

Assembly and Enzymatic Properties of the Catalytic Domain of Human Complement Protease C1r*

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The catalytic properties of C1r, the protease that mediates activation of the C1 complex of complement, are mediated by its C-terminal region, comprising two complement control protein (CCP) modules followed by a serine protease (SP) domain. Baculovirus-mediated expression was used to produce fragments containing the SP domain and either 2 CCP modules (CCP1/2-SP) or only the second CCP module (CCP2-SP). In each case, the wild-type species and two mutants stabilized in the proenzyme form by mutations at the cleavage site (R446Q) or at the active site serine residue (S637A), were produced. Both wild-type fragments were recovered as two-chain, activated proteases, whereas all mutants retained a single-chain, proenzyme structure, providing the first experimental evidence that C1r activation is an autolytic process. As shown by sedimentation velocity analysis, all CCP1/2-SP fragments were dimers (5.5–5.6 S), and all CCP2-SP fragments were monomers (3.2–3.4 S). Thus, CCP1 is essential to the assembly of the dimer, but formation of a stable dimer is not a prerequisite for self-activation. Activation of the R446Q mutants could be achieved by extrinsic cleavage by thermolysin, which cleaved the CCP2-SP species more efficiently than the CCP1/2-SP species and yielded enzymes with C1s-cleaving activities similar to their active wild-type counterparts. C1r and its activated fragments all cleaved C1s, with relative efficiencies in the order C1r < CCP1/2-SP < CCP2-SP, indicating that CCP1 is not involved in C1s recognition.

C1¹ is the multimolecular protease that triggers the classical pathway of complement, a system that plays a major role in innate immunity against pathogenic microorganisms (2–4). Following recognition of the target microorganism by its C1q subunit, internal activation of the C1 complex is mediated by

C1r, a modular serine protease that first activates itself, and then activates C1s, a second serine protease that is responsible for the specific proteolytic activity of C1 toward its substrates C4 and C2. Both C1r autolytic activation and C1s activation involve limited proteolysis of a single Arg-Ile peptide bond in each protease, converting single-chain proenzymes into active proteases. Both these reactions are mediated by the C-terminal (γ -B)₂ catalytic domain of C1r, a non-covalent homodimer that forms the core of the C1r-C1r dimer (5). Each γ -B monomer comprises, from the N terminus, two complement control protein (CCP) modules and a chymotrypsin-like serine protease domain (6–9). CCP modules are protein motifs of about 60 residues characterized by a β -sandwich structure with N and C termini at opposite ends of an ellipsoid shape (10). This module type is particularly abundant in complement control proteins, where it occurs most frequently in tandem arrays. The solution structure of CCP module pairs has been solved by NMR spectroscopy (11, 12), revealing that these differ very significantly with respect to the degree of flexibility at the module–module interface. In addition to C1r, a number of proteases, including C1s, also feature a CCP module pair preceding a C-terminal serine protease domain, and it has been proposed that the second CCP module and the protease domain form a rigid assembly in this protein family (13–15). Indeed, the crystal structure of the C-terminal fragment from the catalytic domain of human C1s, comprising the second complement control protein module and the serine protease domain, has been solved recently, showing that the ellipsoidal CCP2 module is oriented perpendicularly to the surface of the SP domain and that this arrangement is maintained through a rigid module–domain interface (16).

An intriguing question that is central to the activation mechanism of C1 lies in the presumed ability of the C1r catalytic domains to successively adopt at least two distinct conformations allowing: (i) cleavage of the susceptible Arg-Ile bond in each monomer by the catalytic site of the neighboring monomer; and then (ii) cleavage of a C1s molecule by the active site of each monomer. Recent studies have led to a three-dimensional model of the active form of the dimeric catalytic domain of C1r, in which each active site faces opposite directions toward the outside of the dimer, and lies some 77 Å apart from the Arg⁴⁴⁶-Ile⁴⁴⁷ activation site of the other monomer (14). According to current C1 models (17–19), this conformation is fully consistent with the function of the active form of the protein, but appears not compatible with that of the proenzyme form, providing strong support to the hypothesis that one or several important conformational changes take place upon activation of the C1r catalytic domains.

The objective of the present study was to generate well-defined recombinant material representative of the proenzyme

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¹ The abbreviations used are: the nomenclature of complement proteins is that recommended by the World Health Organization; the nomenclature of protein modules is that defined by Bork and Bairoch (1); Ac-Gly-Lys-OMe, N-acetylglycine-L-lysine methyl ester; CCP modules, complement control protein modules; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; CUB, module found in complement C1r/C1s, Uegf, and bone morphogenetic protein-1; EGF, epidermal growth factor.

and active forms of the C1r catalytic region, to gain precise structural and functional information about each form. For this purpose, fragments of this region, comprising either one or two CCP modules associated to the serine protease domain (CCP1/2-SP and CCP2-SP) were expressed in a baculovirus/insect cell system, either under the wild-type, activable form, or stabilized in the proenzyme form. The latter species were obtained using two types of mutations, one substituting Gln for Arg at the Arg⁴⁴⁶-Ile⁴⁴⁷ bond normally cleaved upon activation (R446Q mutants) and the other converting the active site Ser⁶³⁷ to Ala (S637A mutants). Functional characterization of the different species shows that the wild-type fragments all undergo activation and retain full enzymatic activity, whereas all mutants are stable in the proenzyme form. Analysis by analytical ultracentrifugation indicates that the CCP1/2-SP fragments are dimers, whereas the shorter CCP2-SP fragments are monomers. Taken together, these data point out the essential role of the CCP1 module in the dimerization process and indicate that formation of a stable dimer is not a prerequisite for activation.

EXPERIMENTAL PROCEDURES

Materials—Diisopropyl phosphorofluoridate was from Acros Organics, France. Thermolysin from *Bacillus thermoproteolyticus* was from Roche Molecular Biochemicals, France. Chymotrypsin and elastase were from Sigma Chemical Co. Polyclonal anti-C1r antiserum was raised in rabbits according to standard procedures. Vent_R polymerase was from New England Biolabs. The pHClr3 plasmid containing the full-length C1r cDNA (8) was kindly provided by Dr. Agnès Journet (CEA-Grenoble, France). The pBS-C1s plasmid containing the full-length C1s cDNA (20) was kindly provided by Dr. Mario Tosi (Institut Pasteur, Paris, France). Oligonucleotides were obtained from Oligo Express (Paris, France).

