THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 285, NO. 16, pp. 11863–11869, April 16, 2010


# Calcium-dependent Conformational Flexibility of a CUB Domain Controls Activation of the Complement Serine Protease C1r*

Received for publication, December 23, 2009, and in revised form, February 19, 2010 Published, JBC Papers in Press, February 23, 2010, DOI 10.1074/jbc.M109.098541

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**C1, the first component of the complement system, is a $Ca^{2+}$-dependent heteropentamer complex of C1q and two modular serine proteases, C1r and C1s. Current functional models assume significant flexibility of the subcomponents. Noncatalytic modules in C1r have been proposed to provide the flexibility required for function. Using a recombinant CUB2-CCP1 domain pair and the individual CCP1 module, we showed that binding of $Ca^{2+}$ induces the folding of the CUB2 domain and stabilizes its structure. In the presence of $Ca^{2+}$, CUB2 shows a compact, folded structure, whereas in the absence of $Ca^{2+}$, it has a flexible, disordered conformation. CCP1 module is $Ca^{2+}$-insensitive. Isothermal titration calorimetry revealed that CUB2 binds a single $Ca^{2+}$ with a relatively high $K_D$ (430 $\mu$M). In blood, the CUB2 domain of C1r is only partially (74%) saturated by $Ca^{2+}$, therefore the disordered, $Ca^{2+}$-free form could provide the flexibility required for C1 activation. In accordance with this assumption, the effect of $Ca^{2+}$ on the autoactivation of native, isolated C1r zymogen was proved. In the case of infection-inflammation when the local $Ca^{2+}$ concentration decreases, this property of CUB2 domain could serve as subtle means to trigger the activation of the classical pathway of complement. The CUB2 domain of C1r is a novel example for globular protein domains with marginal stability, high conformational flexibility, and proteolytic sensitivity. The physical nature of the behavior of this domain is similar to that of intrinsically unstructured proteins, providing a further example of functionally relevant ligand-induced reorganization of a polypeptide chain.**

Conformational flexibility is essential for the function of proteins, or function is even frequently accompanied by large conformational changes carried out by flexible parts of the protein. Such flexible parts can be intrinsically unfolded polypeptide chains without a well defined three-dimensional structure. These disordered elements adopt a folded structure only upon binding to a suitable target molecule that can be another protein, co-enzyme, or sometimes simply a metal ion (1). $Ca^{2+}$ ions often regulate protein function by $Ca^{2+}$-induced conformational changes as in the case of calmodulin (2). It is possible, although rarely reported, that $Ca^{2+}$ induces a conformational switch between the unfolded and folded states (3, 4) and therefore fundamentally influences conformational flexibility.

The complement system, an important component of the innate immune system, is a sophisticated network of enzyme complexes consisting of multidomain protein components. Flexibility is essential for the action and control of these complexes, and $Ca^{2+}$ may play an important role in determining the structure and regulating the stability and flexibility of the individual components and the entire complexes, as well. The first component of complement (C1)[4] is a $Ca^{2+}$-dependent heteropentamer enzyme complex that is responsible for the initiation of the classical pathway of complement activation. It consists of a recognition molecule (C1q) and a tetramer of two distinct serine proteases, C1r and C1s. C1q, like the collectins, has six C-terminal globular recognition domains (heads) attached to collagen-like arms that merge together near the N terminus, forming a bouquet-like structure. According to the prevalent models of the C1 complex, the linear C1s-C1r-C1r-C1s tetramer is wrapped around the collagenous arms of C1q (5). C1r and C1s have an identical domain organization (for review, see Ref. 6). At the N-terminal region there are two CUB domains that encircle an EGF-like module. The CUB1-EGF-CUB2 part is followed by two CCP modules and the catalytic serine protease domain at the C terminus. The function of the noncatalytic modules is to mediate important protein-protein interactions that are essential for the assembly of the $C1r_2C1s_2$ tetramer and for the binding of the tetramer to the arms of C1q (7). The CCP modules, which are closely associated with the catalytic SP domain, can modulate the proteolytic function by binding and positioning the protein substrates. Recombinant expression of human C1r and its fragments (8, 9) opened up the way for identification of the role of the individual modules. The CCP1 module was shown to be responsible for the dimer formation of C1r (10, 11). The serine proteases are present in zymogenic forms and become activated upon binding of the C1q heads to activator structure. The first enzymatic event in the classical pathway

* This work was supported by Hungarian Scientific Research Fund Grants 68464, 81950, and NK77978, Ányos Jedlik Grant NKFP_07_1-MASPOK07, and National Development Agency Grant KMOP-1.1.2–07/1-2008-0003.
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[4] The abbreviations used are: C1, first component of complement; EGF, epidermal growth factor; DSC, differential scanning calorimetry; ITC, isothermal titration calorimetry; MS, mass spectrometry; IUP, intrinsically unstructured protein; CUB, domain found in complement component C1r/C1s, Uegf, and bone morphogenic protein 1; CCP, complement control protein; MASP, mannose-binding lectin-associated serine protease.

