The Ca^{2+}-dependent interaction between complement serine proteases C1r and C1s is mediated by their α regions, encompassing the major part of their N-terminal CUB-EGF-CUB (where EGF is epidermal growth factor) module array. In order to define the boundaries of the C1r domain(s) responsible for Ca^{2+}-binding and Ca^{2+}-dependent interaction with C1s and to assess the contribution of individual modules to these functions, the CUB, EGF, and CUB-EGF fragments were expressed in eucaryotic systems or synthesized chemically. Gel filtration studies, as well as measurements of intrinsic Tyr fluorescence, provided evidence that the CUB-EGF pair adopts a more compact conformation in the presence of Ca^{2+}.

The Ca^{2+} dependence of the interaction between intact C1r with C1s was studied using surface plasmon resonance spectroscopy, yielding K_D values of 10.9–29.7 nM. The C1r CUB-EGF pair bound immobilized C1s with a higher K_D (1.5–1.8 μM), which decreased to 31.4 nM when CUB-EGF was used as the immobilized ligand and C1s was free. Half-maximal binding was obtained at comparable Ca^{2+} concentrations ranging from 5 μM with intact C1r to 10–16 μM for C1rα and CUB-EGF. The isolated CUB and EGF fragments or a CUB + EGF mixture did not bind C1s. These data demonstrate that the C1r CUB-EGF module pair (residues 1–175) is the minimal segment required for high affinity Ca^{2+} binding and Ca^{2+}-dependent interaction with C1s and indicate that Ca^{2+} binding induces a more compact folding of the CUB-EGF pair.

C1^1 (1) is the complex modular protease that triggers the classical pathway of complement in response to the formation of antigen-antibody complexes and to infection by various microorganisms. The human C1 complex comprises three types of proteins (C1q, C1r, and C1s) that form two distinct entities: the recognition subunit C1q and the catalytic subunit C1s-C1r-C1r-C1s, a Ca^{2+}-dependent tetrameric assembly of the two homologous but distinct serine proteases C1r and C1s. Binding of C1 to the target microorganism is mediated by C1q and triggers autocatalytic activation of C1r into C1r, which in turn converts C1s into C1s, the protease responsible for enzymic activity of C1 (2–4). Both steps of the C1 activation process occur through cleavage of a single peptide bond in the proenzymes, generating active proteases comprising two disulfide-linked chains. C1r and C1s are modular proteins exhibiting homologous overall structures comprising, from the N terminal, two CUB modules (5) surrounding a single EGF-like module, two CCP modules (6), a connecting segment homologous to the activation peptide in chymotrypsinogen, and a serine protease domain. In the same way, each monomeric protease is thought to be organized in two functional regions, a C-terminal catalytic region and an N-terminal (α) interaction region.

A particular feature of C1r and C1s is that they exert their catalytic activities within the Ca^{2+}-dependent C1s-C1r-C1r-C1s complex. The non-covalent C1r-C1r homodimer forms the core of the tetramer, with its catalytic regions in the center, and each of its distal α regions is connected in a Ca^{2+}-dependent fashion to the homologous region of a C1s molecule. N-terminal fragments corresponding to the α regions of C1r and C1s, each comprising the first CUB module, the EGF-like module, and a small N-terminal portion of the following second CUB module, have been obtained by limited proteolysis with trypsin in the presence of Ca^{2+} (7, 8). Each of these fragments contains a single high affinity Ca^{2+}-binding site and retains the ability to mediate Ca^{2+}-dependent heterologous (C1r/C1s) interaction (8). The α regions of C1r and C1s both exhibit similar low temperature transitions, with midpoints of 26–31 °C, which are shifted upward at high ionic strength or in the presence of Ca^{2+} ions (7, 9). The α regions of both C1r and C1s also likely participate in the interaction between the C1s-C1r-C1r-C1s tetramer and C1q and therefore represent key elements of the architecture of the C1 complex (10, 11). In addition to their structural role, various studies suggest that the α regions of C1r may control autoactivation of the catalytic regions of the protease (11).

Previous studies performed on C1s have suggested that the structural determinants required for Ca^{2+} binding and inter-
action with C1r are contributed by both the N-terminal CUB and EGF modules (12, 13), an hypothesis that is supported by recent functional studies on the recombinant CUB-EGF module pair (14). In the case of C1r, a deletion mutant lacking the first CUB module was shown to lose the ability to bind C1s in the presence of Ca\textsuperscript{2+} (15). Conversely, the isolated EGF module of C1r, which exhibits the consensus sequence pattern characteristic of the particular subset of EGF modules involved in Ca\textsuperscript{2+} binding (16), retains the ability to bind Ca\textsuperscript{2+} ions. However, compared with the whole C1r fragment, its affinity for Ca\textsuperscript{2+} is decreased about 300-fold (17, 18), strongly suggesting that Ca\textsuperscript{2+} binding by C1r involves residues located outside the EGF module. The objective of the present study was to measure the ability of the N-terminal CUB-EGF module pair of C1r to bind Ca\textsuperscript{2+} ions and to mediate Ca\textsuperscript{2+}-dependent interaction with C1s, as well as to assess the contribution of the individual CUB and EGF modules to these functions. For this purpose, the CUB module and CUB-EGF pair were expressed in eukaryotic systems, and the EGF module was synthesized chemically. Comparative analysis of the physicochemical and functional properties of these recombinant molecules indicates that the EGF-EGF module pair binds Ca\textsuperscript{2+} with high affinity and mediates Ca\textsuperscript{2+}-dependent interaction with C1s and supports the hypothesis that Ca\textsuperscript{2+} induces a more compact conformation of this domain.