Proteins—Activated C1r was purified from human plasma as described previously (21). Proenzyme recombinant C1s was expressed in a baculovirus/insect cell system. A DNA fragment encoding the full-length protein was amplified by PCR and cloned into the pFastBac1 vector (Life Technologies Inc.). The recombinant baculovirus was generated using the Bac-to-Bac system (Life Technologies Inc.). The recombinant protein was purified from the cell culture supernatant by affinity chromatography on a Sepharose-C1s column as described by Thielens *et al.* (22). The concentrations of purified proteins were determined using the following absorption coefficients ($A_{1\%, 1\text{ cm}}$ at 280 nm) and molecular weights: C1r, 12.4 and 86,300 (22), recombinant C1s, 14.5 (22) and 78,000.² The absorption coefficients ($A_{1\%, 1\text{ cm}}$ at 280 nm) used for the recombinant C1r fragments CCP1/2-SP (15.4) and CCP2-SP (14.9) were calculated from the number of Trp, Tyr, and disulfides according to Edelhoch (23), using average molecular weight values of 49,900 and 40,400, respectively, as determined by mass spectrometry (see "Results").

Construction of C1r Fragments CCP1/2-SP-containing Expression Plasmids—DNA fragments encoding residues 280–688 (CCP1/2-SP) of the mature wild-type and R446Q mutant C1r protein were amplified by PCR using Vent_R polymerase and the pHClr3 (8) and QI mutant (24) C1r cDNAs as templates, respectively, according to established procedures. The sequence of the sense primer a (5'-GGTGATCACGGCTGGAAGCTGCGCTA-3') introduced a *Bcl*I restriction site (underlined), and allowed in-frame cloning with the melittin signal peptide of the pNT-Bac expression vector (25). The antisense primer b (5'-CCCAAGCTTTCAGTCTCTCTCTCCA-3') introduced a *Hind*III restriction site (underlined) at the 3'-end of the two PCR products. The S637A mutant fragment was generated using overlap extension as described by Ho *et al.* (26). The mutagenic primers c (5'-CTGC-CAGGGGGATGCCGGCGGCTTTTTCAGTAAG-3') and the complementary primer d were designed to replace the AGT codon for serine in the wild-type sequence by the GCC codon for alanine (boldface) and to introduce a silent *Ngo*MI restriction site (underlined). Two PCR products were generated using the pHClr3 cDNA as a template, with primers a and d, and c and b, respectively. The overlapping PCR products were fused together in a third PCR, including the outside primers a and b to amplify the fused mutated product. The amplified DNAs corresponding to the wild-type sequence, the R446Q and S637A

mutants were purified using the GeneClean II kit (BIO 101, Inc., Vista, CA), digested with *Bcl*I and *Hind*III, and cloned into the *Bam*HI/*Hind*III sites of the pNT-Bac vector. The resulting constructs were characterized by restriction mapping and checked by double-stranded DNA sequencing (Genome Express, Grenoble).

Construction of C1r Fragments CCP2-SP-containing Expression Plasmids—DNA fragments encoding residues 358–688 of the mature wild-type, R446Q, and S637A mutant C1r protein were amplified by PCR using Vent_R polymerase and the corresponding CCP1/2-SP containing expression plasmids as templates. The antisense primer b was used for the three constructs and the sense primer (5'-GAAGATCTT-GACTGTGGGCGAGCCCCGAAA-3') introduced a *Bgl*II restriction site (underlined) and allowed in-frame cloning with the melittin signal peptide of the pNT-Bac vector for all three fragments. The PCR products corresponding to the wild-type sequence, the R446Q and S637A mutants, were digested with *Bgl*II and *Hind*III, purified, and cloned into the *Bam*HI/*Hind*III sites of the pNT-Bac vector. The final constructs were submitted to double-stranded DNA sequencing.

Cells and Viruses—The *Spodoptera frugiperda* insect cells (Ready-Plaque Sf9 cells from Novagen) were routinely grown and maintained in Sf900 II serum-free medium (Life Technologies Inc.) supplemented with 50 IU/ml penicillin and 50 mg/ml streptomycin (Life Technologies Inc.). The *Trichoplusia ni* (High Five) insect cells were provided by Dr. Jadwiga Chroboczek (Institut de Biologie Structurale, Grenoble). These cells were maintained in TC100 medium (Life Technologies Inc.) containing 10% fetal calf serum (Dominique Dutscher SA, Brumath, France) supplemented with 50 IU/ml penicillin and 50 mg/ml streptomycin. Recombinant baculoviruses were generated using the Bac-to-Bac system (Life Technologies Inc.). The bacmid DNA was purified using the Qiagen midprep purification system (Qiagen SA, Courtaboeuf, France) and used to transfect Sf9 insect cells with cellfectin in Sf900 II SFM medium (Life Technologies Inc.) as recommended by the manufacturer. Recombinant virus particles were collected 4 days later, titered by virus plaque assay, and amplified as described by King and Possee (27).

Protein Production and Purification—High Five cells (1.75×10^7 cells/175-cm² tissue culture flask) were infected with the recombinant viruses at a multiplicity of infection of 2 in Sf900 II SFM medium at 28 °C for 48 h (wild-type species) or 72 h (R446Q and S637A mutants). The supernatants containing the CCP1/2-SP fragments were collected by centrifugation, dialyzed against 25 mM ethanolamine hydrochloride, pH 9.4, and loaded at 1.5 ml/min onto a Q-Sepharose-Fast Flow column (Amersham Pharmacia Biotech) (2.8 × 14 cm) equilibrated in the same buffer. Elution was carried out by applying a 1.2-liter linear gradient from 0 to 500 mM NaCl in the same buffer, and fractions containing the recombinant fragments were identified by Western blot analysis and dialyzed against 1.5 M ammonium sulfate, 50 mM ethanolamine hydrochloride, pH 9.0. Further purification was achieved by high pressure hydrophobic interaction chromatography on a TSK-phenyl 5PW column (7.5 × 75 mm) (Beckman). Elution was carried out by decreasing the ammonium sulfate concentration from 1.5 M to 0 in 30 min at a flow rate of 1 ml/min. The purified fragments were dialyzed against 145 mM NaCl, 25 mM ethanolamine hydrochloride, pH 9.0, and concentrated to 0.3 mg/ml by ultrafiltration on a PM-10 membrane (Amicon).