is the autoactivation of the C1r, which results in the cleavage of an Arg-Ile bond in the catalytic domain. Activated C1r cleaves and activates zymogen C1s in the C1 complex. Activated C1s then cleaves C4 and C2, the next components of the cascade, on the exterior of the C1 complex in the solution phase. The spontaneous activation of C1 is prevented by C1 inhibitor (12). During recent years, the rapid progress of the structural biology of the complement proteins made it possible to build and refine structural models for the C1 complex and propose the mechanism of activation (13). Very recently, new C1 models have been proposed based mainly on surface plasmon resonance experiments using different fragments and mutants (14, 15). These models place the tetramer inside the cone formed by the six C1q arms, and each of the six arms is engaged in a binding interaction with the tetramer. A common feature of these and the older models is that they suppose significant conformational changes during the activation process. Both the autoactivation of C1r and the activation of C1s require large scale movements of the different domains of the proteases. In the zymogenic C1r dimer, the active site of one C1r molecule and the cleavable Arg-Ile bond of the other C1r molecule are separated by a distance of 92 Å (16). To span such a large distance, by any mechanism, a high degree of conformational flexibility is required.

The CUB2-CCP1 fragment is the least characterized region of C1r. Limited proteolysis of C1r (and C1s) results in the digestion of the CUB2 module, suggesting that it has a relatively loose structure (17). To gain further insight into the autoactivation of C1r, we decided to characterize the functional properties of the CUB2-CCP1 region. We found that the CUB2-CCP1 fragment can bind a $Ca^{2+}$ ion with a relatively large dissociation constant. We also showed that without calcium the CUB2 domain loses its ordered structure and becomes a flexible unstructured region of the C1r molecule. Based on these results, we propose that the CUB2 domain and its interfaces could be the most flexible region of C1r and are essential for the biological function of this complement serine protease.

## EXPERIMENTAL PROCEDURES

*Recombinant Protein Expression, Refolding, and Purification*—The cDNA encoding the human C1r CUB2-CCP1 was amplified by PCR. We used the CGCGCTAGCATGACTCAGGCTGAGTGCAGCAGC and CGCGAATTCTCAGTCCTTGATCTTGCATCTGGG sequences as forward and reverse primers, respectively. The resulting DNA fragment was digested with NheI and EcoRI restriction endonucleases and ligated into the vector pET-17b (Novagen). The recombinant plasmid was transformed into the *Escherichia coli* BL21 (DE3) pLysS (Novagen) host strain. Protein expression was induced by adding 0.4 mM isopropyl β-D-thiogalactopyranoside. The inclusion bodies were collected and purified as previously described (18). Approximately 0.2 g of the inclusion body pellet was dissolved in 5 ml of 6 M guanidine hydrochloride, 100 mM dithiothreitol, 50 mM Tris-HCl, pH 8.3, to reach a final protein concentration of 15–20 mg/ml. 4 ml of the solution was rapidly diluted by 500 ml of 750 mM arginine, 500 mM guanidine HCl, 5 mM $CaCl_2$, 1 mM GSSG, 3 mM GSH, pH 8.5, renaturation buffer at 15 °C. The solution was stirred for 4–5 h at 15 °C and then incubated for 2 days at 10 °C. The sample was dialyzed twice against 20 mM Tris-HCl, 5 mM NaCl, 5 mM $CaCl_2$, pH 8, at 10 °C and filtered through a 0.22-μm membrane filter (Sartorius, Gottingen, Germany). After refolding, the protein was purified by Q-Sepharose ion exchange chromatography and gel filtration on a Sephacryl S100 column. Recombinant C1r CUB2-CCP1 fragment was identified by immunoblotting.

*Limited Proteolysis, C1r CCP1 Preparation*—C1r CCP1 was prepared from C1r CUB2-CCP1 (320 μM) by proteolytic digestion with thermolysin (8 μM) using a 1:40 enzyme:substrate molar ratio at 37 °C, 1 h. The proteolysis was stopped by the addition of 10 mM EDTA and dialyzed against 50 mM sodium acetate, 10 mM NaCl, 5 mM EDTA, pH 4.0. C1r CCP1 was purified by SP-Sepharose HP cation exchange (GE Healthcare) in the same buffer. C1r CCP1 fragment was verified by mass spectrometry.

*Measurement of C1r Autoactivation by Immunoblotting*—C1 complex and zymogen C1r were isolated from plasma following the protocol of Arlaud and Thielens (19). Zymogen C1r (60 μg/ml) was incubated at 37 °C in 20 mM Tris, 140 mM NaCl, pH 7.4, in the presence of either 100 μM EDTA or 250, 500, 1000, or 2000 μM $Ca^{2+}$. Samples were run on 12.5% SDS-PAGE and blotted to nitrocellulose membrane using a Bio-Rad Trans-Blot system. Anti-human C1r antibody from goat (Bio-Rad 80297) and anti-goat IgG alkaline phosphatase conjugate from rabbit (Sigma A4187) were used as first and second antibodies, respectively. The blots were evaluated by densitometry using Bio-Rad Gel Doc XR system. The autoactivation was followed by the disappearance of the zymogen C1r band. Band intensities were normalized to the zero time values being 100%. Initial autoactivation rates were determined by exponential fitting to the kinetic curve (see Fig. 6*B*) assuming a simple first order decay. Rate of autoactivation was plotted as a function of $Ca^{2+}$ concentration. The $K_D$ of $Ca^{2+}$ binding was determined by fitting with the following equation:

$$y = y(\text{min}) + (y(\text{max}) - y(\text{min}))/(1 + [Ca^{2+}]/K_D), \quad \text{(Eq. 1)}$$

where $y$ is the rate of autoactivation, $y$(max) is the maximal rate of autoactivation that was observed in the absence of $Ca^{2+}$, and $y$(min) is the rate of autoactivation in full $Ca^{2+}$ saturation. The fit will give the $K_D$ and $y$(min) values.

*CD Spectroscopy*—Secondary structures of C1r CUB2-CCP1 and CCP1 were characterized by CD spectroscopy on a Jasco J-720 instrument (Japan Spectroscopic Co., Tokyo, Japan) in 1-mm quartz cells. The protein concentration was 0.1–0.4 mg/ml. Spectra were recorded in the 190–250-nm range at 10 nm/min scanning speed with 1-nm bandwidth and 8- or 16-s response time. Three scans were accumulated.

*Differential Scanning Calorimetry*—DSC measurements were performed on a MicroCal VP-DSC instrument (MicroCal) in the 5–90 °C temperature range with a heating rate of 1 °C/min. The protein concentration was adjusted to 0.1–0.4 mg/ml. Data were evaluated using the Origin 5.0 software supplied with the instrument.

*Isothermal Titration Calorimetry*—$Ca^{2+}$-binding affinity of C1r CUB2-CCP1 was measured by ITC at 30 °C on a MicroCal

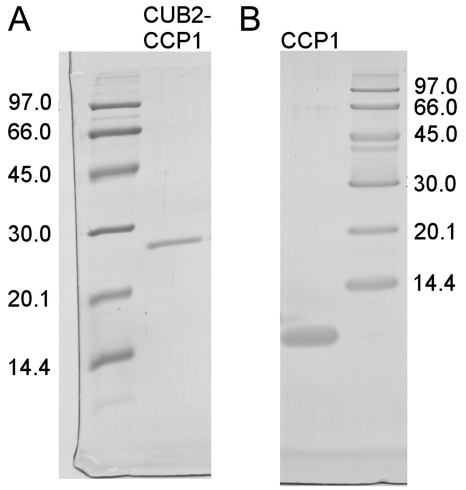


FIGURE 1. **SDS-PAGE of recombinant C1r fragments.** *A*, CUB2-CCP1. *B*, CCP1 produced from CUB2-CCP1 by limited proteolysis.

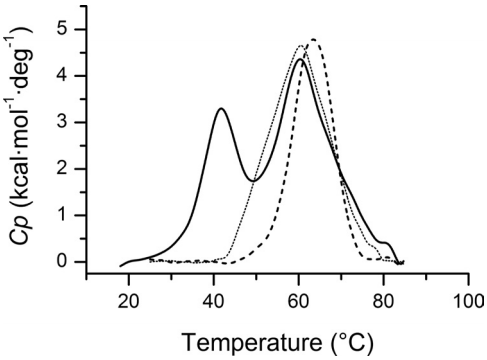


FIGURE 2. **DSC heating profiles of CUB2-CCP1 and CCP1 fragments of C1r as a function of $Ca^{2+}$.** Measurements were carried out in 20 mM Tris, 140 mM NaCl, pH 8.3. The CUB2-CCP1 fragment was measured in the presence of 5 mM $CaCl_2$ (*solid line*) or 5 mM EDTA (*dotted line*). Unfolding of the single CCP1 domain was not affected by $Ca^{2+}$ (*dashed line*).

VP-ITC instrument (MicroCal). The cell was filled with 48 μM protein in 20 mM HEPES, 140 mM NaCl, pH 7.45, and was titrated with 5 mM $CaCl_2$ in the same buffer applying series of injections of 4, 8, and 16 μl. Reference experiments were performed by injecting $Ca^{2+}$-free buffer into the protein solution or $Ca^{2+}$-containing buffer into $Ca^{2+}$-free buffer.

*Hydrogen-Deuterium Exchange by Mass Spectrometry*—CUB2-CCP1, either in the presence or absence of 5 mM $Ca^{2+}$, in a buffer of 20 mM HEPES, 140 mM NaCl, pH 7.45, was frozen in liquid nitrogen and lyophilized. The samples were dissolved in $D_2O$ and incubated at 37 °C. The kinetics of H/D exchange was followed by analyzing the increase in molecular mass by MS. The analysis was performed on a HP-1100 series high pressure liquid chromatography-electrospray ionization-MS system using the flow injection method with 10 mM ammonium formiate in $D_2O$, pH 3.5. The flow rate was 0.2 ml/min. The drying nitrogen gas flow rate was 10 liters/min at 300 °C at a pressure of 30 pounds/square inch. Capillary voltage was 4000 V. Total ion current chromatogram was obtained in positive ion mode by scanning in the 300–2000 mass/charge range. The mass information was evaluated using Agilent ChemStation software.

