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The Role of the Individual Domains in the Structure and Function of the Catalytic Region of a Modular Serine Protease, C1r[1]

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The first enzymatic event in the classical pathway of complement activation is autoactivation of the C1r subcomponent of the C1 complex. Activated C1r then cleaves and activates zymogen C1s. C1r is a multidomain serine protease consisting of N-terminal α region interacting with other subcomponents and C-terminal γB region mediating proteolytic activity. The γB region consists of two complement control protein modules (CCP1, CCP2) and a serine protease domain (SP). To clarify the role of the individual domains in the structural and functional properties of the γB region we produced the CCP1-CCP2-SP (γB), the CCP2-SP, and the SP fragments in recombinant form in *Escherichia coli*. We successfully renatured the inclusion body proteins. After renaturation all three fragments were obtained in activated form and showed esterolytic activity on synthetic substrates similar to each other. To study the self-activation process in detail zymogen mutant forms of the three fragments were constructed and expressed. Our major statement is that the ability of autoactivation and C1s cleavage is an inherent property of the SP domain. We observed that the CCP2 module significantly increases proteolytic activity of the SP domain on natural substrate, C1s. Therefore, we propose that CCP2 module provides accessory binding sites. Differential scanning calorimetric measurements demonstrated that CCP2 domain greatly stabilizes the structure of SP domain. Deletion of CCP1 domain from the CCP1-CCP2-SP fragment results in the loss of the dimeric structure. Our experiments also provided evidence that dimerization of C1r is not a prerequisite for autoactivation. *The Journal of Immunology,* **2001, 167: 5202–5208.**

The first component of the complement system (C1)[4] plays an essential role in the molecular immune response. After binding to activator structures, it initiates the activation of the classical pathway of complement, which results in the destruction and clearance of invading pathogens. C1 is a supramolecular complex, consisting of one C1q, two C1r, and two C1s subunits (for reviews, see Refs. 1–3). C1q is the recognition subunit of the complex, whereas C1r and C1s are highly specific serine proteases (with molecular masses of 86.5 and 80 kDa, respectively), which are responsible for the catalytic function of C1. A specific feature of the C1r and C1s serine proteases is that they form a distinct structural unit, the Ca^{2+}-dependent C1s-C1r-C1r-C1s tetramer, which makes possible the coordinated action of the two enzymes within the C1 complex. This tetramer associates with C1q to yield the heteropentameric C1 complex. C1r and C1s are present in the C1 complex in zymogen form, and become activated after C1q binds to an activator. The first enzymatic event during the activation process is the autoactivation of C1r. Activated C1r then activates zymogen C1s, which in turn cleaves C4 and C2. Both C1r and C1s, like many other serum proteases, are modular serine proteases (4, 5). The protease domain is preceded by five noncatalytic modules, including two CUB, one epidermal growth factor (EGF), and two complement control protein (CCP) ones. The EGF module in the N-terminal half of the molecule is surrounded by the two CUB domains, whereas the two CCP modules are in close contact with the C-terminal chymotrypsin-like serine protease domain. Upon activation, an Arg-Ile bond is cleaved in the catalytic domain of the zymogens, and a disulfide bridge holds together the two (A and B) chains of the activated enzyme. C1r is a noncovalent dimer in the presence or absence of Ca^{2+} forming the core of the C1s-C1r-C1r-C1s tetramer. Dimerization occurs through the C-terminal γB catalytic region of the molecule, whereas the N-terminal α interacting region of C1r (CUB-EGF) binds the C1s subunit in a Ca^{2+}-dependent manner.

The catalytic C-terminal γB fragment of C1r, consisting of the two CCP domains followed by the activation peptide of the protease and the serine protease domain (B chain), can be obtained by autolysis or by limited proteolysis of extrinsic proteases (e.g., thermolysin) (6). The CCP repeat is ~60 residues in length and is widespread among complement proteins. It is likely that the CCP domains significantly contribute to the specificity of the interaction and catalytic properties of the γB fragment. The γB fragment is a dimer, like the intact C1r molecule, and is capable of autoactivation and can cleave and activate zymogen C1s (7). A recent structural model of $(\gamma B)_2$ suggests a loose head-to-tail assembly of the monomers, where the γ-chain (the two CCP modules and the

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[4] Abbreviations used in this paper: C1, the first component of the complement system; C1q, C1r, C1s, subcomponents of C1; CCP, complement control protein; SP, serine protease module including the activation peptide; DSC, differential scanning calorimetry; CUB, module found in C1r/C1s, Uegf, and bone morphogenetic protein-1; EGF, epidermal growth factor; FPLC, fast protein liquid chromatography.

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activation peptide) of one monomer interacts with the serine protease module of the other monomer (8).

To gain detailed information about the role of the individual domains in the structure and function of the catalytic region of human C1r we expressed and characterized three different recombinant fragments in *Escherichia coli*: the γB (CCP1-CCP2-SP), the catalytic domain with one CCP module (CCP2-SP), and the serine protease module including the activation peptide (SP), respectively. Our aim was to determine which domains are responsible for the dimerization and how the CCP domains modulate the stability and the proteolytic action of the serine protease domain. Another objective was to clarify the mechanism of autoactivation of the proenzyme C1r. For this purpose we expressed mutant forms of the recombinant fragments, which were stabilized in the zymogen state. Using these mutants we could obtain new information about the details of the autoactivation process.

Materials and Methods

Construction of recombinant plasmids for expression of the C1r fragments

The cDNA fragments corresponding to the amino acids 309–705 (CCP1-CCP2-SP), 376–705 (CCP2-SP), and 451–705 (SP) amino acids of human C1r were amplified by PCR using the proofreading *Pfu* DNA polymerase enzyme (Stratagene, La Jolla, CA) and the full-length cDNA template (9). For the amplification procedure the following forward primers were obtained from East Port Scientific (Budapest, Hungary): 5′-GCGAAGCTT GCCCCCAGCCCAAGACCCTA-3′, 5′-GCGAAGCTTGTGGGCAGC CCCGAAACCTG-3′, and 5′-GCGAAGCTTGTGGGAAGCCCGTGAA CC-3′ in the case of CCP1-CCP2-SP, CCP2-SP, and SP, respectively. The reverse primer was 5′-GCGGTCGACTCAGTCCTCCTCCTCCATCT-3′ for each fragment. The PCR products were digested with *Hin*dIII and *Sal*I (cleavage sites are underlined) and were ligated into the *Hin*dIII/*Xho*I site of the pET-17b expression vector (Novagen, Madison, WI) in frame with the following Tag sequence 5′-ATGTGCACCCAAGCT-3′. As a result of this the recombinant proteins contained four extra amino acids (Ser-Thr-Gln-Ala) at their N terminus. The constructs were verified by DNA sequencing. In vitro mutagenesis experiments were conducted by means of the QuickChange site-directed mutagenesis kit (Stratagene). The primer pairs (only the sense sequence is shown) were: GTGGAACAGAGGCAG CAGATAATCGGAGGGCAAAAAG for the Arg^{463}Gln and GCCTGC CAGGGGGATGCTGGGGGCGTTTTTGCA for the Ser^{654}Ala mutations.