The supernatants containing the CCP2-SP fragments were treated in the same way, except that elution of the Q-Sepharose-Fast Flow column was carried out by applying a 1-liter linear gradient from 0 to 350 mM NaCl and that the pH of the hydrophobic interaction chromatography buffer and of the storage buffer was decreased to 8.5.

Chemical Characterization of Recombinant Proteins—N-terminal sequence analyses were performed either on soluble proteins or after SDS-PAGE analysis and electrotransfer, using an Applied Biosystems model 477 A protein sequencer as described previously (13). Mass spectrometry analysis of the recombinant proteins was performed using the matrix-assisted laser desorption ionization technique on a Voyager Elite XL instrument (PerSeptive Biosystems, Cambridge, MA), under conditions described previously (14).

Polyacrylamide Gel Electrophoresis and Immunoblotting—SDS-PAGE analysis was performed as described previously (22). Western blot analysis and immunodetection of the recombinant proteins were performed as described (13), using a rabbit polyclonal anti-C1r antiserum (1:400 dilution).

Analytical Ultracentrifugation—Sedimentation velocity analysis was performed using a Beckman XL-I analytical ultracentrifuge and an AN-60 TI rotor (Beckman Instruments). Experiments were carried out at 20 °C in 145 mM NaCl, 25 mM ethanolamine hydrochloride, pH 9.0 (CCP1/2-SP fragments) or pH 8.5 (CCP2-SP fragments). Protein concentrations ranged from 0.14 to 0.23 mg/ml (CCP1/2-SP fragments) and

² S. Pelloux, I. Bally, N. M. Thielens, V. Rossi, and G. J. Arlaud, unpublished data.

from 0.14 to 0.30 mg/ml (CCP2-SP fragments). Samples of 400 μ l were loaded into 12-mm path-length double-sector cells and centrifuged at 42,000 rpm. Absorbance of the samples was recorded at 275 nm. The solvent density and viscosity were calculated as 1.00493 g/ml, and 0.0103 poise, respectively, and the partial specific volume of the proteins was estimated from the amino acid composition at 0.726 ml/g, and 0.721 ml/g for the CCP1/2-SP and CCP2-SP fragments, respectively, using the SEDNTERP program (V1.01; developed by D. B. Haynes, T. Laue, and J. Philo; available at www.bbri.org/RASMB/rasmb.html). Using the SEDFIT program (www.biochem.uthscsa.edu/auc/software), the sedimentation coefficients (s) and molecular masses (M) were modeled from the sedimentation velocity profiles with the non-interacting single component model, taking advantage of a systematic noise evaluation procedure (28, 29). The continuous distributions were obtained considering 200 particles of frictional ratio 1.25 with sedimentation coefficients comprised between 0.2 and 8.0 S, without regularization procedure (F ratio 0.5) (30), and their relevance was evaluated using a Monte Carlo statistical analysis. The maximal axial ratio of the proteins was evaluated using the Teller empirical method of the SEDNTERP program, assuming a prolate ellipsoid shape and a hydration coefficient of 0.4 g of H_2O/g of protein (31).

Activation of the R446Q Mutants by Thermolysin—The R446Q CCP1/2-SP mutant fragment (0.39 mg/ml) was incubated in 145 mM NaCl, 25 mM ethanolamine hydrochloride, pH 9.0, for 5 h at 37 °C in the presence of thermolysin. The initial thermolysin/protein ratio was 2% (w/w), and equivalent amounts of thermolysin were added after every hour of incubation, to reach a final ratio of 10% (w/w). The CCP2-SP mutant fragment was activated under the same conditions, at pH 8.5, for 3 h using a final thermolysin/protein ratio of 6% (w/w). The reaction was stopped by addition of 1 mM 1,10-phenanthroline and 1 mM EDTA.

Functional Assays—Activation of proenzyme C1s was measured by incubation of the protein (4.5 μ M) in the presence of activated C1r, each of the recombinant CCP1/2-SP and CCP2-SP fragments, or the thermolysin-activated R446Q mutant CCP1/2-SP and CCP2-SP fragments (protease/C1s molar ratio = 0.045 or 0.022) for various periods at 37 °C in 145 mM NaCl, 50 mM triethanolamine hydrochloride, pH 7.4. Activation was monitored by SDS-PAGE analysis under reducing conditions and subsequent quantification by gel scanning of the A and B chains characteristic of activated C1s.

The esterolytic activity of C1r and of its recombinant CCP1/2-SP and CCP2-SP fragments was measured on the synthetic ester Ac-Gly-Lys-OMe (Sigma Chemical Co.) using an assay based on the spectrophotometric measurement of methanol released upon hydrolysis (32). The tests were conducted at 30 °C using substrate concentrations varying from 0.2 to 1 mM in 100 mM K_2HPO_4 , pH 8.0.

RESULTS

Expression and Purification of the Recombinant C1r Fragments—The modular structures of human C1r and of the recombinant fragments used in the present study are depicted in Fig. 1. The recombinant baculoviruses for expression of the wild-type, R446Q, and S637A mutant C1r fragments were generated as described under “Experimental Procedures” and used to infect High Five insect cells for various periods at 28 °C. Secretion of the recombinant proteins into the culture medium was monitored by SDS-PAGE and Western blot analysis (not shown). Under non-reducing conditions, the anti-C1r antibody labeled a band of apparent molecular mass 50 or 40 kDa in the supernatants containing the CCP1/2-SP or CCP2-SP fragments, respectively. In all cases, the band became detectable after 24 h of infection and its intensity reached a maximum at 48 h for the wild-type and R446Q species, and at 72 h for the S637A mutants. Under reducing conditions, the 50- or 40-kDa bands were still observed in the case of the R446Q and S637A mutants. In contrast, reduction of the wild-type CCP1/2-SP and CCP2-SP fragments yielded a band at 35 kDa and bands at 19 and 13 kDa, respectively, indicating that these fragments were produced in the activated, two-chain form. The concentrations of recombinant proteins in the 48/72-h culture supernatants were estimated at ~2–4 mg/liter for all fragments.