EXPERIMENTAL PROCEDURES

Materials.—Disopropyl phosphorofluoridate and trypsin from bovine pancreas (treated with 1-chloro-4-phenyl-3-(L-tosylamido)-butan-2-one) were from Sigma. Peptide-N-glycosidase F was purified from cultures of Flavobacterium meningosepticum according to the method of Tarentino et al. (19), modified as described by Aude et al. (20). Polyclonal anti-C1r antiserum was raised in rabbits according to standard procedures. Restriction enzymes were from Boehringer Mannheim. Vent\textsubscript{r} polymerase was from New England Biolabs. The pHC1r3 plasmid containing full-length C1r cDNA (21) was kindly provided by Dr Agnes Journet (Commissariat à l`Energie Atomique, Grenoble, France). Antibiotics and molecular biology reagents were from Appligene Oncor. Oligonucleotides were obtained from Life Technologies, Inc. (Cergy-Pontoise, France).

Protocols.—Activated C1r and C1s were purified from human plasma as described previously (22). Their C1rc and C1sc fragments were obtained by limited proteolysis with trypsin and purified as described previously (8). The EGF-like module from C1r was synthesized chemically and characterized as described (17). The concentrations of purified proteins were determined using the following absorption coefficients obtained by limited proteolysis with trypsin and purified as described (8). The sequences of the sense (5\textsuperscript{'}-GGGTACCTAGGGaaGAGTGTTAAGA-3\textsuperscript{'}) and antisense (5\textsuperscript{'}-GGGTTACCTAGGGaaGAGTGTTAAGA-3\textsuperscript{'}) primers introduced an EcoRI site (underlined) at the 5\textsuperscript{'} end of the PCR product and a stop codon (boldface) followed by a KpnI site (underlined) at the 3\textsuperscript{'} end. The amplified DNA was purified using the GeneClean kit (Bio 101) and subcloned into the pCR-Script Amp SK(+) intermediate vector (Stratagene) according to the manufacturer’s instructions. The fragment was excised by digestion with EcoRI and KpnI and cloned into the EcoRI/KpnI sites of the pFastBac baculovirus transfer vector (Life Technologies, Inc.). The resulting construct was characterized by restriction mapping and checked by double-stranded DNA sequencing (Genome Express, Grenoble, France).

The recombinant baculovirus was generated using the Bac-to-BacTM system (Life Technologies, Inc.). The bacmid DNA was purified using the Qiagen midiprep purification system (Qiagen S.A., Courtaboeuf, France) and used to transfect Sf9 insect cells with Cellfectin (Life Technologies, Inc.) in SF900 II SFM medium as described by the manufacturer. Recombinant virus particles were collected 4 days later, titered by virus plaque assay, and amplified as described by King and Possee (25).

High Five cells (1.75 \times 10^6 cells/175-cm\textsuperscript{2} tissue culture flask) were infected with the recombinant virus at a multiplicity of infection of 2 in SF900 II SFM medium for 84 h at 28 °C. The supernatant was collected by centrifugation, and diisopropyl phosphorofluoridate was added to a final concentration of 1 mM.

Cloning and Expression of the C1r CUB Fragment in P. pastoris.—A DNA fragment encoding the CUB module of C1r (amino acids 1–124 of the mature protein) was amplified by PCR using the Vent\textsubscript{r} polymerase and pHClr3 as a template. The sequence of the sense primer (5\textsuperscript{'}-GGGTACCTAGGGaaGAGTGTTAAGA-3\textsuperscript{'}) introduced an SnaB1 restriction site (underlined) at the 5\textsuperscript{'} end of the PCR product and allowed in-frame cloning with the N- and C-terminal yeast secretion signal. The antisense primer (5\textsuperscript{'}-GGGTTACCTAGGGaaGAGTGTTAAGA-3\textsuperscript{'}) introduced a stop codon (boldface) followed by an AvrII restriction site (underlined) at the 3\textsuperscript{'} end of the PCR product. The purified amplified DNA fragment was subcloned into the pCR-Script Amp SK(+) intermediate vector (Stratagene), excised with SnaB1 and AvrII, and ligated with the pPIC9K yeast expression vector (Invitrogen). The final construct was submitted to double-stranded DNA sequencing. Ten \mu g of recombinant plasmid DNA were linearized with SacI and used for transformation of P. pastoris GS115 cells by electroporation as described in the user’s manual of the Pichia Expression kit (Invitrogen). Histidine-independent transformants were selected on MD plates, replicated on MM plates, and allowed to grow for 2 days at 30 °C. Pure methanol (0.5% v/v) was supplied every 24 h inside the lid of the plates. The colonies were then screened for CUB expression by overlaying the plates with a 0.2-\mu m nitrocellulose disc (Schleicher & Schuell) overnight at 30 °C and probing it with polyclonal anti-C1r antibodies. Screening for Mut “Mut” phenotypes indicated that the clone yielding the strongest CUB immunoreactive signal was Mut\textsuperscript{+}. An optimal methanol induction time of 30 h for liquid medium expression was determined for this clone as described in the Pichia Expression kit manual (Invitrogen).