Production, renaturation, and purification of the recombinant proteins

The expression plasmids were transformed into the BL21(DE3) pLysS host strain, and the transformants were selected on Luria-Bertani medium plates containing ampicillin and chloramphenicol. The expression was conducted according to the manufacturer's instructions (10). After induction with isopropyl β-D-thiogalactoside, the cells were collected in a 1/10 volume Tris-EDTA buffer and frozen at −20°C. The cells were then thawed and incubated for 30 min at room temperature in the presence of 0.5% Nonidet P-40. The viscous solution was sonicated to shear the DNA, and the inclusion bodies were collected by centrifugation (12,000 × *g*, 15 min, 4°C). The supernatant was discarded and the pellet was washed three times with Tris-EDTA buffer (1/10 of the culture volume). The inclusion bodies were solubilized in 6 M GuHCl, 0.1 M Tris-HCl (pH 8.3), 100 mM DTT for 3 h at room temperature. The solution contained ~10 mg/ml protein. The solubilized proteins were diluted to 400-fold into the refolding buffers. The refolding buffers contained 50 mM Tris-HCl (pH 8.3), 3 mM reduced glutathione, 1 mM oxidized glutathione, 5 mM EDTA, and 2 M GuHCl in the case of the CCP1-CCP2-SP fragment, or 0.5 M arginine in the case of the CCP-SP and SP fragments. The renaturation process was conducted at 15°C overnight. The renatured protein solutions were then dialyzed against 50 mM Tris-HCl pH 7.4, 145 mM NaCl, filtrated on a 0.45-μm nitrocellulose membrane and concentrated.

The renatured proteins were purified on Q-Sepharose-Fast Flow column (Pharmacia Biotech, Uppsala, Sweden). The samples were dialyzed against and the column was equilibrated in a buffer containing 20 mM NaCl and 20 mM Tris-HCl, pH 8.3, for the CCP1-CCP2-SP and CCP2-SP or pH 7.4 for the SP fragments. The samples were loaded onto the column and the elution was conducted with a linear NaCl gradient from 20 to 400 mM. Fractions were identified by SDS-PAGE. The recombinant proteins were further purified by gel filtration using a Superose-12 fast protein liquid chromatography column (Pharmacia Biotech) in 50 mM NaCl, 20 mM Tris-HCl (pH 8.3) for CCP1-CCP2-SP and CCP2-SP, or pH 7.4 for SP. The concentration of the recombinant proteins was determined by absorbance at 280 nm using the absorption coefficients 15.2, 15.8, and 15.4 (1%, 1 cm) for the CCP1-CCP2-SP, CCP2-SP, and SP fragments, respectively. For calculation of absorption coefficients we used the method of Gill et al. (11) taking disulfide bridges into account. The molecular masses calculated from the amino acid sequences were 45,532, 37,670, and 28,976 Da for CCP1-CCP2-SP, CCP2-SP, and SP fragments, respectively.

N-terminal sequencing

After SDS-PAGE and blotting to polyvinylidene difluoride membrane, the N-terminal amino acid sequences of the recombinant proteins were determined by a pulsed-liquid phase protein sequencer ABI 471A.

Gel filtration chromatography

Gel filtration experiments were conducted using a Superose-12 FPLC column (Pharmacia Biotech). In the 4.0–6.0 pH range the column was equilibrated with 20 mM citrate, 100 mM NaCl buffer. At pH 7.0 and 8.0 buffers containing 20 mM Tris and 100 mM NaCl were used. Cytochrome *c*, carbonic anhydrase, albumin, and human IgG were used as m.w. standards. Relative m.w. of the C1r fragments were calculated using linear fitting of the elution volume vs m.w. of the standard proteins in logarithmic scale at the appropriate pHs.

Enzymatic assays

Esterolytic activity. The rates of hydrolysis were measured on the Z-Lys-S-Bzl and Z-Gly-Arg-S-Bzl thioesters. The release rate of HS-Bzl was measured through its reaction with 4,4′-dithiodipyridine (12), and was followed with a Jasco V-550 spectrophotometer at a wavelength of 324 nm. Assays were conducted following the method of McRae et al. (13) at 30°C in 20 mM Tris buffer at pH 7.5 containing 145 mM NaCl. Values of k_{cat}/K_M were directly determined from the catalytic rate at low substrate concentrations (10–30 μM).

C1s cleavage. Proenzyme C1s was expressed in baculovirus expression system using High Five insect cell culture (Invitrogen, Carlsbad, CA). Functionally pure (80%) C1s proenzyme was obtained by purifying the cell culture supernatant on a DEAE Sepharose FF column (Amersham Pharmacia Biotech, Piscataway, NJ) as described in Ref. (14). C1s preserved its proenzyme state during the purification and storage. Proenzyme C1s cleavage ability of the C1r fragments was tested by means of the esterolytic activity of the generated active C1s molecules on the Z-Lys-S-Bzl thioester substrate. An enzyme/C1s molar ratio of 1:50 was used for most of the experiments. C1s proenzyme solution (10–15 ml) with a protein concentration of ~0.1 μM in 20 mM Tris, 145 mM NaCl buffer pH 7.4 was thermostated at 30°C. The C1r SP, CCP2-SP, and CCP1-CCP2-SP fragments were added to it at a final concentration of $1–3 \times 10^{-9}$ M. At 1-min intervals, 1 ml of the mixture was withdrawn and the esterolytic activity was measured by the addition of the Z-Lys-S-Bzl substrate at a final concentration of 100 μM. The maximal specific activity value of totally activated C1s of 182 s^{-1} at 100 μM Z-Lys-S-Bzl concentration was used for the calculation of the actual concentration of C1s. Values of k_{cat}/K_M were calculated by linear fitting for the first five to eight points where the amount of the cleaved, active C1s was <10% of the total proenzyme concentration.