## RESULTS

*Production of Recombinant C1r CUB2-CCP1 Fragment*—Our objective was to characterize the CUB2-CCP1 fragment of C1r because we lacked any structural or functional information about this region of C1r, although we suspected that the flexibility of this region could be crucial for the function of the C1 complex. To achieve this goal, we expressed the CUB2-CCP1 fragment in *E. coli*. The inclusion body was renatured, and the protein was purified on ion exchange (Q-Sepharose) and gel filtration (Sephacryl S100) columns (Fig. 1*A*). Because the expression of individual CUB2 and CCP1 domains failed, we prepared the CCP1 domain by limited proteolysis of the CUB2-CCP1 fragment using thermolysin. As in the case of the intact serum C1r, thermolysin digested the CUB2 domain but let the CCP1 module intact. The CCP1 module was purified on SP-Sepharose HP column. The integrity of the single CCP1 module was verified by mass spectrometry. The purity of the recombinant proteins was checked by SDS-PAGE analysis (Fig. 1*B*).

*Effect of $Ca^{2+}$ on the Structural Stability of the CUB2 Domain*—The thermal stability of the refolded C1r CUB2-CCP1 and CCP1 fragments was investigated by DSC (Fig. 2). In the presence of 5 mM $Ca^{2+}$, the CUB2-CCP1 fragment exhibited two heat-denaturation transitions at 41.5 °C and 60.4 °C. In the absence of $Ca^{2+}$ or presence of EDTA, only the second denaturation peak was observed at 60.4 °C. The isolated C1r CCP1 domain showed a single melting transition at 63.4 °C, independently from the presence or absence of $Ca^{2+}$. We can conclude that the second, $Ca^{2+}$-insensitive melting transition of the CUB2-CCP1 fragment around 60 °C is due to the unfolding of the CCP1 domain, whereas the first, $Ca^{2+}$-dependent denaturation peak at 41.5 °C originates from the melting of the CUB2 domain. In the absence of $Ca^{2+}$, the lack of the first peak indicates that the CUB2 domain has no folded structure without $Ca^{2+}$. Adding of $Ca^{2+}$, however, restores the structure of the CUB2 domain. We can point out that at physiological temperature (37 °C), in the presence of $Ca^{2+}$, the CUB2 domain is at its border of stability. It is important to note that the melting transition of CUB2 is reversible, which was proven by repeated heating cycles to 48 °C. The calorimetric enthalpy change of the unfolding of C1r CUB2 domain was 144 kJ/mol. The CCP1 domain exhibited an irreversible unfolding profile that was sharper and appeared at a slightly higher temperature in the case of the isolated CCP1 domain than the corresponding peak of the CUB2-CCP1 fragment. It seems likely that in the case of the CUB2-CCP1 fragment the covalently linked, already unfolded CUB2 domain decreases the stability of the CCP1 domain which is reflected in the decrease of $T_m$ and broadening of the transition.

*Effect of $Ca^{2+}$ on the Structure of CUB2*—The secondary structure of C1r CUB2-CCP1 and C1r CCP1 in the presence or absence of $Ca^{2+}$ was investigated by CD spectroscopy. The spectrum of the single CCP1 domain, which was proven to be $Ca^{2+}$-insensitive, was subtracted from that of CUB2-CCP1 to calculate the spectral contribution of the CUB2 domain. The results are presented in Fig. 3. In the presence of $Ca^{2+}$, C1r CUB2 showed a spectrum that is characteristic for proteins

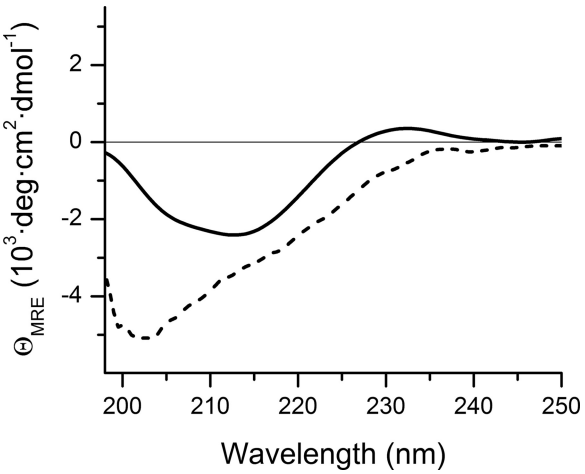


FIGURE 3. **CD spectra of CUB2 domain in the presence or absence of 5 mM $Ca^{2+}$ in 20 mM Tris, 140 mM NaCl, pH 8.3.** These spectra were obtained by subtracting the $Ca^{2+}$-independent spectrum of the CCP1 domain from that of CUB2-CCP1. In the absence of $Ca^{2+}$ the CUB2 domain shows random like secondary structure (*dashed line*), whereas it is more ordered in the presence of 5 mM $Ca^{2+}$ (*bold solid line*). *Light solid line* represents the zero value.

