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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

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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)3 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 Clq, two C1r, and two C1s subunits (for reviews, see Refs. 1–3). Clq 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 Ca2+-dependent Cls-C1r-C1r-C1s tetramer, which makes possible the coordinated action of the two enzymes within the Cl complex. This tetramer associates with Clq to yield the heteropentameric C1 complex. C1r and C1s are present in the C1 complex in zymogen form, and become activated after Clq 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 activates 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 thezymogens, and a disulfide bridge holds together the two (A and B) chains of the activated enzyme. C1r is a covalent dimer in the presence or absence of Ca2+ forming the core of the Cls-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 Cls subunit in a Ca2+-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 (γB)2 suggests a loose head-to-tail assembly of the monomers, where the γ-chain (the two CCP modules and the
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 theimerization and how the CCP domains modulate the stability and the proteolytic activity of the serine protease domain. Another objective was to clarify the mechanism of autoactivation of the proenzyme C1r. For this purpose we expressed mutant fragments 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′-GGCAAGCCTTGCCCACGCCCACCGCCAAGCCCAAGCTTA-3′, 5′-GGGAAGCTTGTGGGAAGCCCGTGAA-3′, and 5′-GGCAAGCTTGTGGGAAGCCCGTGAA-3′ in the case of CCP1-CCP2-SP, CCP2-SP, and SP, respectively. The reverse primer was 5′-GGGGGTGCTCACTGCTCCCTCCTCTCCTACT-3′ for each fragment. The PCR products were digested with HindIII and Solf (cleavage sites are underlined) and were ligated into the HindIII/XhoI site of the pET-17b expression vector (Novagen, Madison, WI) in frame with the following Tag sequence 5′-ATGTTGACCCAAAAGCT-3′. As a result of this the recombinant proteins contained four extra amino acids (Ser-Thr-Gln-Val) at their N terminus. The constructs were verified by DNA sequence. 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: GTTGGGACAGGCGAG CAGATAATCGGAGGGCAAAAAG for the Arg463Gln and GCCTGCAGAA-3′, and CAGATAATCGGAGGGCAAAAAG for the Arg463Gln and GCCTGCAGAA-3′, and CAGATAATCGGAGGGCAAAAAG for the Arg463Gln and GCCTGCAGAA-3′, and CAGATAATCGGAGGGCAAAAAG for the Arg463Gln and GCCTGCAGAA-3′, and CAGATAATCGGAGGGCAAAAAG for the Arg463Gln and GCCTGCAGAA-3′.

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 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 kcat/KM were directly determined from the catalytic rate at low substrate concentrations (10–30 μM).

C1r cleavage. Proenzyme C1s was expressed in baculovirus expression system using High Five insect cell culture (Invitrogen, Carlsbad, CA). Purification of proenzyme (90% pure) of 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. The release rate of HS-Bzl was measured through its reactive rate towards 4,4′-dithiobis (12), and was followed with a Jasco V-550 spectrophotometer at a wavelength of 324 nm. Assays were conducted following the method of Gill et al. (15) at 30°C in 20 mM Tris buffer at pH 7.5 containing 145 mM NaCl. Values of kcat/KM were directly determined from the catalytic rate at low substrate concentrations (10–30 μM).

Calorimetric measurements

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, which were stabilized in the zymogen state. Using these mutants we could obtain new information about the details of the autoactivation process.

Esterolytic activity.

The rates of hydrolysis were measured on the 2-Z-Lys-S-Bzl and 2-Z-Gly-Arg-S-Bzl thioesters. The release rate of HS-Bzl was measured through its reactive rate towards 4,4′-dithiobis (12), and was followed with a Jasco V-550 spectrophotometer at a wavelength of 324 nm. Assays were conducted following the method of Gill et al. (15) at 30°C in 20 mM Tris buffer at pH 7.5 containing 145 mM NaCl. Values of kcat/KM were directly determined from the catalytic rate at low substrate concentrations (10–30 μM).

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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 7.4. 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 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 molecule was followed by SDS-PAGE. The proteins were electroblotted onto a nitrocellulose membrane and concentrated.