C1r autoactivation experiments. The S654A mutant proenzyme SP, CCP2-SP, and CCP1-CCP2-SP fragments were used as substrates to investigate the autoactivation ability of the wild-type active C1r fragments. Measurements were conducted in 20 mM Tris, 145 mM NaCl buffer at pH 8.3. Reaction was started by the addition of the active enzyme to the S654A mutant proenzyme solution thermostated at 37°C. An enzyme/zymogen ratio of 1:10–1:50 was used. Ten-microliter aliquots were removed at 10–15 time points in the range of 0.5 min to 1 h and added to 10 μl of 5% SDS sample buffer (15) containing 5% 2-ME and were immediately incubated for 3 min at 100°C. The cleavage at the activation site of the proenzyme molecules was followed by reducing SDS-PAGE. The acrylamide gels were stained with Coomassie brilliant blue. The concentration of the uncleaved proenzyme vs time was calculated from the density of its bands recorded by a Bio-Rad GelDoc2000 imaging system (Hercules, CA). After curve fitting and derivation in Origin 5.0 data analyzing software (MicroCal, Northampton, MA) the cleavage rate vs proenzyme concentration was calculated and proved to be linear in the concentration range used; therefore, k_{cat}/K_M values could be obtained from the slope of the curve.

Differential scanning calorimetry (DSC). Calorimetric measurements were performed on a VP-DSC (MicroCal) differential scanning calorimeter. Denaturation curves were recorded between 10 and 80°C at a pressure

of 2.5 atm, using a scanning rate of 1°C/min. The protein concentration was set to 0.1 mg/ml. Samples were dialyzed against 20 mM Tris pH 8.3, 145 mM NaCl, and the dialysis buffer was used as a reference. Heat capacities were calculated as outlined by Privalov (16).

Results

Expression and renaturation of recombinant proteins

Three cDNA fragments from the catalytic region of C1r (Fig. 1) have been cloned into a modified pET-17b vector in fusion with the (Met)-Ser-Thr-Gln-Ala sequence. Each insert begins with a Cys (Cys^{309}, Cys^{376}, Cys^{451} in the case of CCP1-CCP2-SP (γB), CCP2-SP, and SP, respectively) at the N terminus and ends with Asp^{705} at the C terminus. The mature proteins have an N-terminal sequence Ser-Thr-Gln-Ala-(Cys)... as verified by protein sequencing. To prevent autoactivation, stabilized mutant constructs were also expressed. In one series we introduced the Arg^{463}Gln mutation into the cDNAs, whereas in another series we changed the active site Ser^{654} to Ala for all the three fragments. The expression plasmids were transformed into the *E. coli* BL21(DE3) pLysS strain, and the recombinant protein expression was induced by adding isopropyl β-D-thiogalactoside. After induction, the cells were lysed and the soluble and insoluble fractions were separated by centrifugation and analyzed on SDS-PAGE (data not shown). In the soluble fraction we could not detect recombinant proteins using Coomasie blue staining, whereas the pellet contained almost exclusively the recombinant C1r fragments (purity ~80%). Because the recombinant proteins were present as inclusion bodies, renaturation procedures were needed to generate the native, folded structure. The inclusion bodies were solubilized in 6 M guanidine-HCl solution, which contained 100 mM DTT to reduce all the disulfide bridges. The solubilized recombinant proteins (~10 mg/ml) were then diluted 400-fold (final concentration ~25 μg/ml) using different refolding buffers and incubated at 15°C overnight. Many different refolding solutions containing various additives and different oxido shuffling reagents were tested for the three fragments and the best ones were selected for large scale renaturation (17, 18). We found the highest renaturation yield using 2 M guanidine-HCl in the case of CCP1-CCP2-SP fragment and 0.5 M L-arginine in the case of CCP2-SP and SP fragments. The optimal oxido shuffling system was the mixture of reduced and oxidized glutathione in a ratio of 3 mM reduced glutathione/1 mM oxidized glutathione at pH 8.3 in all experiments. The efficiency of the folding process could be estimated by reducing SDS-PAGE, because native, functionally active C1r can cleave itself into two chains (γ 18-kDa and B 30-kDa chains in the case of the CCP1-CCP2-SP fragment). Because the denatured recombinant proteins have single-chain structure in the inclusion bodies, the appearance of two chains on the reducing gel is a good indicator of autoactivation and of successful renaturation of the wild-type fragments (Fig. 2*A*). Edman degradation of the large (30-kDa) chains yielded the Ile-Ile-Gly-Gly-Gln sequence in all cases, indicating that the correct autolytic cleavage at the Arg^{463}-Ile^{464} bond between the γ- and B chains had occurred during the activation process. The efficiency of the renaturation was ~10–20%, allowing us to obtain enough material for all subsequent physicochemical and functional studies. After the renaturation process the aggregated material was removed by filtration on a 0.45-μm nitrocellulose membrane, and the refolded recombinant proteins were purified by anion-exchange and gel-filtration chromatography as described in *Materials and Methods*. On the Q-Sepharose Fast Flow column most of the contaminants did not bind to the resin at low ionic strength (20 mM NaCl and 20 mM Tris-HCl) and could be removed by washing the column with the low salt buffer. The correctly folded, native recombinant fragments eluted as single peaks detected at 280 nm during the ascending salt gradient. There was no difference between the elution volume of the wild-type (activated) and the R463Q or S654A mutant (zymogen) fragments, although the recombinant fragments were essentially pure after the ion-exchange chromatography to remove the traces contaminants we performed a gel filtration chromatography on a Superose-12 FPLC column. After this step, the protein solutions were concentrated and the

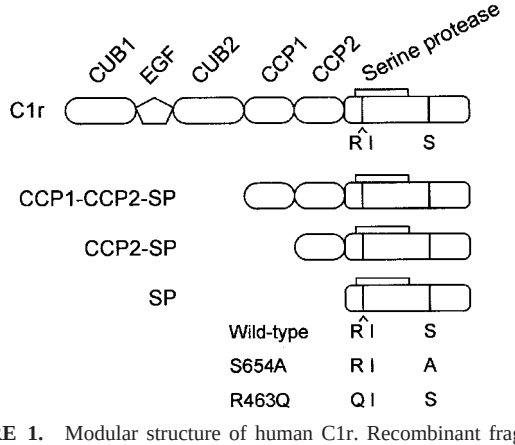

FIGURE 1. Modular structure of human C1r. Recombinant fragments of the catalytic region used in this study were CCP1-CCP2-SP, CCP2-SP, and SP. The γB fragment that can be obtained from the entire C1r molecule by limited proteolysis and our recombinant CCP1-CCP2-SP fragment have the same domain structure. The S654A catalytic site mutant fragment and the R463Q activation site mutant fragments are also presented.

