation loop	443–446 (P4–P1 residues)	436, 438, 443–446 (P4–P1 residues)
"Product" SP domain	644, 646–648, 650	647–650
"Enzyme" SP domain	448, 485	448, 485
	LoopA (469)	
	LoopD (581–586)	LoopD (581–586)
	Loop3 (612)	Loop3 (610, 612)
	Loop1 (631–635, 637)	Loop1 (631–635, 637)
	Loop2 (657–663)	Loop2 (657–663)
	667–670	667–670
CCP2–SP interactions		
"Product" CCP2 module	391, 395	391, 392, 395
"Enzyme" SP domain	Loop2 (665)	Loop2 (665)

[a] Donor–acceptor geometry was checked using Coot program (distance limit: 3.5 Å).

This arrangement, however, is ideal for the cleavage of C1s since the active sites of the C1r SP domains facing outwards are easily accessible for the cleavable C1s SP domains. It is reasonable to presume that for autoactivation a weaker CCP1/SP interaction is more advantageous because the head-to-tail dimer should be disrupted during autoactivation in order to make possible the contact between the zymogen SP domains. Our results are fully consistent with this picture. After autoactivation the head-to-tail dimer arrangement is restored with tighter CCP1/SP interaction. Comparing the structure of the active C1r dimer with that of the zymogen (PDB code 1GPZ) we can see a larger CCP1/SP contact surface with more noncovalent interactions in the case of the active species (Table 2). Moreover, loop B in the active SP domain is more ordered which also indicates a tighter intermolecular interaction. It is very probable therefore that the active C1r dimer is more stable than the zymogen one and it is fixed during the subsequent steps of the cascade, *i.e.* C1s activation and the cleavage of C4 and C2 by activated C1s. The C1r–C1r core provides a stable frame for activated C1s, which has to be exposed to the solution to carry out its function.

4.2. Enzyme–product complex in the crystal

The recombinant CCP1–CCP2–SP fragment – as well as the natural γB fragment – autoactivates rapidly in solution phase. The mechanism of the solution phase autoactivation should be somewhat different from that of the autoactivation of C1r inside the C1 complex. It seems obvious that in solution phase an interdimer mechanism is dominant. Our C1r structure is fully consistent with the interdimer mechanism of autoactivation. In the crystal, there is a symmetric enzyme/product relationship between the C1r dimers (Fig. 2A). The crucial S1–P1 salt bridge between Asp631 and Arg446 is detectable in the crystal structure. Our structure is the first example for an autoactivating enzyme/product complex of an immunologically important serine protease. An intriguing novel feature of this enzyme/product complex is the presence of the intermolecular CCP2/SP interaction. It was shown earlier that the presence of the CCP2 module greatly enhances the rate of the autoactivation and the C1s cleavage, while the catalytic efficiency towards small synthetic substrates remains unchanged (Kardos et al., 2001). It is very probable that the CCP modules mediate protein–protein interactions in the enzyme/substrate complex. The C4-cleaving ability of the structurally related C1s and MASP-2 proteases is also largely dependent on the presence of the CCP modules (Ambrus et al., 2003; Gál et al., 2005; Rossi et al., 1998; Rossi et al., 2005). The CCP2 domain has at least two functions in the catalytic mechanism of C1r: (i) it has exosite on its surface through which it can strengthen the stability of the enzyme/substrate complex, and (ii) due to the flexible CCP2/SP intramolecular interface it can contribute to the correct positioning of the scissile bond to the active site of the enzyme during proteolysis. We believe that the CCP2–SP interactions, both the inter- and intramolecular ones, play important roles in the autoactivation mechanism of C1r, not only in the solution phase but also inside the C1 complex. Based on our structural data we propose a new molecular model for C1 activation which takes into account the intermolecular CCP2/SP interactions and resolves some contradictions of the former model.