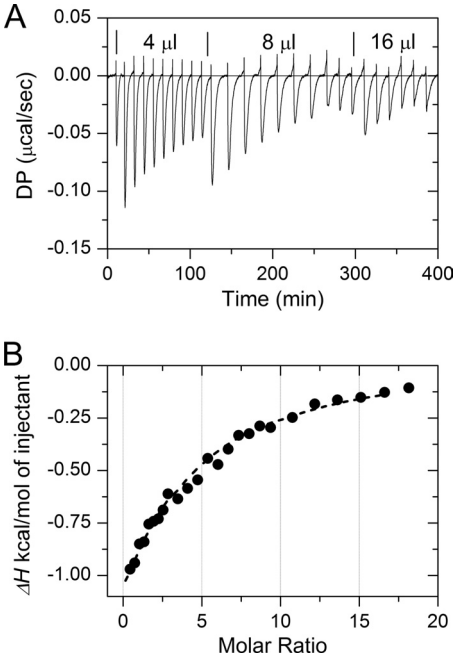


FIGURE 4. **$Ca^{2+}$ binding of C1r CUB2-CCP1, as followed by ITC. 4-, 8-, and 16-μl aliquots of 5 mM $Ca^{2+}$ in 20 mM HEPES, 140 mM NaCl, pH 7.45, were injected into the calorimeter cell containing 48 μM C1r CUB2-CCP1 in the same buffer lacking $Ca^{2+}$.** *A*, titration profile showing an exothermic reaction leading to the gradual saturation of CUB2 by $Ca^{2+}$. *B*, calculated enthalpy changes of the titration reaction with the best fit resulting 1:1 binding stoichiometry and a $K_D$ value of 430 ± 20 μM.

containing β-sheet and turn structure, whereas in the absence of $Ca^{2+}$, the corresponding spectrum was random-like.

*CUB2 Domain Binds a Single $Ca^{2+}$ Ion*—The $Ca^{2+}$ binding affinity of the CUB2 domain was studied by ITC. The buffer, containing 5 mM $Ca^{2+}$, was injected into the calorimeter cell containing 48 μM C1r CUB2-CCP1 solution (Fig. 4*A*). The titration profile indicated 1:1 binding stoichiometry (Fig. 4*B*), which is in accordance with the work of Gregory *et al.* (20), reporting that a CUB domain binds one $Ca^{2+}$ ion. The $K_D$ of $Ca^{2+}$ binding of the CUB2 domain proved to be 430 ± 20 μM. The 1.2 mM free (ionized) $Ca^{2+}$ concentration observed in the human blood (21) is close to this value, resulting in a theoretical 74% saturation of the CUB2 domain. It is possible that the unstable, $Ca^{2+}$-unbound form of the CUB2 domain provides the flexibility required for the function of the C1r molecule.

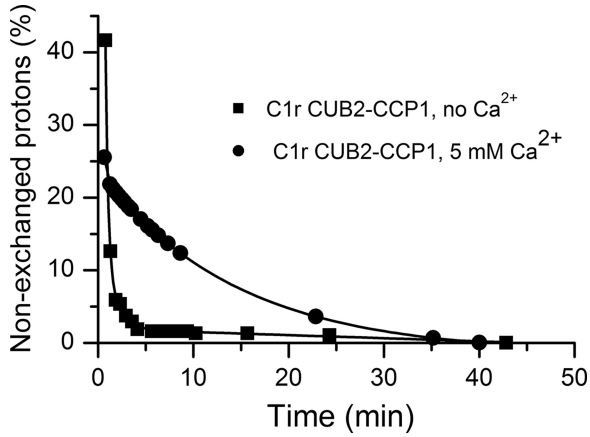


FIGURE 5. **H/D exchange of C1r CUB2-CCP1 in the presence or absence of 5 mM $Ca^{2+}$ in 20 mM HEPES, 140 mM NaCl, pH 7.45.** The nonexchanged amide proton fraction is presented as a function of time. The exchange kinetics of C1r CUB2-CCP1 in the absence of $Ca^{2+}$ is rapid, suggesting an unfolded, random-like structure with low protection of amide-protons (■). In the presence of 5 mM $Ca^{2+}$, a significantly slower exchange can be observed indicating the burial of amide protons in a more rigid, compact structure (●). Lines are guiding for the eye.

We compared the enthalpy changes corresponding to the unfolding of CUB2 measured by DSC with that of $Ca^{2+}$ binding measured by ITC. From the DSC, it was possible to calculate the $\Delta H$ of unfolding at 30 °C, taking into account an average of 50 J·mol$^{-1}$·K$^{-1}$/residue unfolding heat capacity change of a globular protein (22), resulting in 75 kJ·mol$^{-1}$. The 43.5 kJ·mol$^{-1}$ enthalpy change of $Ca^{2+}$ binding indicates that the absence of $Ca^{2+}$ causes significant loss of interactions, *i.e.* the structure of C1r CUB2 domain is loose whereas it becomes folded upon $Ca^{2+}$ binding.