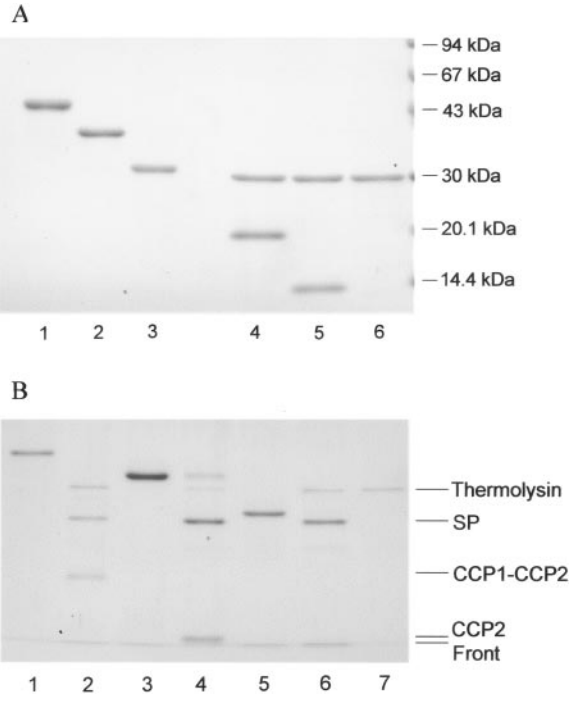

FIGURE 2. SDS-PAGE of the renatured and purified C1r fragments. *A*, Wild-type C1r fragments, nonreducing conditions: *lane 1*, CCP1-CCP2-SP; *lane 2*, CCP2-SP; *lane 3*, SP; reducing conditions: *lane 4*, CCP1-CCP2-SP; *lane 5*, CCP2-SP; *lane 6*, SP. Proteins were analyzed on a 12.5% polyacrylamide gel. The enzymes are fully activated after purification. *B*, R463Q mutant zymogen fragments and their thermolysin activated two-chain forms (10 U thermolysin/mg C1r fragment, 2 h, 30°C), reducing conditions: *lanes 1* and *2*, R463Q CCP1-CCP2-SP; *lanes 3* and *4*, R463Q CCP2-SP; *lanes 5* and *6*, R463Q SP, zymogen, and activated forms, respectively; *lane 7*, thermolysin.

concentrations of the recombinant proteins were measured from the absorbance at 280 nm. The final yields for the CCP1-CCP2-SP, CCP2-SP, and SP fragments were 2, 5, and 2 mg/l of culture, respectively. Both the wild-type and the zymogen mutant fragments yielded a single band on nonreducing SDS-PAGE analysis, although with different apparent molecular mass (~45 and ~39 kDa for wild-type CCP1-CCP2-SP and zymogen CCP1-CCP2-SP, respectively; ~38 and ~34 kDa for wild-type and zymogen CCP2-SP; and ~31 and ~27 kDa for the wild-type and zymogen SP fragment). However, on the reducing gel the wild-type fragments exhibited the activated two-chain forms (~30 kDa for the B chain and ~18 kDa for the γ-chain), whereas the zymogen mutants retained a single-chain structure. To prove the correct folding of the zymogen mutants, the renatured proteins were converted into the two-chain form. The Arg^{463}Gln mutants could be specifically cleaved at the Gln^{463}-Ile^{464} and activated by thermolysin (Fig. 2*B*). After thermolysin treatment all the three R463Q fragments showed proteolytic and esterolytic activity similar to that of the wild-type autoactivated proteases (Table I). The Ser^{654}Ala mutants cannot autoactivate themselves, but wild-type C1r fragments could cleave them at Arg^{463}-Ile^{464} activation site, as verified by protein sequencing.

Physicochemical characterization

Gel permeation chromatography: relative molecular mass. To determine the relative molecular mass and investigate the dimerization properties of the expressed and renaturated C1r fragments, the relative molecular mass of the three C1r fragments vs pH were analyzed by gel permeation chromatography. The molecular mass of the three C1r fragments were determined relative to the standards at every pH (Fig. 3).

The SP and the CCP2-SP fragments showed molecular masses of 28–30 and 36–38 kDa, respectively. These values were independent of pH and were in accord with the molecular mass determined from SDS-PAGE analysis. The relative molecular mass of the CCP1-CCP2-SP fragment was found to be ~90 kDa at neutral or alkaline pH. Below pH 6.0 it showed a sigmoidal shaped decrease to 44–47 kDa, which is the molecular mass of the monomer CCP1-CCP2-SP, and is in accord with the SDS-PAGE.

DSC. DSC measurements were performed, on the one hand, to check the native structure of the fragments and, on the other hand, to investigate the role of the individual domains in the conformational stability of the catalytic region of C1r.

The SP fragment showed a sharp, cooperative melting transition at a relatively low temperature (47.5°C), indicating a compact, stable structure of the molecule (Fig. 4).

Fragment CCP2-SP showed a cooperative unfolding curve with a melting point at 55.4°C. The larger calorimetric enthalpy change and the significantly higher melting temperature compared with that of SP indicate that the CCP2 module establishes tight interactions with the SP domain and significantly improves its stability.

Table I. *Catalytic efficiency of wild-type C1r fragments and the thermolysin-activated R463Q mutants for synthetic substrates*[a]

	SP	CCP2-SP	CCP1-CCP2-SP
Z-Lys-S-Bzl	1,600[b]	1,900[b]	1,300[b]
Z-Gly-Arg-S-Bzl	164,000[b]	210,000[b]	174,000[b]
Z-Gly-Arg-S-Bzl	112,000[c]	147,000[c]	124,000[c]

[a] k_{cat}/K_M ($s^{-1}M^{-1}$). Measurements were carried out in 20 mM Tris, 145 mM NaCl, pH 7.4, at 30°C. Values of k_{cat}/K_M are the averages of at least three independent experiments.
[b] Wild-type C1r fragments.
[c] C1r R463Q fragments, after a treatment with 10 U thermolysin (Sigma) per milligram of C1r fragment, pH 8.0, 30°C, 2 h.

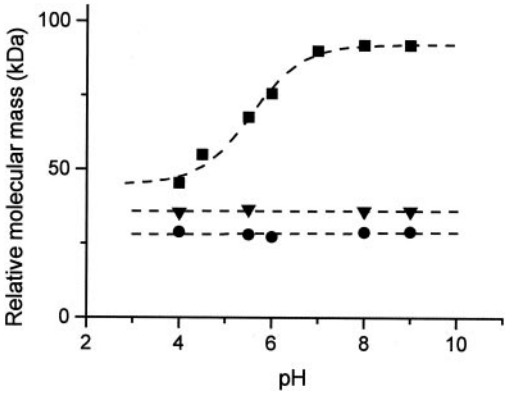

FIGURE 3. Relative molecular mass of C1r fragments vs pH determined by gel filtration chromatography. SP (●), CCP2-SP (▼), and CCP1-CCP2-SP (■). CCP1-CCP2-SP shows a dimer-monomer transition with a midpoint at pH 5.5. Molecular masses were determined on a Superose-12 column using a protein standard series at every pH, at 25°C, as described in *Materials and Methods*.