4.3. An improved model for the C1r autoactivation in the C1 complex

In the latest C1r autoactivation model (Fig. 3A), Budayova-Spano et al. hypothesized that the head-to-tail assembly of the dimeric zymogen C1r molecules (Fig. 3C) is disrupted by mechanical stress upon multivalent binding of the C1q heads to their targets (Budayova-Spano et al., 2002a; Budayova-Spano et al., 2002b; Arlaud et al., 2007). In the C1r dimer the distance between the activation site of one molecule and the active site of the other is 92 Å. The model presents a hypothetical linear activation complex which is achieved through a mechanical pulling apart of the monomers in well-defined direction. This model leaves several questions unanswered: (i) our present active dimeric structure and a number of other observations (Arlaud et al., 1980; Arlaud et al., 1986; Kardos et al., 2001) indicate that the active form of C1r is a dimer, having similar molecular arrangement to the zymogen C1r as it was suggested based on a 4 Å resolution electron density map (Budayova-Spano et al., 2002a). C1r/C1s complexes with monovalent C1q binding site such as C1s/C1rα (Busby and Ingham, 1990) or C1-inh/C1r/C1s/C1-inh (Wong et al., 1999) fail to bind to C1q. It is plausible to presume that prolonged dissociation of the C1r dimer would lead to the disassembly of the C1 complex. If the binding of the C1q heads to their targets causes a strong mechanical stress that disrupts the zymogen C1r dimer how can the C1r molecules re-associate while the mechanical pull is constantly maintained due to the fixed C1q heads? (ii) Budayova-Spano's model supposes that the mechanical stress acts in a proper direction, although the C1q heads bind to an irregular pattern of binding sites resulting in an indefinite change of the distances between them, even not excluding the possibility of shortening. (iii) This "linear" activation complex model requires 92 Å relative movement of the catalytic domain of C1r in the long axis of the molecules. It is not clear whether sufficient space could be created for such a large movement between C1q arms upon binding of the C1q heads at the surface of the invading pathogen. Gaboriaud et al. (2004) proposed a model of C1 complex with C1r binding sites in the middle of the C1q arms. Because the length of the half of a C1q arm is approximately 70 Å (Perkins, 1989) and the dimension of the dimeric catalytic domain in itself is about 120 Å, a 92 Å movement, if ever, could be achieved only by fully flattened C1q arms. (iv) The previous models do not take into consideration the role of the CCP2 module as secondary binding site in the autoactivation process, which is suggested by previous results and our present X-ray structure.

Here, we propose a new model for C1r activation (Fig. 3B) based on the first atomic resolution structure of the functional, activated, dimeric C1r catalytic domain (Fig. 3D and E) taking into account and harmonizing with most of the previous observations and facts. Previously it was reported that there is an exchange of subunits between C1r dimers suggesting that there