The colony expressing the recombinant CUB fragment was grown overnight in 10 ml of BMGY. This culture was inoculated into 150 ml of BMGY and grown at 30 °C in a shaking incubator until the A\textsubscript{600} reached a value of 6.0. The cells were collected by centrifugation at 1500 \times g, resuspended in 600 ml of BMGY, and cultured for 30 h, and pure methanol was added to a final concentration of 0.5% after 24 h of induction. The supernatant was separated from the cells by centrifugation, and diisopropyl phosphorofluoridate was added to a final concentration of 1 mM.

Purification of the Recombinant CUB-EGF Fragment.—The culture supernatant containing the CUB-EGF fragment was dia lyzed against 50 mM NaCl, 1 mM CaCl\textsubscript{2}, 50 mM Tris-HCl, pH 8.5, and loaded at 1.5 ml/min onto a Q-Sepharose-Fast Flow column (Amersham Pharmacia Biotech) (2.8 \times 14 cm) equilibrated in the same buffer. Elution was carried out by applying a 1.2-liter linear gradient from 50 to 500 mM NaCl in the same buffer, and fractions containing the recombinant fragments were identified by Western blot analysis.
The CUB-containing culture supernatant was dialyzed against 25 mM MES, pH 6.0, and loaded at 1 ml/min onto a carboxymethylcellulose CM52 column (2.8 × 14 cm) (Whatman) equilibrated in the same buffer. Elution was carried out by applying a 1-liter linear gradient from 0 to 150 mM NaCl in the same buffer. Fractions containing the recombinant fragment were identified by Western blot analysis.

Fractions containing the CUB-EGF or CUB fragments were pooled and dialyzed against 1.0 mM ammonium sulfate, 1 mM CaCl₂, 0.1 mM triethanolamine hydrochloride, pH 7.4. Further purification of both fragments 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 1.0 to 0 in 20 min at a flow rate of 1 ml/min. The purified fractions were dialyzed against 145 mM NaCl, 50 mM triethanolamine hydrochloride, pH 7.4, and concentrated if necessary to 0.1 mg/ml by ultrafiltration on Microsep microconcentrators (molecular weight cutoff = 3,000) (Milltron).

**Chemical Characterization of Recombinant Proteins—**N-terminal sequence analyses were performed after SDS-PAGE and electrophoresis, using an Applied Biosystems model 477 A protein sequencer as described previously (26). Mass spectrometry analysis of the recombinant proteins was performed using the matrix-assisted laser desorption ionization technique on a Voyager Elite XI instrument (PerSeptive Biosystems, Cambridge, MA), under conditions described previously (27).

**Elution of the Reimmobilized Proteins—**A 10-fold concentrated culture supernatants containing the CUB-EGF or CUB fragments were incubated with 0.5 μg of peptide-N-glycosidase F for 4 h at 25 °C. Deglycosylation of the recombinant fragments was monitored by SDS-PAGE and Western blot analysis of the samples. The purified CUB-EGF fragment (0.1 mg/ml) in 145 mM NaCl, 50 mM triethanolamine hydrochloride, pH 7.4, was incubated in the presence of 20% (v/w) peptide-N-glycosidase F for 20 h at 25 °C. Protein deglycosylation was monitored by SDS-PAGE analysis of the samples.

**Polycrylamide Gel Electrophoresis and Immunoblotting—**SDS-PAGE analysis was performed as described previously (8). Western blot analysis and immunodecoration of the recombinant proteins were performed as described (28), using rabbit polyclonal anti-C1r antisera (1:400 dilution).

**High Pressure Gel Permeation—**The C1r CUB-EGF and α fragments (190 pmols each), either alone or in equimolar mixture with C1α, were analyzed by high pressure gel permeation on a TSK G3000 SWG column (7.5 × 600 mm) (Toso Haas) equilibrated in 145 mM NaCl, 50 mM triethanolamine hydrochloride, pH 7.4, containing EDTA (2.5 mM) or CaCl₂ (2.5 or 0.2 mM), and run at 1 ml/min. Samples volumes ranged from 42 to 56 μl. Proteins were detected from their absorbance at 280 nm.