The CCP1-CCP2-SP fragment showed an unfolding transition at 57.5°C, which is in good agreement with that of the equivalent CCP1-CCP2-SP produced in baculovirus expression system (58.3°C) (19). These data prove that renatured CCP1-CCP2-SP is in a native form and that its conformational stability is similar to that of a CCP1-CCP2-SP with a somewhat larger N-terminal and with carbohydrate side-chains. The stability of CCP1-CCP2-SP is somewhat higher than that of CCP2-SP. The presence of the CCP1 module and the dimerization of the CCP1-CCP2-SP fragment exert less effect on the stability of the protein as compared with the significant stabilizing effect of the CCP2 module in the interaction with SP domain in the CCP2-SP construct.

Functional characterization of the recombinant proteins

Esterolytic activity on synthetic substrates. The values of the catalytic efficiency (k_{cat}/K_M) for the reaction of the C1r fragments with the Z-Lys-S-Bzl and Z-Gly-Arg-S-Bzl thioesters are presented in Table I. Z-Lys-S-Bzl is not a "good" substrate for C1r, but its spontaneous hydrolysis rate is very low; therefore, measurements of low catalytic activity was possible. Z-Gly-Arg-S-Bzl, a more sensitive thioester substrate of C1r, was hydrolyzed at a

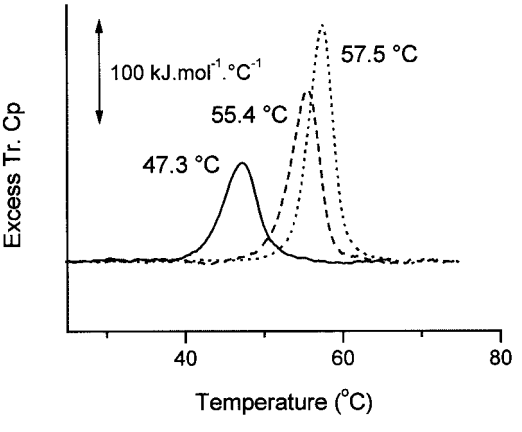

FIGURE 4. DSC melting curves of the C1r fragments. SP (——), CCP2-SP (- - - -), and CCP1-CCP2-SP (. . . . . .) in 20 mM Tris (pH 8.3) buffer containing 145 mM NaCl. The protein concentration was 0.1 mg/ml. A heating rate of 1°C/min was used.

high rate by the fragments. The three C1r fragments showed similar esterolytic activities on the thioester substrates indicating similar active site conformations in the SP, CCP2-SP, and CCP1-CCP2-SP fragments. However, the CCP2-SP fragment proved to be slightly more potent as compared with the others. The observed k_{cat}/K_M values on the Z-Gly-Arg-S-Bzl substrate are four times higher than those described previously for the entire C1r molecule isolated from human serum (13).

The catalytic efficiency of C1s cleavage by the recombinant C1r fragments. The ability of the C1r fragments to cleave proenzyme C1s was tested through the esterolytic activity of the activated C1s molecules on the Z-Lys-S-Bzl thioester substrate. Although C1r also cleaves this substrate (see Table I), its catalytic efficiency is about two orders of magnitude less than that of C1s. An enzyme-substrate ratio of 1:50 assures that the activity of C1r on the Z-Lys-S-Bzl was negligible compared with that of C1s. The proenzyme C1s concentration (0.04–0.1 μM) was orders of magnitude below the K_M value, and this allowed direct calculation of the k_{cat}/K_M values from the linear part of the C1s activation curve. Kinetic analysis of the activation of C1s by CCP1-CCP2-SP is shown on Fig. 5. The slope of the line is proportional to the catalytic efficiency. The results with the three C1r fragments are summarized in Table II. All the three fragments efficiently cleaved proenzyme C1s. The CCP2-SP fragments exhibited an exceptionally high k_{cat}/K_M value.

Cleavage of the S654A mutant fragments with the wild-type enzymes. Proenzyme S654A mutant fragments were used as substrates in investigating autoactivation properties of the wild-type fragments. The active enzyme cleaves and activates proenzyme S654A mutant that has no catalytic activity even in the two-chain form. Thus, the autoactivation can be studied in a simple enzyme-substrate system. The activity of wild-type SP, CCP2-SP, and CCP1-CCP2-SP on the S654A mutants of the SP, CCP2-SP, and CCP1-CCP2-SP fragments were measured in each combination. Cleavage of the S654A mutants was followed by reducing SDS-PAGE. Catalytic efficiency values are presented in Table III. In all enzyme-substrate pairs a high catalytic efficiency, comparable to the activities on synthetic ester substrates, could be detected. SP fragment activated the three S654A mutants at a similar rate. The highest k_{cat}/K_M values were obtained for the self-activation of the CCP2-SP fragment (i.e., its activity on the S654A mutant CCP2-SP) (Fig. 6).

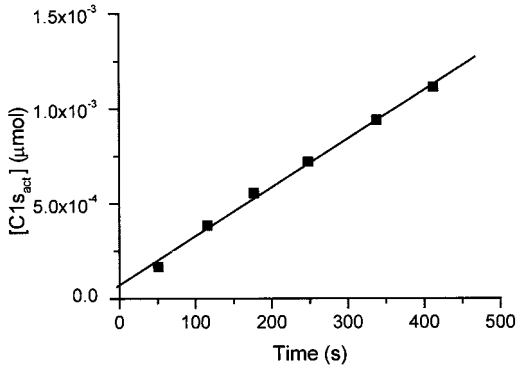

FIGURE 5. Proenzyme C1s cleavage by C1r CCP1-CCP2-SP. The figure shows a typical experiment that was conducted in 20 mM Tris (pH 7.4) containing 145 mM NaCl with an enzyme/substrate ratio of 1:50 at 30°C. The generated active C1s molecules were monitored through their esterolytic activity on Z-Lys-S-Bzl substrate.

Table II. *Efficiency of C1s cleavage*[a]

C1r Fragments	k_{cat}/KM Values
SP	28,000 ± 2,000
CCP2-SP	208,000 ± 10,000
CCP1-CCP2-SP	58,000 ± 4,000

[a] k_{cat}/K_M ($s^{-1}M^{-1}$). Reactions were performed in 20 mM Tris, 145 mM NaCl, pH 7.4, at 30°C.