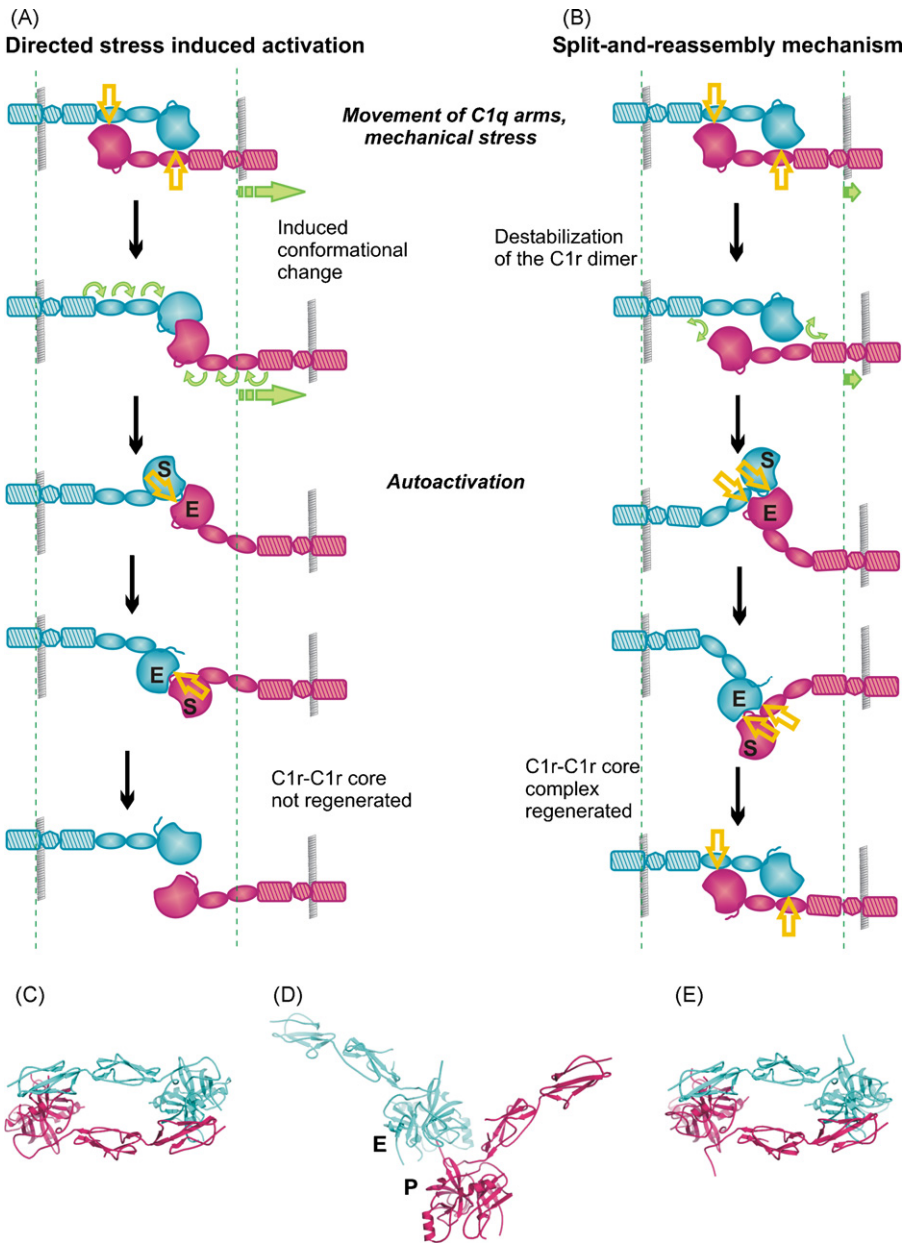

Fig. 3. Model of C1r autoactivation within the C1 complex. (A) and (B) show alternative mechanisms. C1q arms (grey) and the two C1r molecules (cyan and magenta) of the core complex of C1 are depicted schematically. Remaining parts of C1q and C1s molecules are not shown. C1r–C1r intermolecular contacts are marked with orange arrows. Green dashed reference lines show the original positions of the C1q arms. Green arrows refer to the initial displacement of C1q arms upon target binding and consequent conformational changes of C1r. Enzyme, substrate and product of the autoactivation reaction are labeled as E, S, and P, respectively. (A) Former proposed mechanism (Budayova-Spano et al., 2002a,b) includes directed displacement of C1q arms causing induced conformational changes in C1r monomers by pulling them apart. The resulting enzyme/substrate complex contains intermolecular contacts of SP/SP, but not of CCP2/SP. The C1r dimer core cannot be formed following autoactivation. (B) A relatively small displacement of C1q arms (grey) upon target binding can cause destabilization and dissociation of the C1r/C1r core complex. The C1r monomers can rearrange as enzyme/substrate complex, and autoactivation takes place. The C1r/C1r core can be easily re-formed by the activated C1r. In panels (C), (D), and (E) cartoon representation of the CCP1–CCP2–SP fragment of zymogen dimer core (PDB code: 1GPZ), activated enzyme/product complex and activated dimer core of the new structure are shown, respectively. These crystal structures, together with enzyme kinetic studies confirm the split-and-reassembly mechanism (panel A).