**Real Time Surface Plasmon Resonance Measurements and Data Evaluation—**Real-time analysis of the Ca²⁺-dependent interaction between C1r or its fragments and C1s or C1α was performed at 25 °C using an upgraded BIACore™ instrument (BIACore AB). The running buffer for protein immobilization was 145 mM NaCl, 5 mM EDTA, 10 mM HEPES, pH 7.4. Protein ligands were diluted to 10–20 μg/ml either in 10 mM formate, pH 3.0 (C1r, CUB, and C1α), or in 10 mM acetate, 10 mM NaCl, pH 4.8 (CUB-EGF and unglycosylated CUB-EGF), and coupled to the carboxymethylated dextran surface of a CM5 sensor chip (BIACore AB) using the amine coupling chemistry (BIACore AB amine coupling kit) according to the manufacturer’s instructions. Binding of C1r and its fragments was measured over 530 and 3100 resonance units (RU) of immobilized C1s (or 150 and 600 RU of C1α), at a flow rate of 10 μl/min in 145 mM NaCl, 0.25 mM CaCl₂, 50 mM triethanolamine hydrochloride, pH 7.4. Binding of C1s was measured under the same conditions as above, over 1000 RU of immobilized C1r, 150 and 450 RU of CUB-EGF, or 350 RU of unglycosylated CUB-EGF. Binding of C1α was measured over 3200 RU of immobilized C1r. Equivalent volumes of each protein sample were injected over a surface with immobilized bovine serum albumin (instead of C1s or C1r) or ovalbumin (instead of C1α or CUB-EGF) to serve as blank sensorgrams for subtraction of bulk refractive index background. Regeneration of the surfaces was achieved by injection of 10 μl of 20 mM EDTA. The effect of Ca²⁺ concentration on the interaction between C1r or its fragments and C1s was studied in 145 mM NaCl, 50 mM triethanolamine hydrochloride, pH 7.4, containing 1 mM EGTA and varying amounts of CaCl₂ calculated to give the desired free calcium concentrations as described (29).

Sensorgrams were analyzed by non-linear least squares curve fitting using the BIAevaluation 2.1 Software (Amersham Pharmacia Biotech). A single-site binding model was used for kinetic analysis of all interactions. The equation $R_i = R_i(1 - \exp(-k_{on}(t - t_o)))$ was used for the dissociation phase, where $R_i$ is the amount of ligand (in RU) remaining bound at time $t$, and $t_o$ is the beginning of the dissociation phase. The final dissociation rate constant $k_{off}$ was calculated from the mean of the values obtained from a series of injections. To analyze the association phase, the equation $R_i = R_i_0(1 - \exp(-k_{on}(t - t_o)))$ was employed, where $R_i_0$ is the amount of bound ligand (in RU) at equilibrium, $t_o$ is the starting time of injection, and $k_{on}$ is the maximum binding rate constant. Protein deglycosylation was monitored by SDS-PAGE and Western blot analysis of the samples.

The apparent equilibrium dissociation constant ($K_{D(rein)}$) was calculated from the ratio of these two kinetic constants ($k_{on}/k_{off}$).

Whenever this was possible, i.e. when the binding reaction reached or approached equilibrium, the association constant ($K_{D(rein)}$) was also determined from equilibrium levels of the analyte binding to the surface ($R_i_0$). The equation $R_i_0/C = K_{D(rein)} × R_{max} - R_{max} × R_{eq}$ was used, where $R_{max}$ is the maximum capacity of the immobilized ligand, and the association constant $K_{D(rein)}$ was determined from the slope of a Scatchard plot of $R_i/C$ versus $R_{eq}$. The dissociation constant derived from $K_{D(rein)}$ is referred to in the text as $K_{D DispatchQueue}$.

**Fluorescence Measurements—**Intrinsic protein fluorescence was measured at 20 °C using a SLM-Aminco Bowman Series 2 Luminescence Spectrometer at a photomultiplier voltage of 900 V with a 10-mm path length cell. The excitation wavelength was 275 nm, and the band path was 4 nm. Emission spectra were recorded at a scanning rate of 1 nm/s in the 285–355 nm range using a band path of 4 nm. All measurements were corrected for buffer (145 mM NaCl, 50 mM triethanolamine hydrochloride, pH 7.4, containing 0.1 mM EGTA or 0.9 mM CaCl₂) background emission.