*Conformational Flexibility of CUB2*—H/D exchange experiments involve exposing proteins to a deuterated solvent and measuring the exchange of its labile protons with deuterons over time (23). A faster exchange reflects higher accessibility of the amide protons for the solvent, *i.e.* higher conformational flexibility and/or more loose structure and lower number of backbone hydrogen bonds. Every exchange of a proton to deuteron with heavy water results in a 1-unit increase of the molecular mass, which can be followed by MS. C1r CUB2-CCP1 was freeze-dried in 20 mM HEPES, 140 mM NaCl, pH 7.45, either in the presence or in the absence of 5 mM $Ca^{2+}$. For H/D exchange measurement, the samples were dissolved in $D_2O$ at 37 °C. Small aliquots were injected into the electrospray MS instrument at different time points for analysis of the molecular mass. Fig. 5 shows the unexchanged amide proton ratio that was calculated from the molecular mass of the most intensive component in the spectrum. H/D exchange of the $Ca^{2+}$-free C1r CUB2-CCP1 was fast and nearly complete in a few minutes, indicating that in the absence of $Ca^{2+}$, the structure of the protein is loose or unfolded. In the presence of 5 mM $Ca^{2+}$, the H/D exchange was 1 order of magnitude slower, being completed

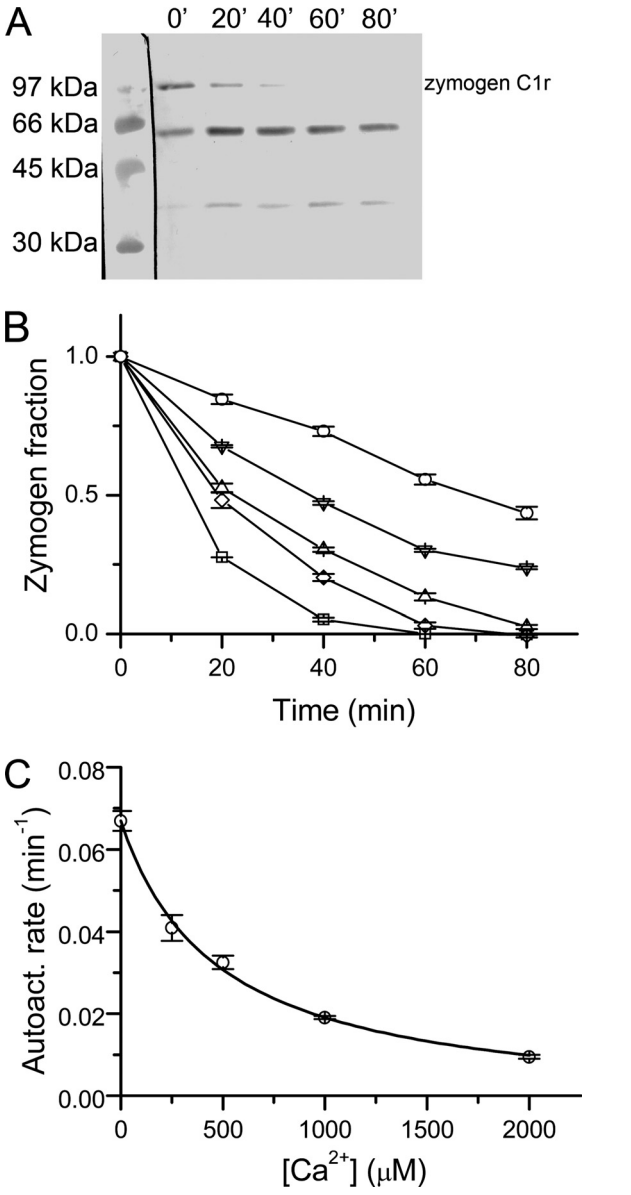


FIGURE 6. **Effect of $Ca^{2+}$ on the autoactivation of zymogen C1r followed by immunoblotting.** Zymogen C1r was incubated at 37 °C in 20 mM Tris, 140 mM NaCl, pH 7.4, in the presence of either 100 $\mu$M EDTA or 250, 500, 1000, or 2000 $\mu$M $Ca^{2+}$. Three independent experiments were carried out. *A*, representative immunoblot showing the autoactivation process in the absence of $Ca^{2+}$. *B*, time course of autoactivation: remaining zymogen C1r fraction as a function of time at 100 $\mu$M EDTA (□), 250 $\mu$M $Ca^{2+}$ (◇), 500 $\mu$M $Ca^{2+}$ (△), 1000 $\mu$M $Ca^{2+}$ (▽), 2000 $\mu$M $Ca^{2+}$ (○). *C*, autoactivation rates as a function of $Ca^{2+}$ concentration. Hyperbolic fit resulted in an apparent $K_D$ value of 474 ± 54 $\mu$M. Detailed description of data analysis is provided under "Experimental Procedures."

after more than 0.5 h, revealing a more compact, folded structure of C1r CUB2-CCP1. Isolated CCP1 showed no difference in H/D exchange kinetics in the function of $Ca^{2+}$ concentration (data not shown).