is a kinetic equilibrium between the associated and dissociated forms although strongly shifted towards the dimer (Dobó et al., 1999). Moreover, it is well known that even without binding to its target, C1 complex activates spontaneously (Ziccardi, 1982) indicating that the dissociation of the zymogen dimer occurs from time to time (One of the role of C1-inhibitor is to neutralize this spontaneous activation.). The binding of C1q heads to their targets results in a change in the relative position of the C1q arms. We propose that this movement destabilizes the zymogen C1r dimer through the C1q binding CUB-EGF-CUB region and the equilibrium between the dimer and the dissociated monomers shifts towards the latter one. After dissociation, the SP modules can find the proper spatial arrangement for autoactivation by accidental rambling, which might be possible because

of the probable flexibility provided by the five N-terminal C1r modules and/or their junctions (This flexibility is essential for both the previous activation model of Budayova-Spano and our present model.). The local concentration of the SP domain inside the cone of C1q is extremely high compared to the concentration applied in experiments where the isolated C1r molecules have already activated each other with considerable efficiency in solution. This large local concentration might facilitate the formation of the enzyme/substrate complex. Moreover, the CCP2/SP secondary enzyme/substrate binding site, similar to that observed in the present structure and revealed by other experiments (Kardos et al., 2001), can help the proper orientation of the SP domains and the temporary stabilization of the activation complex. The approximately 90° angle of the long axes of the molecules observed in the CCP1–CCP2–SP enzyme–product complex (Fig. 3D) can be attained without the need for the 92 Å linear movement of the C1r molecules in well-defined direction as proposed by the previous model (see the schematic representations of the two activation models for comparison in Fig. 3A and B). According to our view, the role of the C1q arms is limited to the disruption of the CCP1/SP intermolecular interactions inducing the dissociation of the head-to-tail dimer and facilitating the formation of the activation complex. After cleavage of the activation sites in the SP domains, the dimeric form with fully active C1r molecules can be easily restored. The equilibrium will be likely shifted toward the functional active dimer form due to the tighter CCP1/SP intermolecular interactions between the monomers.

C1 complex bears a finely tuned function in activating the complement cascade of the immune system. The first step of this process is the conversion of a mechanical signal that is generated by binding of the C1q heads to their target molecules, to enzymatic reaction. This key event is mediated by the autoactivation of C1r. Here, we proposed a model based on the structure of the active, dimeric C1r catalytic domain shedding light on the details of the activation mechanism. Our model presumes significant flexibility in the chain of the CUB2–CCP1–CCP2–SP modules of C1r, similarly to the previous models. It is not clear, however, which modules or intermodule-junctions this flexibility is located on. Future studies could settle this question.

Acknowledgements

The authors acknowledge the Japan Synchrotron Radiation Research Institute (JASRI) and the European Synchrotron Radiation Facility for provision of synchrotron radiation facilities and we would like to thank Masahide Kawamoto for assistance in using the beamline BL41XU at SPring-8 and Hassan Belrahli and Gordon Leonard in using beamlines ID 14 EH1 and ID 29 at ESRF. This research was supported by grants from the Hungarian Scientific Research Foundation (OTKA F67937, K68464, T046444, TS049812, NI61915 and T046412), Medichem 2 and ICGEB Hun04-03 and ETT Grant 555/2006 by the Hungarian Ministry of Health. J.K. is supported by Bolyai János fellowship of the Hungarian Academy of Sciences and Öveges József fellowship of NKTH.

References

Ambrus, G., Gál, P., Kojima, M., Szilágyi, K., Balczer, J., Antal, J., Gráf, L., Laich, A., Moffatt, B., Schwaeble, W., Sim, R.B., Závodszky, P., 2003. Natural substrates and inhibitors of mannan-binding lectin-associated serine protease-1 and -2: a study on recombinant catalytic fragments. J. Immunol. 170, 1374–1382.

Arlaud, G.J., Barlow, P.N., Gaboriaud, C., Gros, P., Narayana, S.V.L., 2007. Deciphering complement mechanisms: the contributions of structural biology. Mol. Immunol. 44, 3809–3822.

Arlaud, G.J., Chesne, S., Villiers, C.L., Colomb, M.G., 1980. A study on the structure and interactions of the C1 subcomponents C1r and C1s in the fluid phase. Biochem. Biophys. Acta 616, 105–115.

Arlaud, G.J., Gaboriaud, C., Thielens, N.M., Rossi, V., Bersch, B., Hernandez, J.-F., Fontecilla-Camps, J.C., 2001. Structural biology of C1: dissection of a complex molecular mashinery. Immunol. Rev. 180, 136–145.

Arlaud, G.J., Gagnon, J., Villiers, C.L., Colomb, M.G., 1986. Molecular characterization of the catalytic domains of human complement serine protease C1r. Biochemistry 25, 5177–5182.