**RESULTS**

**Expression of the Recombinant C1r Fragments—**The modular structures of human C1r and of the various fragments used in this study are depicted in Fig. 1. The recombinant baculovirus for expression of the CUB-EGF fragment of C1r was generated as described under “Experimental Procedures” and used to infect Sf9 and High Five insect cells for various periods at 28 °C. Secretion of the recombinant protein into the culture medium was monitored by SDS-PAGE and Western blot analysis, as illustrated in Fig. 2. The anti-C1r antibody labeled a band of apparent molecular mass 20 kDa in both cell culture supernatants, a major, broader band of about 22 kDa in the case of High Five cells (Fig. 2B), and two minor bands of 21 and 22 kDa in the case of Sf9 cells (Fig. 2A). All bands became detectable after 24 h of infection, and their intensity reached a maximum at 96 h. Incubation of the 96-h Sf9 culture medium with CUB-EGF and CUB fragments was achieved under the same conditions as sample were injected over a surface with immobilized bovine serum albumin. Elution was carried out by decreasing the ammonium sulfate concentration from 1.0 to 0 in 20 min at a flow rate of 1 ml/min. The purified fragments were dialyzed against 145 mM NaCl, 50 mM triethanolamine hydrochloride, pH 7.4, and concentrated if necessary to 0.1 mg/ml by ultrafiltration on Microsep microconcentrators (molecular weight cutoff = 3,000) (Milltron).
The amounts of purified proteins obtained were typically about 0.5 mg/ml culture medium for the glycosylated and unglycosylated CUB-EGF species, respectively. Both were very sensitive to aggregation and could not be concentrated over 0.1 mg/ml.

The recombinant CUB fragment was purified by fractionation of the culture supernatant by cation-exchange chromatography, followed by the same hydrophobic interaction chromatography step as above. The CUB fragment also was hydrophobic, as it eluted between the glycosylated and unglycosylated CUB-EGF species and was prone to aggregation upon concentration above 0.1 mg/ml. The amount of purified fragment obtained was typically 0.5 mg/ml culture.

The recombinant CUB fragments were essentially pure as judged from SDS-PAGE analysis (Fig. 3). Each yielded a single band, which was more diffuse in the case of the glycosylated CUB-EGF fragment, with apparent molecular masses of 21.5 (glycosylated CUB-EGF), 20 (unglycosylated CUB-EGF), and 14 kDa (CUB), consistent with expected values. Incubation of the purified glycosylated CUB-EGF fragment with 7% (w/w) peptide: N-glycosidase F for 1 h at 25 °C, i.e., under conditions known to remove both N-linked oligosaccharides at positions 108 and 204 of C1r (8), did not yield significant deglycosylation. Incubation of the fragment with 20% (w/w) enzyme for 20 h at 25 °C led to the appearance of a 20-kDa deglycosylated species, but deglycosylation remained partial (~50%) under these conditions. No sign of proteolytic degradation of the recombinant proteins was detected by SDS-PAGE analysis upon storage of the recombinant proteins for several weeks at 0 °C.

**Chemical Characterization of the Recombinant C1r Fragments**—Edman degradation of both purified CUB-EGF species yielded a single sequence Ser-Ile-Pro-Ile-Pro-Gln-Lys-Leu-Phe-Gly . . . , corresponding to the N-terminal end of C1r. The CUB fragment also yielded a single sequence Tyr-Val-Ser-Ile-Pro-Ile-Pro-Gln-Lys-Leu . . . , corresponding to the above sequence preceded by the two residues Tyr-Val expected to be added at the N terminus, due to in-frame cloning with the signal sequence of the yeast a-factor (see “Experimental Procedures”).

**Analysis** by matrix-assisted laser desorption ionization mass spectrometry of the various recombinant CUB-EGF species is summarized in Table I. Both the unglycosylated and the enzymatically deglycosylated fragment exhibited a major peak (approximately 70% of the total material) with a mass consistent with the sequence of the N-terminal Ser–Glu<sup>179</sup> C1r fragment (calculated mass = 19,790 Da). A minor peak with an average extra mass of 164 ± 10 Da was consistently observed in both cases. This difference probably accounts for covalent attachment of a diisopropyl phosphate group (164 Da), considering that diisopropylphosphorofluoridate was present at all steps of the purification procedure and that nonspecific labeling of the C1r interaction region with radioactive diisopropyl phosphorofluoridate has been previously reported (30). Analysis of the
glycosylated CUB-EGF species yielded a wide, heterogeneous peak comprising four major components (Table I), with deduced mass values for the non-polypeptide component of the fragment of 1,539 ± 10, 1,694 ± 10, 1,880 ± 10, and 2,016 ± 10 Da. The first three values are compatible with the presence of an heterogeneous high mannose oligosaccharide comprising 2 N-acetylglucosamine and 7, 8, or 9 mannose residues (calculated masses 1,542, 1,704, and 1,866 Da, respectively), whereas additional binding of a diisopropyl phosphate group would account for the highest value 2,016 ± 10 Da. Mass spectrometry analysis of the CUB fragment yielded a single peak with a mass of 14,243 ± 7 Da, consistent with a sequence comprising residues Tyr-Val followed by the N-terminal segment Ser1-Val124 of C1r (calculated mass = 14,243 Da).

In summary, mass spectrometry analyses clearly showed that the recombinant CUB fragment was unglycosylated, whereas the CUB-EGF fragment was only partially glycosylated, with a heterogeneous high mannose oligosaccharide attached to Asn108 within the CUB module.