*Autoactivation of the Zymogen C1r*—To investigate the functional relevance of the $Ca^{2+}$ binding of the CUB2 domain, we isolated zymogen, full-length C1r from plasma and studied its autoactivation as a function of $Ca^{2+}$ concentration. Autoactivation results in a proteolytic cleavage of the linker region between the CCP2 and serine protease domains of C1r, which can be detected by SDS-PAGE and immunoblotting. The free (ionized) $Ca^{2+}$ concentration is ~1.2 mM in the human blood, thus we studied the autoactivation process in the submillimolar-millimolar range. As shown in Fig. 6, autoactivation is fast in the absence of $Ca^{2+}$ or at 250 $\mu$M $Ca^{2+}$. At 500 $\mu$M $Ca^{2+}$, autoactivation needs more than 60 min, whereas it is not completed even after 80 min at 1 or 2 mM $Ca^{2+}$. A $K_D$ value of 474 ± 54 $\mu$M was estimated from autoactivation rates (see "Experimental Procedures"), which is in good agreement with the $K_D$ value measured for $Ca^{2+}$ binding affinity of the CUB2 domain by ITC (430 ± 20 $\mu$M).

Full-length C1r has more $Ca^{2+}$ binding sites. Besides CUB2, other sites are located very probably at the CUB1 and EGF domains of the C1r$\alpha$ fragment. This fragment binds $Ca^{2+}$ with high affinity ($K_D$ = 32 $\mu$M) (17). Because the C1r$\alpha$ fragment is already completely saturated at the lowest $Ca^{2+}$ concentration (250 $\mu$M) used in this study, the observed effect on autoactivation must be due to the $Ca^{2+}$ binding of the CUB2 domain.

## DISCUSSION

The purpose of our work was to study the role of the CUB2-CCP1 region of C1r in the mechanism of the autoactivation of C1r and the activation of the C1 complex. In the recent years, a considerable body of structural information accumulated concerning the structures of different fragments of the subcomponents of the C1 complex. Structures of different fragments of C1q (24), C1r (16, 25–27), and C1s (20, 28) are available which served as base to create functional models for the C1 complex. A consensus element of all existing models is that significant flexibility of the $C1r_2s_2$ tetramer is needed for the autoactivation of C1r and the subsequent activation of C1s in the C1 complex (5). The crystal structures provide little information about the flexibility of the subcomponents; however, it seems likely that the CCP2-SP interface of C1r (and that of the cognate proteases MASP-1 and MASP-2) exhibits limited flexibility (26, 27, 29, 30). This flexibility, however, is not enough to account for the large conformational changes that must take place during the activation process. The CCP1-CCP2 interdomain junction contains conserved stabilizing interactions and shows similar conformation in the known structures of C1r, MASP-1, and MASP-2; therefore, it is unlikely that this region would be a source of considerable flexibility (27). The CUB1-EGF domains are responsible for the C1r-C1s interaction (and dimerization of MASPs) holding together the tetramer and make intensive contacts with each other, which also precludes the flexible movements of these domains. The centrally positioned CUB2-CCP1 fragment of C1r is highly sensitive for proteolytic cleavage indicating a relaxed, flexible structure. Limited proteolysis of C1r and C1s yields two fragments: the $\alpha$-fragment containing the CUB1 and EGF modules and the N-terminal third of CUB2 and the $\gamma$B fragment consisting of the CCP1, CCP2, and SP domains (31). It means that the proteolysis occurs in the CUB2 domain and in the CUB2-CCP1 interface. There is absolutely no structural information available about this region possibly because of the difficulties arising in producing this fragment.

In our study we chose the CUB2-CCP1 region as a probable source of flexibility required for the function of C1r. We managed to produce the CUB2-CCP1 fragment and the CCP1 domain in recombinant form using a bacterial expression sys-

tem. Despite our attempts, the recombinant expression of the individual domains failed, probably because of the small size and the proteolytic cleavage inside the bacterial cells. However, the expression and renaturation of the CUB2-CCP1 fragment were successful, and proteolytic cleavage of this fragment yielded the single CCP1 module. This experiment indicated that the CUB2 domain is not as compactly folded as the CCP1 module in accordance with the earlier limited proteolysis experiments using serum C1r.

This study provides the first direct evidence that CUB2 domain of C1r binds calcium. It was demonstrated earlier by x-ray crystallography that the CUB1 domain of C1s contains a calcium binding site (20), and it was predicted from the amino acid sequence that the CUB domains of C1r also may have potential binding sites for this ion. Thielens *et al.* (17) demonstrated that the $\alpha$-fragment of C1r that contains the CUB1 and EGF modules binds $Ca^{2+}$ with high affinity. We managed, for the first time, to prove the calcium-binding ability of C1r CUB2 and quantitatively measure its affinity by means of ITC. Comparing the $K_D$ value of the C1r CUB2 calcium binding (430 $\mu$M) with that of the C1r$\alpha$ fragment (32 $\mu$M), we can conclude that the binding affinity of C1r CUB2 is weaker with 1 order of magnitude. The weaker binding might be the consequence of the mutation at position 226 which changed the conserved Glu to Asp in the Glu-Asp-Asp consensus sequence of the CUB domain $Ca^{2+}$ binding site (14). The large difference in $Ca^{2+}$ binding affinity can result in significant difference in stability between the N-terminal domains (CUB1-EGF, CUB2) of C1r that can have functional consequences as discussed below.