The supernatants containing the recombinant fragments were initially fractionated by anion-exchange chromatography. The wild-type and mutant fragments eluted at the same posi-

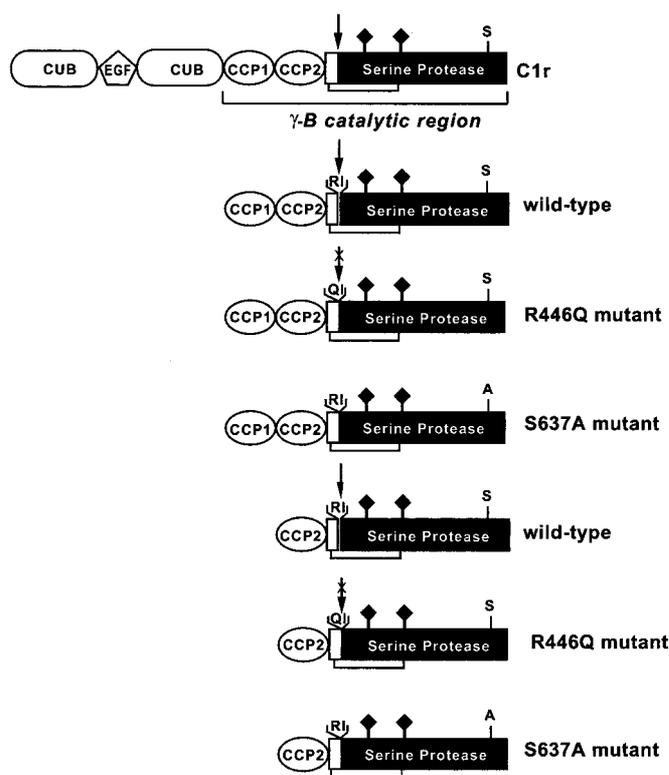


FIG. 1. Schematic representation of the modular structures of human C1r and of the recombinant fragments used in this study. The nomenclature and symbols used for protein modules are those defined by Bork and Bairoch (1). *CUB*, module found in complement C1r/C1s, Uegf, and bone morphogenetic protein-1; *EGF*, epidermal growth factor-like module; the unlabeled segment corresponds to the activation peptide in chymotrypsinogen. The arrow indicates the Arg⁴⁴⁶Ile⁴⁴⁷ bond cleaved upon activation. *S*, active site serine residue. The only disulfide bridge shown is that connecting the activation peptide to the serine protease domain. ♦, oligosaccharides linked to Asn⁴⁹⁷ and Asn⁵⁶⁴.

tion during the ascending salt gradient, along with a major contaminant of mass 67 kDa. Final purification was achieved by hydrophobic interaction chromatography, yielding amounts of purified material of typically 0.4–1 mg/liter of culture.

The recombinant fragments were essentially pure as judged from SDS-PAGE analysis (Fig. 2). The CCP1/2-SP fragments all yielded a single band under non-reducing conditions (Fig. 2A, lanes 1–3), although with quite different apparent mass values (56 and 47 kDa for the wild-type and mutant species, respectively). Under reducing conditions, both mutants retained a single-chain structure (Fig. 2A, lanes 5 and 6) whereas, as already observed by Western blot analysis, the wild-type species yielded two chains (Fig. 2A, lane 4) corresponding to the serine protease domain (35 kDa) and the CCP1/2 moiety (19 kDa). The latter appeared as a doublet in some preparations, with a minor species migrating slightly faster than the major one. Incubation of the wild-type CCP1/2-SP fragment for periods up to 6 h at 37 °C did not significantly modify the proportion of the two species. Analysis of the shorter CCP2-SP fragments yielded comparable results, with apparent mass values of 42 and 39 kDa under non-reducing conditions for the wild-type and mutant fragments, respectively (Fig. 2B, lanes 1–3), and the appearance of two chains at 35 and 13.5 kDa upon reduction of the wild-type fragment (Fig. 2B, lane 4). It was clear, therefore, that both the shorter and larger wild-type species were recovered in a fully activated, two-chain form, whereas all mutants retained a single-chain, proenzyme structure. As judged from SDS-PAGE analysis, no sign of proteolytic degradation was detected upon storage of the

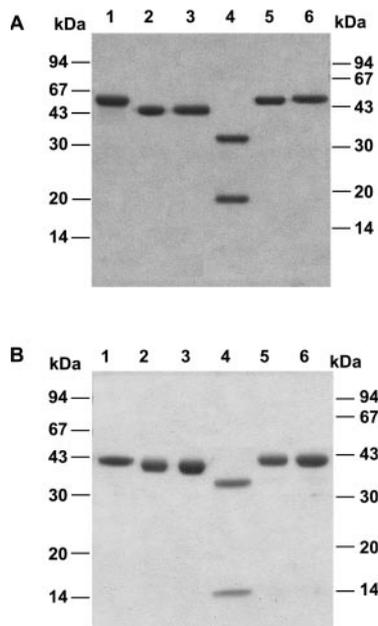


FIG. 2. SDS-PAGE analysis of the recombinant proteins. A, CCP1/2-SP fragments; B, CCP2-SP fragments. Lanes 1 and 4, wild-type fragments; lanes 2 and 5, R446Q mutants; lanes 3 and 6, S637A mutants; lanes 1–3 contain unreduced samples, and lanes 4–6 contain reduced samples. The molecular masses of unreduced and reduced standard proteins are shown on the left and right sides of the gel, respectively.

recombinant proteins for several months at -20°C .