Size-exclusion Chromatography—The ability of the recombinant CUB-EGF fragment to mediate Ca2+-dependent interaction with the α fragment of C1s was first investigated using high pressure size-exclusion chromatography, as described previously for the C1ro fragment (8). When mixtures containing equimolar amounts of C1sa and either C1ro or the glycosylated CUB-EGF fragment were applied to the gel filtration column in the presence of 2.5 mM CaCl2, the peaks corresponding to C1ro or CUB-EGF (retention times 15.2 and 17.3 min, respectively) disappeared to yield peaks with respective retention times of 14.1 and 14.3 min. The observed shifts and the relative intensity of the resulting peaks were consistent with the formation of a (CUB-EGF)-C1sa heterodimer, analogous to the previously observed C1ro-C1sa complex (8). Identical results were obtained when the CaCl2 concentration was decreased to 0.2 mM. Formation of such a heterodimer was also observed with the unglycosylated CUB-EGF fragment, although no significant peak was eluted when the isolated fragment was injected on the column, indicating that most of the unglycosylated species was likely adsorbed to the matrix, as previously observed with deglycosylated C1ro (8).

Size-exclusion chromatography of the isolated glycosylated CUB-EGF pair in the presence of CaCl2 (0.2 or 2.5 mM) or EDTA indicated a 0.9-min Ca2+-induced shift of its elution position (from 16.4 to 17.3 min). A similar but less important shift (0.3 min) was observed in the case of C1ro. This observation showed that the CUB-EGF and C1ro fragments behaved in a similar way with respect to Ca2+ binding and suggested that the CUB-EGF pair adopts a more compact conformation upon Ca2+ binding. No evidence for complex formation between the isolated CUB and EGF fragments was obtained when an equimolar mixture of both fragments was applied to the column in the presence of 2.5 mM CaCl2.

Effect of Ca2+ on Protein Intrinsic Fluorescence—With a view to detect potential conformational modifications induced upon Ca2+ binding to the interaction region of C1r, the intrinsic fluorescence spectra of different fragments from this region were recorded in the presence of either EGTA or CaCl2. As the α region of C1r contains no tryptophan residue, the intrinsic fluorescence is contributed only by the 8, 3, 10, and 15 tyrosine residues present in the CUB, EGF, CUB-EGF, and C1r fragments, respectively. As depicted in Fig. 4, maximum emission occurred at 306 nm for C1ro and at about 301 nm for the other fragments, and no significant shift in the wavelength was observed in the presence of Ca2+. In contrast, Ca2+ induced a small (7–10%) but highly reproducible decrease in the fluorescence intensity of C1ro (Fig. 4A) and both the glycosylated and unglycosylated CUB-EGF fragments (Fig. 4B). The isolated CUB and EGF modules, and an equimolar mixture of these fragments, exhibited no detectable fluorescence change in the presence of calcium (Fig. 4C). These experiments were consistent with a Ca2+-induced quenching of intrinsic tyrosine fluorescence within

### Table I

| Glycosylated CUB-EGF | Deglycosylated CUB-EGF | Unglycosylated CUB-EGF |
|----------------------|------------------------|-----------------------|
| Da                   | Da                     | Da                    |
| 21,329 ± 10          | 19,802 ± 10            | 19,795 ± 10           |
| 21,484 ± 10          | 19,969 ± 10            | 19,956 ± 10           |
| 21,670 ± 10          |                        |                       |
| 21,806 ± 10          |                        |                       |

*The glycosylated CUB-EGF fragment was treated with peptide N-glycosidase F as described under “Experimental Procedures.”*
C1r and Ca\(^{2+}\) Binding to C1r CUB-EGF Module Pair

**Figure 5. Analysis by surface plasmon resonance spectroscopy of the interaction between C1r or various C1r fragments and immobilized C1s.** C1s was immobilized on the sensor chip as described under “Experimental Procedures.” Fifty μl of each analyte were injected in the running buffer containing 1 mM CaCl\(_2\) at a flow rate of 10 μl/min. The plots are as follows from top to bottom: A, C1r (100 nM) and C1rα fragment (1 μM); B, recombinant CUB-EGF, glycosylated (1.6 μM), recombinant CUB-EGF, unglycosylated (1 μM), recombinant CUB (2.8 μM), chemically synthesized EGF (3 μM). The sensor chip was regenerated after each experiment by a 10-μl injection of 20 mM EDTA. The specific binding signal shown was obtained by subtracting the background signal as described under “Experimental Procedures.”