Discussion

In the present study we used an *E. coli*-based expression system for recombinant production of fragments representing the catalytic region of human C1r. Previously the baculovirus-insect cell system was used to produce recombinant C1r and C1s and their fragments (14, 20, 21). The major advantage of the baculovirus-insect cell system is that the native, functionally active recombinant proteins are secreted into the cell culture medium. However, the yield of the secreted recombinant proteins is usually low and the posttranslational modifications are not complete. Furthermore, the level of β-hydroxylation of the Asn residue in the EGF domain (Asn^{167} in C1r and Asn^{149} in C1s) is very low (14, 22) and the glycosylation patterns differ significantly from the complex type glycosylation found in the case of serum proteins. However, these differences in the posttranslational modifications do not affect the functional properties of the recombinant proteins, because even aglycosylated proteins retain their biological activity (14, 21). It has been reported that simple (single-domain) serine proteases (trypsin, chymotrypsin) can be produced in *E. coli* and the inclusion bodies can be successfully renatured to obtain natively folded, functionally active recombinant proteins with a high yield (17, 23–26). Based on these results we chose to express fragments from human C1r in bacteria. The γB fragment of C1r, which can be obtained from the intact molecule by limited proteolysis retains the proteolytic activity of the molecule. It consists of three domains, two CCP modules and the serine protease domain, and it is a dimer at physiological pH, like the entire molecule. It is also capable of autoactivation. Because this fragment preserves several basic functions of C1r, it is an attractive candidate to study the role and significance of the CCP modules in the function and regulation of C1r. To explore the contribution of the individual domains to the above-mentioned properties of C1r we successively deleted the CCP domains preceding the SP domain from the cDNA. As a result, we made and expressed three cDNA constructs. The recombinant proteins accumulated as inclusion bodies inside the *E. coli* cells. After disruption of the cells we purified and renatured these proteins. The renatured fragments were purified to homogeneity by ion-exchange chromatography and gel filtration. After these procedures all the three fragments were in a correctly folded, functionally active form as confirmed by subsequent physicochemical and enzymatic measurements. After renaturation the wild-type

Table III. *Self-cleavage efficiency of the C1r fragments*[a]

	SP S654A	CCP2-SP S654A	CCP1-CCP2-SP S654A
SP	71,000	75,000	72,000
CCP2-SP	68,000	130,000	74,000
CCP1-CCP2-SP	45,000	15,900	6,200

[a] k_{cat}/K_M ($s^{-1}M^{-1}$). The S654A inactive zymogen mutants were used as substrates for the wild-type fragments. Reactions were performed in 20 mM Tris, 145 mM NaCl, pH 8.3, at 37°C. The values are the averages of two to four independent measurements. Variations between the results of the individual measurements were <10%.

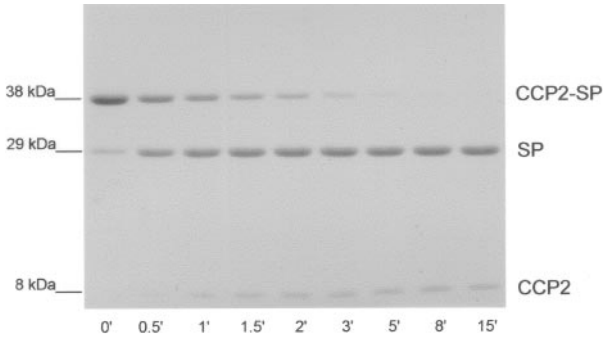

FIGURE 6. Cleavage of CCP2-SP S654A zymogen mutant by wild-type CCP2-SP. The reaction was performed in 20 mM Tris, 145 mM NaCl, pH 8.3 at 37°C. An enzyme/substrate ratio of 1:15 was used. The activation process was followed by 12.5% SDS-PAGE under reducing conditions. Reaction times for the appropriate lanes are indicated. After cleavage, the CCP2 and SP domains are separated under reducing conditions.

fragments were present in the activated, two-chain form. Autoactivation is a good indicator of correct folding. Indeed, by the end of the renaturation procedure the activation was complete. To study the zymogen form of the enzymes, as well as the autoactivation procedure, we constructed and expressed mutant C1r fragments, i.e., stable proenzymes. In one set of experiments we mutated the Arg^{463}-Ile^{464} cleavage site to Gln-Ile (27), whereas in another set we changed the catalytic site Ser^{654} to Ala. In the first case the zymogen cannot be activated by a trypsin-like serine protease; however, it could be cleaved and converted to an active enzyme by thermolysin. In the case of the Ser^{654}Ala mutants the Arg-Ile bond can be cleaved by wild-type C1r, but the mutant itself cannot function as an active protease. All of these C1r fragments were produced with a similar yield except the CCP2-SP fragments where the renaturation efficiency was significantly higher. This can be interpreted as the CCP2 domain closely associates with the SP domain forming a compact cooperative folding unit. The SP domain alone is less stable as it is indicated by the DSC measurements. The CCP1-CCP2-SP fragment contains the CCP1 module, which associates loosely to the CCP2-SP. The larger, more complex structure of CCP1-CCP2-SP can account for its lower renaturation yield.

The DSC measurements indicate that all of the renatured fragments have compact, folded structures. The calorimetric heat denaturation curves (excess heat capacity against temperature) show cooperative unfolding of the native structures in the case of the three fragments. The heat capacity curve of the recombinant CCP1-CCP2-SP is essentially identical with the curve of CCP1-CCP2-SP expressed in the baculovirus insect cell system. According to the DSC curves the SP fragment has the least stable structure. The CCP2 module exerts a dramatic stabilizing effect, as the midpoint of the heat denaturation peak of SP (47.5°C) is being shifted to 55.4°C in the case of the CCP2-SP fragment (Fig. 4). The presence of the CCP1 module stabilizes the structure further, but this effect is much less significant than that of CCP2. These results are in agreement with the homology model of the catalytic region of C1r (8) and with the crystal structure of the CCP2-SP fragment of C1s (28). According to the crystal structure the CCP2 module associates to SP domain through a rigid module-domain interface involving intertwined proline and tyrosine-rich polypeptide segments. Such a rigid CCP-SP assembly is conserved in other extracellular proteases (29).

Gel filtration experiments show that the SP and CCP2-SP fragments are monomers at all pH values, whereas the CCP1-CCP2-SP fragment is a dimer at neutral and basic pH (Fig. 3). Like serum C1r (30), our recombinant CCP1-CCP2-SP dimers dissociate under slightly acidic conditions. These results provide straightforward experimental evidence that the CCP1 domain is involved in the dimerization of C1r. Previously a three-dimensional model of activated $(\gamma B)_2$ has been constructed, which was based on chemical cross-linking and homology modeling (8). Chemical cross-linking of the $(\gamma B)_2$ fragment produced by autolytic cleavage of the active serum C1r indicated the existence of converse salt bridges between Lys^{299} in the N-terminal region of the γ segment of one monomer, and Glu^{510} of the serine protease domain of the other monomer. However, our CCP1-CCP2-SP construct begins with the Cys^{309} of C1r preceded by the Ser-Thr-Gln-Ala N-terminal fusion peptide and therefore lacks Lys^{299}. The fact that our recombinant CCP1-CCP2-SP is a stable dimer indicates that formation of salt bridges between Lys^{299} and Glu^{510} is not a prerequisite of dimerization. Future site-directed mutagenesis experiments could reveal the amino acid residues of the CCP1 module that are involved in C1r dimerization.