Beinrohr, L., Harmat, V., Dobó, J., Lőrincz, Zs., Gál, P., Závodszky, P., 2007. C1 inhibitor serpin domain structure reveals the likely mechanism of heparin potentiation and conformational disease. J. Biol. Chem. 282, 21100–21109.

Berman, H.M., Westbrook, J., Feng, Z., Gilliland, G., Bhat, T.N., Weissig, H., Shindyalov, I.N., Bourne, P.E., 2000. The Protein Data Bank. Nucleic Acids Res. 28, 235–242.

Bersch, B., Hernandez, J.-F., Marion, D., Arlaud, G.J., 1998. Solution structure of the epidermal growth factor-like module of human complement protease C1r, an atypical member of the EGF family. Biochemistry 37, 1204–1214.

Budayova-Spano, M., Grabarse, W., Thielens, N.M., Hillen, H., Lacroix, M., Schmidt, M., Fontecilla-Camps, J.C., Arlaud, G.J., Gaboriaud, C., 2002b. Monomeric structures of the zymogen and active catalytic domain of complement protease C1r: further insights into the C1 activation mechanism. Structure 10, 1509–1519.

Budayova-Spano, M., Lacroix, M., Thielens, N.M., Arlaud, G.J., Fontecilla-Camps, J.C., Gaboriaud, C., 2002a. The crystal structure of the zymogen catalytic domain of complement protease C1r reveals that a disruptive mechanical stress is required to trigger activation of the C1 complex. EMBO J. 21, 231–239.

Busby, T.F., Ingham, K.C., 1990. NH_2-terminal calcium-binding domain of human complement C1s mediates the interaction of C1r with C1q. Biochemistry 29, 4613–4618.

Collaborative Computational Project, Number 4, 1994. The CCP4 suite: programs for protein crystallography. Acta Crystallogr. D50, 760–763.

DeLano, W.L., 2002. The PyMOL Molecular Graphics System. DeLano Scientific, San Carlos, CA, USA.

Dobó, J., Gál, P., Szilágyi, K., Cseh, S., Lőrincz, Zs., Schumaker, V.N., Závodszky, P., 1999. One active C1r subunit is sufficient for the activity of the complement c1 complex: stabilization of C1r in the zymogen form by point mutations. J. Immunol. 162, 1108–1112.

Emsley, P., Cowtan, K., 2004. Coot: model-building tools for molecular graphics. Acta Crystallogr. D60, 2126–2132.

Evans, P.R., 1993. Data reduction. In: Proceedings of the CCP4 Study Weekend on Data Collection and Processing. CLRC Daresbury Laboratory, UK, pp. 114–122.

Gaboriaud, C., Juanhuix, J., Gruez, A., Lacroix, M., Darnault, C., Pignol, D., Verger, D., Fontecilla-Camps, J.C., Arlaud, G.J., 2003. The crystal structure of the globular head of complement protein C1q provides a basis for its versatile recognition properties. J. Biol. Chem. 278, 46974–46982.

Gaboriaud, C., Rossi, V., Bally, I., Arlaud, G.J., Fontecilla-Camps, J.C., 2000. Crystal structure of the catalytic domain of human complement C1s: a serine protease with a handle. EMBO J. 19, 1755–1765.

Gaboriaud, C., Thielens, N.M., Gregory, L.A., Rossi, V., Fontecilla-Camps, J.C., Arlaud, G.J., 2004. Structure and activation of the C1 complex of complement: unraveling the puzzle. Trends Immunol. 25, 368–373.

Gál, P., Barna, L., Kocsis, A., Závodszky, P., 2007. Serine proteases of the classical and lectin pathways: similarities and differences. Immunobiology 212, 267–277.

Gál, P., Harmat, V., Kocsis, A., Bián, T., Barna, L., Ambrus, G., Végh, B., Balczer, J., Sim, R.B., Náray-Szabó, G., Závodszky, P., 2005. A true autoactivating enzyme. Structural insights into mannose-binding lectin-associated serine protease-2 activation. J. Biol. Chem. 280, 33435–33444.