The ability of C1r and its fragments to bind C1s in the presence of Ca\(^{2+}\) was studied using surface plasmon resonance spectroscopy. As shown in Fig. 5A, both C1r and C1rα bound to immobilized C1s in the presence of 1 mM CaCl\(_2\), as shown by the increase in the resonance units during the association phase of the sensorgrams. However, when the running buffer was substituted for the analyte, the proteins exhibited very different dissociation phases; only very slow dissociation was observed in the case of C1r, whereas almost complete dissociation of C1rα occurred over the same period. As expected in view of the Ca\(^{2+}\) requirement of the C1r/C1s interaction, bound C1r could be eluted at the end of the dissociation phase by a pulse injection of EDTA, and no binding of the C1rα fragment was observed when the running buffer contained 2 mM EDTA instead of CaCl\(_2\). Binding of the glycosylated and unglycosylated C1r CUB-EGF fragments to C1s was then tested in the same way, and the resulting sensorgrams were quite comparable to that observed for C1rα, as shown in Fig. 5B. In contrast, no binding to C1s was observed in the case of the isolated CUB or EGF modules (Fig. 5B) or for an equimolar mixture of these fragments (not shown). In the same way, the CUB + EGF mixture was unable to compete with either C1r or the CUB-EGF fragment for binding to C1s, even at molar ratios of 32:1 and 7:1, respectively. In contrast, prior incubation of the C1r CUB-EGF and α fragments with the α fragment of C1s (1:1 molar ratio) almost abolished their ability to bind to immobilized C1s. These results clearly indicated that the C1r CUB-EGF module pair mediates specific, Ca\(^{2+}\)-dependent interaction with the C1sα region, in agreement with gel filtration experiments.

We next studied the Ca\(^{2+}\) dependence of the binding of C1r or its fragments to immobilized C1s. Sensorgrams were recorded at different Ca\(^{2+}\) concentrations, and resonance units at equilibrium (R\(_{eq}\)) were determined for each analyte (C1r, C1rα, glycosylated, and unglycosylated CUB-EGF) by fitting the data as described under “Experimental Procedures.” Binding of the four proteins to C1s increased with Ca\(^{2+}\) concentration to reach a plateau, at Ca\(^{2+}\) concentrations ranging from 100 μM for C1r to 300 μM for the fragments. Increasing the Ca\(^{2+}\) concentration to 10 mM resulted in reduced binding, possibly due to Ca\(^{2+}\)-induced aggregation of C1r and its fragments. No binding of the fragments was observed at Ca\(^{2+}\) concentrations below 100 nM, but residual C1r binding was still observed in the absence of Ca\(^{2+}\). In order to determine the Ca\(^{2+}\) concentration yielding half-maximal binding (EC\(_{50}\)), R\(_{eq}\) values were normalized for each fragment to the maximal value (R\(_{eq(max)}\)) obtained at 300 μM CaCl\(_2\) (Fig. 6). Half-maximal Ca\(^{2+}\)-dependent binding to C1s was found to occur at similar CaCl\(_2\) concentrations, namely 5 μM for C1r, and 10, 16, and 14 μM for C1rα, the glycosylated and unglycosylated CUB-EGF fragments, respectively.

Further experiments were aimed at determining the kinetic parameters of the C1r/C1s interaction. This was achieved by recording sensorgrams at varying protein concentrations with a fixed, saturating CaCl\(_2\) concentration of 250 μM. Fig. 7A shows the association and dissociation curves from a representative series of experiments performed with five C1r concentrations (13–103 nM). The association phase was analyzed by nonlinear least squares fitting as described under “Experimental Procedures” to yield k\(_a\) values at each concentration. A plot of k\(_a\) versus C1r concentration produced a straight line (Fig. 7B) with a slope corresponding to the association rate constant k\(_{on}\) (Table II). The dissociation phase (415–675 s) was also analyzed by nonlinear least squares curve fitting as described under “Experimental Procedures” to yield the dissociation rate constant k\(_{off}\) (Table II). The apparent equilibrium dissociation constant K\(_D\) determined from the (k\(_{off}\)/k\(_{on}\)) ratio was 10 nM. This experiment was repeated on two different chips, and the resulting mean K\(_D\) value was 10.9 ± 0.9 nM (Table II). As Ca\(^{2+}\)-dependent binding of C1r to C1s is known to involve the

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**Figure 6. Ca\(^{2+}\) dependence of the interaction between C1r or its fragments and C1s.** Sensorgrams were recorded in the running buffer containing 1 mM EGTA and varying amounts of CaCl\(_2\) to give free Ca\(^{2+}\) concentrations ranging from 10 nM to 1 mM, as described under “Experimental Procedures.” One hundred μl of 50 nM C1r (○), 1 μM C1rα (□), 1 μM CUB-EGF, glycosylated (■), and 750 nM CUB-EGF, unglycosylated (□), were injected over immobilized C1s at a flow rate of 10 μl/min. R\(_{eq}\) values were determined from the association phases of the sensorgrams as described under “Experimental Procedures.” These values were normalized for each analyte to the maximal value obtained at 300 μM CaCl\(_2\) and plotted as a function of the free Ca\(^{2+}\) concentration.
C1s interaction region, a similar experiment was performed using the fragment of C1s immobilized on the sensor chip. As shown in Table II, comparable k\textsubscript{on} values were obtained for both immobilized ligands, whereas the k\textsubscript{off} increased about 2-fold in the case of C1s \textalpha, resulting in a 2-fold increase in the apparent KD value.