Regarding enzymatic activity it is somewhat surprising that the serine protease domain of C1r alone can cleave C1s with a rate comparable with that of the activation by the CCP1-CCP2-SP fragment. This indicates that the SP domain contains all the structural elements necessary for C1s binding and cleavage. However, the presence of the CCP2 domain alone in the CCP2-SP fragment causes a dramatic increase (one order of magnitude) of the k_{cat}/K_m value of the reaction. We can conclude that the CCP2 domain is responsible for the enhancement of the efficiency of the C1s cleaving activity of C1r. We suggest that although the SP domain alone can cleave C1s, the CCP2 domain provides with additional contact surfaces for binding and orienting the substrate. As we mentioned above, our results obtained by DSC indicate that the CCP2 domain strongly stabilizes the structure of the SP domain. The increase of the k_{cat}/K_m value could be explained in principle by this domain-domain interaction. However, the similar esterolytic activity of the recombinant C1r fragments shows that this is not the case. It is obvious that all the three recombinant C1r fragments contain a fully functional active site. The changes in stability caused by the addition of CCP modules to the serine protease domain do not affect the catalytic power of the serine protease active site on the synthetic substrates. We suspect that the dramatic increase of the C1s cleaving ability of the CCP2-SP fragment is due to additional substrate binding sites present on the surface of the CCP2 domain, and not to the stabilizing effect. However, the corresponding k_{cat}/K_m value of the CCP1-CCP2-SP fragment for C1s cleavage is smaller than that of the CCP2-SP fragment. As we showed above, the CCP1 domain is responsible for the dimerization of C1r. The catalytic site of C1r or the substrate binding residues on the CCP2 domain can be less accessible for the C1s in the CCP1-CCP2-SP dimers than in the CCP2-SP and SP monomers. It is also very likely that the CCP1 domain does not contain additional substrate binding sites for C1s. We should keep in mind that we are dealing with fluid phase reactions in our present study. However, inside the C1 complex the catalytic domains of C1r and C1s are precisely positioned; therefore, the efficiency of C1s cleavage by the C1r dimer can be significantly higher (31).

Our work with recombinant fragments provided valuable information concerning the autoactivation of C1r. The S654A mutant retains its zymogen form during the renaturation and purification procedure, whereas the wild-type C1r fragments are fully activated after the same treatment. We can conclude that this activation is a true autoactivation and that extrinsic (i.e., *E. coli*) proteases do not contribute to it. Because all three wild-type fragments autoactivate, two important conclusions can be drawn: 1) dimerization is not a prerequisite for autoactivation, and 2) autoactivation is an inherent property of the serine protease domain. Previously, autoactivation was shown to be a property of the dimers both in the case of the

entire molecule and its CCP1-CCP2-SP fragment (7). Under acidic conditions (pH <5.5) the C1r dimer dissociates and the resulting monomers lose their ability of autoactivation (32). Because at acidic pH the catalytic activity of the serine protease active center is expected to decrease it was not clear which phenomenon was responsible for the loss of the autoactivation ability. Our results indicate that under physiological conditions the monomeric CCP2-SP fragment is capable of autoactivation, i.e., dimerization is not required for autoactivation. Because the SP domain itself retains the ability to autoactivate, the presence of even one CCP domain is not a prerequisite for autoactivation.

Autoactivation of C1r is supposed to be a two-step process. In the initial step zymogen molecules activate zymogens, whereas in the second step the generated active enzymes cleave zymogen molecules. The fact that our wild-type fragments can autoactivate shows the existence of the initial step. The second step could be studied in detail using our zymogen mutants. The Ser^{654}Ala mutants, which have an inactive catalytic center but have a cleavable Arg-Ile bond, were used as substrates for the wild-type fragments. We determined the kinetic constants for these type of reactions (Table III). Each Ser^{654}Ala mutant was cleaved by its own wild-type counterpart. The SP fragment showed effective self-cleavage ability. The higher catalytic efficiency of the CCP2-SP construct compared with that of the SP domain can be interpreted assuming that the CCP2 domain, like in the case of the C1s cleavage, orients the Arg-Ile bond of one C1r in a favorable position to be cleaved by the active site of the other C1r. The CCP1-CCP2-SP fragment possesses significantly lower k_{cat}/K_m values than the other two fragments. Because on $(CCP1\text{-}CCP2\text{-}SP)_2$ we follow intermolecular (interdimeric) cleavage, we may conclude that dimer formation partially blocks the accessibility of either the catalytic site of the protease, or the activation site of the proenzyme and therefore decreases the efficiency of the proteolysis. To clarify this question we conducted experiments with combinations of the different fragments. The fact that the wild-type SP fragment exerts similar proteolytic activity on the dimeric CCP1-CCP2-SP fragment and on the smaller monomer fragments indicates that the Arg-Ile bond to be cleaved is accessible for extrinsic cleavage. The catalytic efficiency of CCP2-SP on CCP1-CCP2-SP also supports this observation. In a complementary experiment the wild-type CCP1-CCP2-SP fragment showed decreasing catalytic efficiency with the increasing size of the substrate proenzyme SP, CCP2-SP, and CCP1-CCP2-SP S654A fragments. It is very likely that the catalytic site of one CCP1-CCP2-SP is pointed to the "inside" (i.e., toward the other CCP1-CCP2-SP molecule) in the dimer, and this positioning facilitates the intramolecular autolytic cleavage.

The major conclusion of this work is that the serine protease module itself is an autonomous folding unit with inherent serine protease activity similar to that of intact C1r. The SP module has the ability to cleave C1s, the natural substrate of C1r, and autoactivation property is also retained.

Comparative measurements highlighted the role of the CCP modules in C1r as modulators of the catalytic functions through allosteric effects occurring upon binding to natural substrates and dimerization. The intimate interaction of the SP domain with the CCP2 domain is reflected in the sizeable stabilizing effect observed if CCP2 is attached to the SP module.