Gál, P., Sárvári, M., Szilágyi, K., Závodszky, P., Schumaker, V.N., 1989. Expression of hemolytically active human complement component C1r in insect cells using a baculovirus vector. Complement Inflamm. 6, 433–441.

Gregory, L.A., Thielens, N.M., Arlaud, G.J., Fontecilla-Camps, J.C., Gaboriaud, C., 2003. X-ray structure of the Ca^{2+}-binding interaction domain of C1s. Insights into the assembly of the C1 complex of complement. J. Biol. Chem. 278, 32157–32164.

Guex, N., Peitsch, M.C., 1997. SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. Electrophoresis 18, 2714–2723.

Kardos, J., Gál, P., Szilágyi, L., Thielens, N.M., Szilágyi, K., Lőrincz, Zs., Kulcsár, P., Gráf, L., Arlaud, G.J., Závodszky, P., 2001. The role of the individual domains in the structure and function of the catalytic region of a modular serine protease C1r. J. Immunol. 167, 5202–5208.

Lacroix, M., Ebel, C., Kardos, J., Dobó, J., Gál, P., Závodszky, P., Arlaud, G.J., Thielens, N.M., 2001. Assembly and enzymatic properties of the catalytic domain of human complement protease C1r. J. Biol. Chem. 276, 36233–36240.

Laskowski, R.A., MacArthur, M.W., Moss, D.S., Thornton, J.M., 1993. PROCHECK: a program to check the stereochemical quality of protein structures. J. Appl. Crystallogr. 26, 283–291.

Lee, B., Richards, F.M., 1971. The interpretation of protein structures: estimation of static accessibility. J. Mol. Biol. 55, 379–400.

Leslie, A.G.W., 1992. Recent changes to the MOSFLM package for processing film and image plate data. Joint CCP4 + ESF-EAMCB Newsletter on Protein Crystallography, No. 26.

Lorand, L., Mann, K.G., 1993. Proteolytic enzymes in coagulation, fibrinolysis, and complement activation. Methods Enzymol., v222. Academic Press, San Diego.

Murshudov, G.N., Vagin, A.A., Dodson, E.J., 1997. Refinement of macromolecular structures by the maximum-likelihood method. Acta Crystallogr. D53, 240–255.

Perkins, S.J., 1989. Models for the C1 complex determined by physical techniques. Behring Inst. Mitt. 84, 129–141.

Perona, J.J., Craik, C.S., 1997. Evolutionary divergence of substrate specificity within the chymotrypsin-like serine protease fold. J. Biol. Chem. 272, 29987–29990.

Read, R.J., 2001. Pushing the boundaries of molecular replacement with maximum likelihood. Acta Cryst. D57, 1373–1382.

Rossi, V., Bally, I., Thielens, N.M., Esser, A.F., Arlaud, G.J., 1998. Baculovirus mediated expression of truncated modular fragments from the catalytic region of human complement serine protease C1s. Evidence for the involvment of both complement control protein modules in the recognition of the C4 protein substrate. J. Biol. Chem. 273, 1232–1239.

Rossi, V., Teillet, F., Thielen, N.M., Bally, I., Arlaud, G.J., 2005. Functional characterization of complement proteases C1s/mannan-binding lectin-associated serine protease-2 (MASP-2) chimeras reveals the higher C4 recognition efficacy of the MASP-2 complement control protein modules. J. Biol. Chem. 280, 41811–41818.

Schumaker, V.N., Závodszky, P., Poon, P.H., 1987. Activation of the first component of complement. Annu. Rev. Immunol. 5, 21–42.

Winn, M., Isupov, M., Murshudov, G.N., 2001. Use of TLS parameters to model anisotropic displacements in macromolecular refinement. Acta Crystallogr. D57, 122–133.

Wong, N.K.H., Kojima, M., Dobó, J., Ambrus, G., Sim, R.B., 1999. Activities of the MBL-associated serine proteases (MASPs) and their regulation by natural inhibitors. Mol. Immunol. 36, 853–861.

Ziccardi, R.J., 1982. Spontaneous activation of the first component of human complement (C1) by an intramolecular autocatalytic mechanism. J. Immunol. 128, 2500–2504.