Chemical Characterization of the Recombinant C1r Fragments—Edman degradation of the purified R446Q and S637A CCP1/2-SP fragments yielded a single sequence *Asp-His-Gly-Trp-Lys-Leu-Arg-Tyr-Thr-Thr* . . . , corresponding to the segment *Gly²⁸⁰-Thr²⁸⁷* of human C1r (32) preceded by the two residues *Asp-His* expected to be added at the N terminus, due to in-frame cloning with the signal sequence of melittin (see “Experimental Procedures”) (Table I). In addition to the above sequence, the wild-type species yielded approximately equal amounts of a second sequence *Ile⁴⁴⁷-Ile-Gly-Gly-Gln-Lys-Ala-Lys⁴⁵⁴* . . . , corresponding to the N-terminal end of the serine protease domain of C1r (6), thereby confirming that activation of the fragment had occurred through cleavage of the *Arg⁴⁴⁶-Ile⁴⁴⁷* bond. A third, minor sequence *Tyr²⁸⁵-Thr-Thr-Glu-Ile-Ile-Lys²⁹¹* . . . was also observed in some preparations, indicating partial cleavage at the *Arg²⁸⁴-Tyr²⁸⁵* bond, resulting in removal of the first seven N-terminal residues of the fragment. Partial cleavage at this position was previously shown to occur upon autolytic cleavage of active C1r isolated from human serum (33), and this likely explains the occurrence of the minor CCP1/2 segment species observed by SDS-PAGE analysis of the reduced wild-type CCP1/2-SP fragment. Edman degradation of the purified R446Q and S637A mutant CCP2-SP fragments yielded a single sequence *Asp-Leu-Asp-Cys-Gly-Gln-Pro-Arg-Asn-Leu* . . . , corresponding to the segment *Asp³⁵⁸-Leu³⁶⁵* of human C1r (33) preceded by the two residues *Asp-Leu* expected to be added at the N terminus, due to in-frame cloning with the signal sequence of melittin (see “Experimental Procedures”). The wild-type species also yielded a second sequence, corresponding to the N-terminal end of the serine protease domain of C1r (Table I), thereby confirming the activated state of this fragment.

Analysis of the recombinant proteins by matrix-assisted laser desorption ionization mass spectrometry consistently yielded a wide, heterogeneous peak centered on mass values ranging from $49,657 \pm 25$ to $50,079 \pm 25$ (CCP1/2-SP species)

and from $40,362 \pm 20$ to $40,490 \pm 20$ Da (CCP2-SP species) (Table I). The deduced masses corresponding to the sum of the two oligosaccharides attached to *Asn⁴⁹⁷* and *Asn⁵⁶⁴* (14), range from 2762 ± 20 to 3234 ± 25 Da), and are compatible with the presence of two heterogeneous high mannose oligosaccharides each comprising 2 *N*-acetylglucosamine and 6, 7, or 8 mannose residues (calculated masses 1379, 1541, and 1704 Da). In summary, N-terminal sequence and mass spectrometry analyses clearly showed that all recombinant C1r fragments had the expected sequences, with high mannose oligosaccharide chains attached to *Asn⁴⁹⁷* and *Asn⁵⁶⁴*.

Extrinsic Activation of the Mutant Fragments—As judged from SDS-PAGE analysis under reducing conditions, the four mutants retained a single-chain structure upon prolonged incubation at 37°C , indicating that the preparations were free of contaminating proteases. We next addressed the question whether it was possible to cleave the *Gln⁴⁴⁶-Ile⁴⁴⁷* activation site in the R446Q mutants by means of an extrinsic protease. For this purpose, the CCP1/2-SP R446Q mutant was incubated in the presence of either chymotrypsin, thermolysin, or elastase, using enzyme/protein ratios of 2% (w/w) for 1 h at 37°C . Of these three proteases, only thermolysin yielded the expected activation pattern on SDS-PAGE analysis, *i.e.* a band of decreased mobility under non-reducing conditions, and two bands corresponding to the SP and CCP1/2 fragments under reducing conditions (see Fig. 2A for comparison). Complete activation of the fragment by thermolysin could only be achieved after prolonged incubation (5 h) in the presence of increasing amounts of enzyme (final enzyme/protein ratio = 10%, w/w) (Fig. 3A). Edman degradation of the thermolysin-activated mutant yielded two sequences, *Asp-His-Gly²⁸⁰-Trp-Lys-Leu-Arg-Tyr-Thr-Thr* . . . , and *Ile⁴⁴⁷-Ile-Gly-Gly-Gln-Lys-Ala-Lys* . . . , indicating that specific cleavage had indeed occurred at the *Gln⁴⁴⁶-Ile⁴⁴⁷* bond, in agreement with the known ability of thermolysin to cleave *Xaa-Ile* peptide bonds. The CCP2-SP R446Q mutant fragment could also be cleaved by thermolysin to yield the two-chain activated species. In that case, generation of the fully activated fragment required less thermolysin (final enzyme/protein ratio = 6%, w/w) and a shorter incubation time (3 h) than necessary to achieve full activation of the larger fragment (Fig. 3B).

We next checked whether it was possible to cleave the *Arg⁴⁴⁶-Ile⁴⁴⁷* activation site in the S637A mutant fragments by means of an extrinsic protease. For this purpose, both fragments were incubated in the presence of either trypsin, clostripain, thrombin, or plasmin, using enzyme/protein ratios of 2% (w/w) for 2 h at 37°C . Only clostripain yielded the expected two-chain activation pattern on SDS-PAGE analysis, with cleavage extents of 50 and 80% for the CCP1/2-SP and CCP2-SP fragments, respectively. Trypsin also initially gave the correct fragmentation pattern, but further degradation of both the SP and CCP2 moieties occurred upon further incubation. We also checked whether the CCP2-SP S637A mutant could be activated by the wild-type CCP2-SP species. Incubation for 1 h at 37°C at an enzyme/protein ratio of 2% (w/w) resulted in complete activation of the S637A mutant.