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References

1. Arlaud, G. J., M. G. Colomb, and J. Gagnon. 1987. A functional model of the human C1 complex. *Immunol. Today 8:106.*
2. Schumaker, V. N., P. Závodszky, and P. H. Poon. 1987. Activation of the first component of complement. *Annu. Rev. Immunol. 5:21.*
3. Gál, P., and G. Ambrus. 2001. Structure and function of complement activating enzyme complexes: C1 and MBL-MASPs. *Curr. Prot. Pept. Sci. 2:43.*
4. Arlaud, G. J., and N. M. Thielens. 1993. Human complement serine proteases C1r and C1s and their proenzymes. *Methods Enzymol. 223:61.*
5. Volanakis, J. E., and G. J. Arlaud. 1998. Complement enzymes. In *The human Complement System in Health and Disease,* M. Frank, and J. E. Volanakis, eds. Marcel-Dekker, New York, p. 49.
6. Arlaud, G. J., J. Gagnon, C. L. Villiers, and M. G. Colomb. 1986. Molecular characterization of the catalytic domains of human complement serine protease C1r. *Biochemistry 25:5177.*
7. Lacroix, M. B., C. A. Aude, G. J. Arlaud, and M. G. Colomb. 1989. Isolation and functional characterization of the proenzyme form of the catalytic domains of human C1r. *Biochem. J. 257:885.*
8. Lacroix, M., V. Rossi, C. Gaboriaud, S. Chevallier, M. Jaquinod, N. M. Thielens, J. Gagnon, and G. J. Arlaud. 1997. Structure and assembly of the catalytic region of human complement protease C1r: a three-dimensional model based on chemical cross-linking and homology modeling. *Biochemistry 36:6270.*
9. Leytus, S. P., K. Kurachi, K. S. Sakariassen, and E. W. Davie. 1986. Nucleotide sequence of the cDNA coding for human complement C1r. *Biochemistry 25:4855.*
10. pET System Manual TB055, 7th Ed. 4/97. Novagen, Madison, WI.
11. Gill, S. C., and P. H. von Hippel. 1989. Calculation of protein extinction coefficients from amino acid sequence data. *Anal. Biochem. 182:319.*
12. Castillo, M. J., K. Nakajima, M. Zimmerman, and J. C. Powers. 1979. Sensitive substrates for human leukocyte and porcine pancreatic elastase: a study of the merits of various chromophoric and fluorogenic leaving groups in assays for serine proteases. *Anal. Biochem. 99:53.*
13. McRae, B. J., T. Y. Lin, and J. C. Powers. 1981. Mapping the substrate binding site of human C1r and C1s with peptide thioesters. *J. Mol. Biol. 256:12362.*
14. Luo, C., N. M. Thielens, J. Gagnon, P. Gál, M. Sárvári, Y. Tseng, M. Tosi, P. Závodszky, G. J. Arlaud, and V. N. Schumaker. 1992. Recombinant human complement subcomponent C1s lacking β-hydroxyasparagine, sialic acid, and one of its two carbohydrate chains still reassembles with C1q and C1r to form a functional C1 complex. *Biochemistry 31:4254.*
15. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T1. *Nature 227:680.*
16. Privalov, P. L. 1979. Stability of proteins: small globular proteins. *Adv. Protein Chem. 33:167.*
17. Rudolph, R., and H. Lilie. 1996. In vitro folding of inclusion body proteins. *FASEB J. 10:49.*
18. Clark, E. D. B. 1998. Refolding of recombinant proteins. *Curr. Opin. Biotechnol. 9:157.*
19. Thielens, N. M., M. Lacroix, J. Dobó, P. Gál, P. Závodszky, and G. J. Arlaud. 1998. Expression and characterization of the proenzyme and activated forms of the catalytic region of human C1r. *Mol. Immunol. 35:332.*
20. Gál. P., M. Sárvári, K. Szilágyi, P. Závodszky, and V. N. Schumaker. 1989. Expression of hemolytically active human complement component C1r proenzyme in insect cells using a baculovirus vector. *Complement Inflamm. 6:433.*
21. Rossi, V., I. Bally, N. M. Thielens, A. F. Esser, and G. J. Arlaud. 1998. Baculovirus-mediated expression of truncated modular fragments from the catalytic region of human complement serine protease C1s. *J. Biol. Chem. 273:1232.*
22. Monkovic, D. D., W. J. VanDusen, C. J. Petroski, V. M. Garsky, M. K. Sardana, P. Závodszky, A. M. Stern, and P. A. Friedman. 1992. Invertebrate aspartyl/asparaginyl β-hydroxylase: potential modification of endogenous epidermal growth factor-like modules. *Biochem. Biophys. Res. Commun. 189:233.*
23. Orsini, G., and M. E. Goldberg. 1978. The renaturation of reduced chymotrypsinogen A in guanidine HCl: refolding versus aggregation. *J. Biol. Chem. 253:3453.*
24. Gráf, L., C. S. Craik, A. Patthy, S. Roczniak, R. J. Fletterick, and W. J. Rutter. 1987. Selective alteration of substrate specificity by replacement of aspartic acid-189 with lysine in the binding pocket of trypsin. *Biochemistry 26:2616.*
25. Peterson, F. C., N. C. Gordon, and P. G. Gettins. 2001. High-level bacterial expression and ^{15}N-alanine-labeling of bovine trypsin: application to the study of trypsin-inhibitor complexes and trypsinogen activation by NMR spectroscopy. *Biochemistry 40:6275.*
26. Szilágyi, L., E. Kénesi, G. Katona, Gy. Kaslik, G. Juhász, and L. Gráf. 2001. Comparative in vitro studies on native and recombinant human cationic trypsins. *J. Biol. Chem. 276:24574.*
27. Dobó, J., P. Gál., K. Szilágyi, S. Cseh, Z. Lõrincz, V. N. Schumaker, and P. Závodszky. 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.*
28. Gaboriaud, C., V. Rossi, I. Bally, G. J. Arlaud, and J. C. Fontecilla-Camps. 2000. Crystal structure of the catalytic domain of human complement C1s: a serine protease with a handle. *EMBO J. 19:1755.*
29. Gaboriaud, C., V. Rossi, J. C. Fontecilla-Camps, and G. J. Arlaud. 1998. Evolutionary conserved rigid module-domain interactions can be detected at the sequence level: the examples of complement and blood coagulation proteases. *J. Mol. Biol. 282:459.*
30. Arlaud, G. J., S. Chesne, C. L. Villiers, and M. G. Colomb. 1980. A study on the structure and interactions of the C1 sub-components C1r and C1s in the fluid phase. *Biochim. Biophys. Acta 616:105.*
31. Lõrincz, Zs., P. Gál, J. Dobó, S. Cseh, K. Szilágyi, G. Ambrus, and P. Závodszky. 2000. The cleavage of two C1s subunits by a single active C1r reveals substantial flexibility of the C1s-C1r-C1r-C1s tetramer in the C1 complex. *J. Immunol. 165:2048.*
32. Arlaud, G. J., C. L. Villiers, S. Chesne, and M. G. Colomb. 1980. Purified proenzyme C1r: some characteristics of its activation and subsequent proteolytic cleavage. *Biochim. Biophys. Acta 616:116.*