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 (Cys309, Cys376, Cys451) in the case of CCP1-CCP2-SP (γB), CCP2-SP, and SP, respectively) at the N terminus and ends with Asp705 at the C terminus. The mature proteins have an N-terminal CCP2-SP, and SP, respectively) at the N terminus and ends with glutathione at pH 8.3 in all experiments. 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.](http://www.jimmunol.org/) 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.](http://www.jimmunol.org/) 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 thezymogen 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 andzymogen CCP1-CCP2-SP, respectively; ∼38 and ∼34 kDa for wild-type andzymogen CCP2-SP; and ∼31 and ∼27 kDa for the wild-type andzymogen SP fragment). However, on the reducing gel the wild-typefragments exhibited the activated two-chain forms (∼30 kDa for the B chain and ∼18 kDa for the γ-chain), whereas thezymogen mutants retained a single-chain structure. To prove the correct folding of thezymogen mutants, the renatured proteins were converted into the two-chain form. The Arg463 Gln mutants could be specifically cleaved at theGln 463 –Ile 464 and activated by thermolysin (Fig. 2B). After thermolysin treatment all the three R463Q fragments showed proteolytic and esterolytic activity similar to that of the wild-type autoactivatedproteases (Table I). The Ser654 Ala mutants cannot autoactivate themselves, but wild-type C1r fragments could cleave them at Arg463 –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 renatured C1r fragments, the relative molecular mass of the three C1r fragments was determined by gel permeation chromatography. The molecular mass of the three C1r fragments was 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.

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 (kcat/Km) for the reaction of the C1r fragments with the Z-Lys-S-BzI and Z-Gly-Arg-S-BzI thioesters are presented in Table I. Z-Lys-S-BzI 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-BzI, a more sensitive thioester substrate of C1r, was hydrolyzed at a

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**Table 1.** Catalytic efficiency of wild-type C1r fragments and the thermolysin-activated R463Q mutants for synthetic substrates

<table>
<thead>
<tr>
<th></th>
<th>SP</th>
<th>CCP2-SP</th>
<th>CCP1-CCP2-SP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z-Lys-S-BzI</td>
<td>1,600b</td>
<td>1,900b</td>
<td>1,300b</td>
</tr>
<tr>
<td>Z-Gly-Arg-S-BzI</td>
<td>164,000b</td>
<td>210,000b</td>
<td>174,000b</td>
</tr>
<tr>
<td>Z-Gly-Arg-S-BzI</td>
<td>112,000b</td>
<td>147,000b</td>
<td>124,000b</td>
</tr>
</tbody>
</table>

* kcat/Km (s−1.M−1). Measurements were carried out in 20 mM Tris, 145 mM NaCl, pH 7.4, at 30°C. Values of kcat/Km 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.
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](image-url) 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

<table>
<thead>
<tr>
<th>C1r Fragments</th>
<th>$k_{cat}/K_M$ Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP</td>
<td>28,000 ± 2,000</td>
</tr>
<tr>
<td>CCP2-SP</td>
<td>208,000 ± 10,000</td>
</tr>
<tr>
<td>CCP1-CCP2-SP</td>
<td>58,000 ± 4,000</td>
</tr>
</tbody>
</table>

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 (Asn167 in C1r and Asn149 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...
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\textsuperscript{465}-Ile\textsuperscript{464} cleavage site to Gln-Ile (27), whereas in another set we changed the catalytic site Ser\textsuperscript{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\textsuperscript{654}Ala mutants the Arg-Ile bond can be cleaved 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.

FIGURE 6. Cleavage of CCP2-SP Ser\textsuperscript{654}A 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.

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 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 Ser\textsuperscript{654}A 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 stepzymogen molecules activatezymogens, whereas in the second step the generated active enzymes cleavezymogen molecules. The fact that our wild-typefragments can autoactivate shows the existence of the initial step. The second step could be studied in detail using ourzymogen mutants. The Ser654Alamutants, which have an inactive catalytic center but have a cleavable Arg-Ile bond, were used as substrates for the wild-typefragments. We determined the kinetic constants for these type of reactions (Table III). Each Ser654Alamu tant was cleaved by its own wild-type counterparts. 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-CCP2-SP)2 we follow intermolecular (interdimeric) cleavage, we may conclude that dimerization of the SP domain itself 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.

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References

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