Analytical Ultracentrifugation Analysis of the Recombinant Fragments—The recombinant fragments produced in this study were submitted to sedimentation velocity analysis, and the data were analyzed as described under “Experimental Procedures.” Fig. 4 shows a selection of sedimentation profiles obtained under the same experimental conditions for one CCP1/2-SP fragment (wild-type species, Fig. 4A) and one CCP2-SP fragment (S637A mutant, Fig. 4A’), as well as their modeling using a single component with the corresponding residuals (Fig. 4, B and B’). The corresponding results for all

TABLE I
Mass spectrometry and N-terminal sequence analysis of the recombinant C1r fragments used in this study

C1r fragment	Experimental mass	Calculated mass ^a	Deduced mass of carbohydrates	N-terminal sequence ^b
Wild-type CCP1/2-SP	49,657 ± 25	<i>Da</i> 46,891	2766 ± 25	<i>DHGWKLRYTT</i> . . . IIG GQKAK. . . YTTEIHK. . .
R446Q mutant CCP1/2-SP	50,079 ± 25	46,845	3234 ± 25	<i>DHGWKLRYTT</i> . . .
S637A mutant CCP1/2-SP	49,974 ± 25	46,856	3118 ± 25	<i>DHGWKLRYTT</i> . . .
Wild-type CCP2-SP	40,490 ± 20	37,635	2855 ± 20	<i>DLDCGQPRNL</i> . . . IIGGQKAK. . .
R446Q mutant CCP2-SP	40,405 ± 20	37,588	2816 ± 20	<i>DLDCGQPRNL</i> . . .
S637A mutant CCP2-SP	40,362 ± 20	37,600	2762 ± 20	<i>DLDCGQPRNL</i> . . .

^a Value derived from the amino acid sequence.

^b Residues in italics were introduced due to in-frame cloning with the signal sequence of melittin (see "Experimental Procedures").

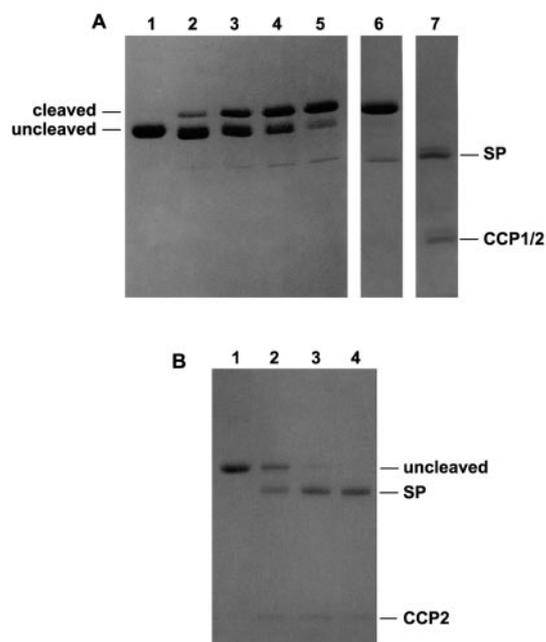


FIG. 3. **Extrinsic activation of the R446Q mutants with thermolysin.** *A*, the mutant CCP1/2-SP fragment was incubated with thermolysin (2%, w/w) added at time 0 and after every hour of incubation at 37 °C in 145 mM NaCl, 25 mM ethanolamine hydrochloride, pH 9. *Lanes 1–6*, unreduced samples, at time 0 and after 1, 2, 3, 4, and 5 h of incubation; *lane 7*, same sample as in *lane 6*, analyzed under reducing conditions. The band migrating above the SP domain is thermolysin. *B*, the mutant CCP2-SP fragment was incubated with thermolysin (2%, w/w) added at time 0 and after every hour of incubation at 37 °C in 145 mM NaCl, 25 mM ethanolamine hydrochloride, pH 8.5. *Lanes 1–4*, reduced samples, at time 0 and after 1, 2, and 3 h incubation.

fragments are given in Table II. All CCP1/2-SP fragments had very similar sedimentation coefficients (5.47–5.59 S), and the deduced molecular masses (86–93 kDa) clearly indicate, when compared with the experimental masses described in Table I, that these fragments are dimers. All CCP2-SP fragments had sedimentation coefficients in the range 3.25–3.35 S, with molecular masses corresponding to monomers (36–39 kDa, compare Tables I and II). In addition, analysis of the sedimentation profiles in terms of a continuous distribution of globular particles showed single narrow peaks in all cases (Fig. 4, *C* and *C'*). This clearly indicates that the samples were homogeneous and did not undergo association-dissociation processes and, thereby, confirms that all CCP1/2-SP and CCP2-SP fragments behave exclusively as dimers and monomers, respectively.

Functional Characterization of the Recombinant Fragments—The esterolytic activity of the wild-type and mutant C1r fragments was tested against the synthetic ester Ac-Gly-Lys-OMe, as described under "Experimental Procedures," and

compared with that of activated C1r isolated from human serum. Activated C1r and its wild-type CCP1/2-SP and CCP2-SP fragments all cleaved the synthetic substrate, with values of K_m and k_{cat} ranging from 1.3 ± 0.2 to 2.1 ± 0.2 mM and 8.2 ± 1.1 to 9.2 ± 1.8 s⁻¹, respectively, *i.e.* values not significantly different from each other. In contrast, none of the single-chain R446Q and S637A species showed detectable esterolytic activity.

To check whether the recombinant fragments displayed proteolytic activity, their ability to activate proenzyme C1s was measured and again compared with that of activated human C1r. As shown in a comparative kinetic analysis (Fig. 5), the wild-type CCP1/2-SP and CCP2-SP fragments were both able to specifically cleave C1s, with initial cleavage rates about 1.5- and 2-fold greater than that of intact active C1r, respectively. This relative increase in C1s cleavage efficiency observed upon decreasing the size of the C1r molecule was observed at two different C1r/C1s molar ratios (0.022 and 0.045). No detectable C1s cleavage was observed when C1s was incubated for 90 min at 37 °C in the presence of either the R446Q mutant (Fig. 6, *A* and *B*) or the S637A mutant (not shown). We next tested the ability of the thermolysin-activated R446Q mutants to mediate C1s cleavage. Both the activated CCP1/2-SP (Fig. 6*A*) and CCP2-SP (Fig. 6*B*) R446Q mutants cleaved C1s very efficiently, with activation kinetics superimposable to that obtained with the corresponding wild-type fragment, indicating that, in both cases, thermolytic cleavage yielded a fully active enzyme with unmodified cleavage specificity.