The most important finding of our work is that $Ca^{2+}$ ion induces the folding of C1r CUB2 domain and stabilizes its structure. DSC measurements, CD spectroscopy, and H/D exchange experiments demonstrated unambiguously that in the absence of $Ca^{2+}$ the C1r CUB2 domain has a loose, disordered structure. Moreover, the effect is reversible; that is, the removal of $Ca^{2+}$ destroys the folded structure, whereas adding $Ca^{2+}$ to the disordered CUB2 domain restores the correct folding. One can speculate that the $Ca^{2+}$ ion can serve as a switch between the folded and disordered forms of C1r CUB2 domain. The unfolded CUB2 domain can provide high flexibility that is needed for the autoactivation process. To address the possible *in vivo* relevance of this phenomenon, we investigated the effect of $Ca^{2+}$ on the autoactivation of full-length zymogen C1r isolated from plasma (32). The $K_D$ value estimated from the autoactivation of zymogen C1r is comparable with the $K_D$ of CUB2 $Ca^{2+}$ binding determined by ITC. Our functional measurements proved that at low $Ca^{2+}$ concentration (250 $\mu$M), where the CUB1-EGF region is still saturated with $Ca^{2+}$ but the CUB2 domain is not, the autoactivation of isolated C1r is ~4 times faster than at high $Ca^{2+}$ concentration (2 mM). This effect could be more important in the C1 complex, where the C1r dimer is tightly packed inside the cone formed by the C1q arms. If we consider that at free $Ca^{2+}$ concentration of serum (~1.2 mM) the CUB2 is not fully saturated, we can conclude that the C1r CUB2 domain works at the margin of its stability providing flexibility for the C1r dimer and probably for the entire $C1r_2s_2$ tetramer. This effect could be more pronounced in case of infection-inflammation, where the local $Ca^{2+}$ concentration decreases (33). It is possible that the decreased $Ca^{2+}$ level facilitates the activation of C1 via increasing the ratio of the $Ca^{2+}$-unbound disordered form of C1r CUB2 domains.

In contrast to the CUB2 domain, the structure of C1r CUB1 domain proved to be totally insensitive to $Ca^{2+}$ (34). The intrinsic fluorescence spectrum of recombinant CUB1 remained unchanged in the presence of either EGTA or $CaCl_2$. This observation further emphasizes the special role of the $Ca^{2+}$-sensitive, marginally stable CUB2 domain in the function of C1r.

According to the very latest C1 models, the entire $C1r_2s_2$ tetramer is packed inside the C1q cone, and the C1r CUB2 domains provide important binding sites not only for $Ca^{2+}$ ion but also for C1q (14, 15). Mutation of the $Ca^{2+}$ binding site also abolishes the C1q binding. It is not clear whether the $Ca^{2+}$ binding site is directly involved in the C1q binding (35) or the loss of C1q binding is the consequence of the disruption of the folded structure. If the $Ca^{2+}$ ion dissociates from one of the C1r CUB2 domains, the interaction between the C1q and the tetramer can weaken, which makes possible large conformational changes of the tetramer inside the C1q cone. We can hypothesize that the tetramer can switch between the C1q arms to occupy the most favorable position after (some of the) C1q arms has bound to the target surface and the cone adopted an asymmetrical shape. The involvement of the marginally stable C1r CUB2 domain in the C1q-$C1r_2s_2$ tetramer interaction further emphasizes its possible role in the dramatic conformational changes that must take place during the activation of the C1 complex.

In the lack of $Ca^{2+}$, the CUB2 domain resembles intrinsically unstructured (natively unfolded) proteins (IUPs) in several properties such are the unstable, disordered structure, fast H/D exchange, proteolytic sensitivity. We investigated if CUB2 could be classified as a member of this protein family. The amino acid sequence of CUB2 was checked by IUPred (36, 37) and PONDR (38–40) for intrinsically disordered segments. The results showed no evident signs of IUPs, suggesting that CUB2 does not belong to them and might exist in globular fold under suitable conditions. Thus, CUB2 and possible other similar proteins with high conformational plasticity will not be picked out from sequence databases searching for IUPs. Based on our findings we might establish a separate, new family of protein domains as "flexibility domain." A member of this group is characterized by its function providing the flexibility or plasticity required for the proper function of a larger protein molecule or complex. They have marginal stability, high conformational flexibility, might be sensitive for proteolysis, and they seem globular proteins in their primary sequence. Compared with IUPs, these proteins might be evolved from the opposite direction, from the ordered proteins.

*Acknowledgment—We thank Ferenc Tölgyesi (Semmelweis University, Institute of Biophysics) for help with ITC measurements.*

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