DISCUSSION

In this study, a baculovirus/insect cell system was used to express different fragments from the C-terminal catalytic region of human C1r, comprising the serine protease domain and either one or two of the preceding CCP modules, both with the wild-type sequence and after mutation of Arg⁴⁴⁶ into Gln at the Arg-Ile activation site, or of the active site Ser⁶³⁷ into Ala. In contrast with the full-length C1r molecule, which has proven very difficult to recover from insect cell cultures (34), or with the N-terminal CUB-EGF fragment of C1r, which is synthesized at a low yield (35), each fragment produced in this study was isolated at a satisfactory yield ranging from 0.4 to 1.0 mg/liter of culture. This yield allowed detailed chemical, physicochemical, and functional characterization of the recombinant material. SDS-PAGE, N-terminal sequence, and mass spectrometry analyses indicated that each fragment had the expected sequence and boundaries. The data obtained by the latter technique were consistent with the occurrence at positions Asn⁴⁹⁷ and Asn⁵⁶⁴ in each recombinant protein of high mannose N-linked oligosaccharides comprising 2 N-acetylglucosamine and 6–8 mannose residues.

From a functional point of view, a major finding is that both wild-type species, whether containing one or two CCP modules,

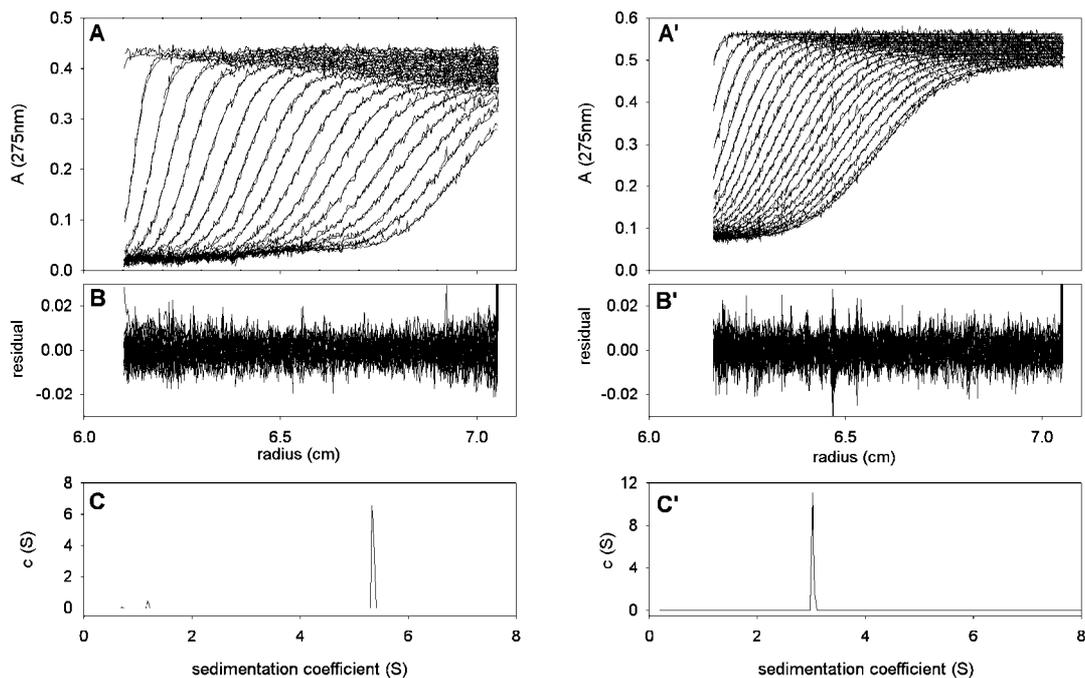


FIG. 4. **Sedimentation velocity analysis of the wild-type CCP1/2-SP and S637A mutant CCP2-SP fragments.** Sedimentation velocity absorbance profiles for the wild-type CCP1/2-SP (A) and S637A mutant CCP2-SP fragments (A') were obtained at 20 °C at a rotor speed of 42,000 rpm and scans were recorded at 275 nm. The last scan shown corresponds to 3.5-h centrifugation. The best-fit profiles corresponding to a single-component model are superimposed to the experimental data, and the corresponding residuals are shown on panels B and B'. Panels C and C' show the continuous distribution of sedimentation coefficients obtained considering globular proteins (see "Experimental Procedures"). The minor peak at 1.2 S on panel C, because non-robust in statistical analysis, is considered as an artifact.

TABLE II

Sedimentation velocity analysis of the recombinant fragments

Sedimentation coefficients and molecular masses were obtained by fitting sedimentation velocity profiles considering the model of one non-interacting species, as described under "Experimental Procedures."

Protein	$s_{20,w}$	Molecular mass
	<i>S</i>	<i>kDa</i>
CCP1/2-SP, wild-type	5.55 ± 0.05	86 ± 9
CCP1/2-SP, R446Q mutant	5.59 ± 0.05	93 ± 9
CCP1/2-SP, S637A mutant	5.47 ± 0.05	87 ± 9
CCP2-SP, wild-type	3.34 ± 0.05	38 ± 4
CCP2-SP, R446Q mutant	3.35 ± 0.05	36 ± 4
CCP2-SP S637A mutant	3.25 ± 0.05	39 ± 4

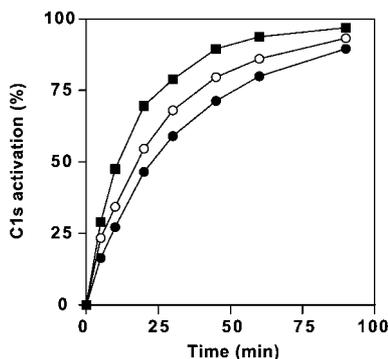


FIG. 5. **Kinetic analysis of the activation of C1s by activated C1r and the wild-type CCP1/2-SP and CCP2-SP fragments.** Proenzyme C1s was incubated with activated C1r (●), the CCP1/2-SP (○), and the CCP2-SP fragments (■) at enzyme/C1s molar ratios of 0.022 for various periods at 37 °C, in 145 mM NaCl, 50 mM triethanolamine hydrochloride, pH 7.4. Activation was monitored by SDS-PAGE quantification of the two-chain structure characteristic of activated C1s.

were recovered as two-chain, active species, indicating that cleavage of the susceptible Arg⁴⁴⁶-Ile⁴⁴⁷ bond had occurred in both cases during the synthesis process. In contrast, the four

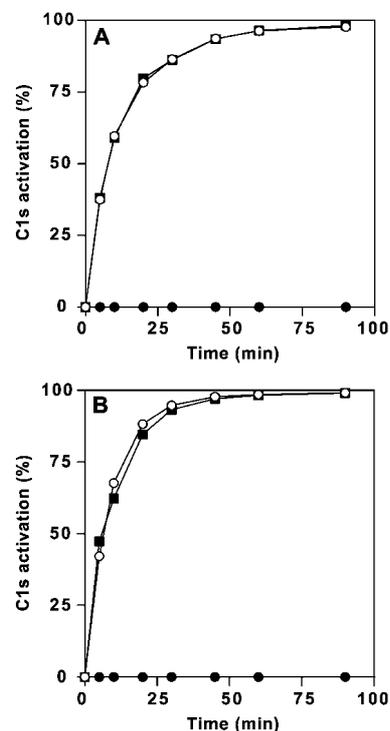


FIG. 6. **Kinetic analysis of the activation of C1s by the wild-type and thermolysin-activated R446Q fragments.** A, CCP1/2-SP fragments; B, CCP2-SP fragments. Proenzyme C1s was incubated with the wild-type (○), R446Q (●), or thermolysin-activated R446Q fragment (■) at enzyme/C1s molar ratios of 0.045. Activation was monitored as described in the legend to Fig. 5.

R446Q and S637A mutants were all recovered in a totally uncleaved, proenzyme form, and were fully resistant to activation when submitted to further incubation after purification. It should be emphasized that the ability of C1r to self-activate has

long been a controversial issue (2), because most of the studies on this question had been conducted on the protease isolated from serum, and hence the involvement of a contaminating protease could not be ruled out. In this respect, the observation that conversion of the Arg-Ile cleavage site into Gln-Ile, or substitution of Ala for the active site Ser totally prevents activation, provides the first experimental evidence that C1r activation is indeed a self-activation process. Thus, substitution of Ser⁶³⁷ for Ala is obviously expected to produce an inactive enzyme, because the reactive hydroxyl group of the active site serine is essential to the activity of a serine protease. In the same way, conversion of Arg⁴⁴⁶ into Gln is also expected to prevent the activation process, because C1r only cleaves a restricted number of arginyl and lysyl bonds (32, 36), and therefore is not expected to cleave a Gln-Ile bond.

A further important finding of this study lies in the observation that, although all larger CCP1/2-SP species form stable dimers as shown by analytical ultracentrifugation analysis, all shorter CCP2-SP species are monomers. This observation has important implications: (i) From a structural point of view, this demonstrates that the N-terminal CCP module plays an essential role in the assembly of the C1r-C1r dimer. This is in complete agreement with previous studies based on chemical cross-linking, suggesting that the catalytic domain of C1r dimerizes through a loose "head-to-tail" association involving contacts between the N-terminal CCP module of one monomer and the serine protease domain of the other monomer (14). (ii) From a functional point of view, it is noteworthy that both wild-type recombinant molecules produced in this study, either containing two CCP modules or only the second CCP module, undergo self-activation during the synthesis process. It may be concluded, therefore, that formation of the stable dimer characterized by ultracentrifugation is not a prerequisite for the self-activation process. On the other hand, it is clear from the three-dimensional homology modeling of the C1r catalytic region (14) and from the x-ray structure of the corresponding region of C1s (16) that activation cannot take place within an isolated C1r catalytic domain, because the active site and the Arg-Ile activation site are located well apart from each other (about 25 Å in the C1s structure). This implies that C1r activation requires cleavage of monomer 1 by monomer 2, and conversely. Therefore it may be hypothesized that activation probably takes place through formation of a transient dimer with a configuration that is distinct from that of the stable dimer requiring the first CCP module. The observation that the CCP2-SP S637A mutant is efficiently cleaved by catalytic amounts of the wild-type CCP2-SP species implies that both monomers must interact transiently and provides full support to the above hypothesis.

The maximal *a/b* axial ratios derived from the *S* values assuming a hydration coefficient of 0.4 g of H₂O/g of protein and a prolate ellipsoid shape were estimated at 3.1–3.5:1 for the monomeric CCP2-SP species, and 4.4–4.7:1 for the dimeric CCP1/2-SP species. These values indicate that both types of molecules are rather elongated and confirm previous observations based on neutron scattering (37) and homology modeling (14) of the C1r catalytic domains, both consistent with a loose, end-to-end association of the monomers. The functional experiments performed in this study yield information about the relative accessibility of the cleavage site and of the active site in the dimeric and monomeric species. Thus, our data clearly indicate that activation of both the R446Q and S637A mutants can be achieved using extrinsic proteases with appropriate cleavage specificities. However, as best illustrated in the case of the R446Q mutant, complete activation by thermolysin requires significantly higher enzyme/C1r ratios and longer incu-

bation periods for the dimeric CCP1/2-SP species than for the monomeric CCP2-SP species. This suggests that access of the cleavage site to an activating extrinsic protease may be restricted in the dimer and made easier in the monomer. However, the above difference possibly arises from the structure and specificity of thermolysin itself rather than from the accessibility of the susceptible bond. We also provide evidence that the wild-type CCP1/2-SP and CCP2-SP species both specifically activate C1s, with relative cleavage efficiencies about 1.5- and 2-fold higher than that of intact C1r. It appears therefore that progressive removal of modules at the N-terminal end of the C1r molecule (namely the CUB-EGF-CUB moiety, and then the first CCP module) significantly increases its C1s-cleaving activity in solution. In the case of the CCP1/2-SP species, the observed increase may be related in part to a better accessibility of the active site, because the CUB-EGF-CUB segment of C1r has no known implication in its proteolytic activity (35), and may even generate steric hindrance in solution due to inter-modular flexibility. As for the shorter CCP2-SP species, the increased C1s-cleaving efficiency may obviously be an indirect consequence of the removal of the first CCP module, because this leads to dissociation into monomers, which individually are possibly more efficient than dimers in solution. In this respect, it should be kept in mind that C1s activation by C1r does not normally take place in solution, but within the C1 complex. It is clear, however, that removal of the first CCP module does not impair C1r ability to cleave C1s. It may be concluded, therefore, that the C-terminal CCP2-SP segment of C1r contains the structural determinants required for efficient recognition and cleavage of C1s within the C1 complex. In contrast, the first CCP module of C1r is not directly involved in its proteolytic activity but plays an essential role in the assembly of the C1r-C1r dimer.

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