Binding of C1r and of the glycosylated and unglycosylated C1r CUB-EGF fragments to C1s, was then analyzed by recording sensorgrams at various protein concentrations in the presence of 250 \( \mu \text{M} \) CaCl\textsubscript{2}. A representative experiment with five concentrations of the glycosylated CUB-EGF fragment (225 nM to 1.2 \( \mu \text{M} \)) is shown in Fig. 8A. Compared with C1r, both the association and dissociation phases for the three fragments were fitted less satisfactorily using monoexponential equations. However, this method allowed us to estimate k\textsubscript{on} and k\textsubscript{off} values, from which KD values ranging from 1.2 to 1.6 \( \mu \text{M} \) were derived (Table II). As the binding of the fragments approached equilibrium, the affinity of the interactions was also estimated by determining the equilibrium dissociation constant from a Scatchard plot of \( R_{eq}/C \) versus \( R_{eq} \), as described under “Experimental Procedures.” A representative plot for the glycosylated CUB-EGF fragment is shown in Fig. 8B. KD values, calculated from two independent experiments for each analyte, were 2.2 \( \pm \) 1.0, 1.8 \( \pm \) 0.6, and 1.5 \( \pm \) 0.1 \( \mu \text{M} \) for C1r, the glycosylated and unglycosylated CUB-EGF pair, respectively (Table II). These values were fully consistent with those estimated using mono-

**TABLE II**

| Soluble analyte | Immobilized ligand | \( k_{on} \) \( \text{M}^{-1} \text{s}^{-1} \) | \( k_{off} \) s\textsuperscript{-1} | \( K_{D_{kin}} \) \( \pm \) S.D.\textsuperscript{a} | \( K_{D_{eq}} \) \( \pm \) S.D.\textsuperscript{a} |
|----------------|--------------------|-----------------|-----------------|-----------------|-----------------|
| C1r           | C1s                | 3.7 \( \pm \) 0.3 \times 10\textsuperscript{4} | 3.7 \( \pm \) 0.8 \times 10\textsuperscript{-4} | 10.9 \( \pm \) 0.9 | ND\textsuperscript{d} |
| C1r           | C1s\textalpha     | 3.9 \( \pm \) 0.9 \times 10\textsuperscript{4} | 7.9 \( \pm \) 0.8 \times 10\textsuperscript{-4} | 20.2           | ND\textsuperscript{d} |
| C1r\textalpha| C1s                | 8.8 \( \pm \) 1.1 \times 10\textsuperscript{3} | 1.4 \( \pm \) 0.4 \times 10\textsuperscript{-2} | 1,600          | 2,200 \( \pm \) 1,000 |
| CUB-EGF       | C1s                | 6.6 \( \pm \) 0.8 \times 10\textsuperscript{3} | 9.3 \( \pm \) 0.8 \times 10\textsuperscript{-3} | 1,400          | 1,800 \( \pm \) 600  |
| CUB-EGF unglycosylated | C1s | 7.3 \( \pm \) 1.5 \times 10\textsuperscript{3} | 8.6 \( \pm \) 0.3 \times 10\textsuperscript{-3} | 1,200          | 1,500 \( \pm \) 100  |
| C1s           | CUB-EGF            | 7.5 \( \pm \) 0.3 \times 10\textsuperscript{4} | 2.7 \( \pm \) 0.6 \times 10\textsuperscript{-3} | 36.0           | 31.4 \( \pm \) 4.2  |
| C1s           | CUB-EGF unglycosylated | 6.6 \( \pm \) 0.4 \times 10\textsuperscript{4} | 3.2 \( \pm \) 0.4 \times 10\textsuperscript{-3} | 48.5           | 56.0 |
| C1s           | C1r                | 3.7 \( \pm \) 0.2 \times 10\textsuperscript{4} | 1.1 \( \pm \) 0.1 \times 10\textsuperscript{-3} | 29.7           | ND\textsuperscript{d} |
| C1s\textalpha| C1r                | 1.4 \( \pm \) 0.1 \times 10\textsuperscript{4} | 2.2 \( \pm \) 0.1 \times 10\textsuperscript{-3} | 157.0          | 133.0 |

\textsuperscript{a} Dissociation constant determined from the kinetic rate constants \( k_{off}/k_{on} \) ratio.

\textsuperscript{b} Mean value of two experiments.

\textsuperscript{c} Dissociation constant determined from the Scatchard plot of \( R_{eq}/C \) versus \( R_{eq} \).

\textsuperscript{d} Not determined.
exponential equations (Table II) and comparable for the three C1r fragments but strikingly differed from the $K_D$ value determined for intact C1r by a factor of 150–200. Interaction of the above three fragments with immobilized C1s was also observed, and the shape of the binding curves was similar to that obtained with immobilized C1s.

The fact that C1ra and CUB-EGF showed a decreased affinity for C1s suggested that these fragments exhibited a decreased stability compared with intact C1r. To test this hypothesis, the CUB-EGF fragment itself was immobilized, whereas C1s was used as the soluble analyte. Under this configuration, C1s was found to bind readily to the C1r CUB-EGF fragment (Fig. 9A), and analysis of the data obtained at varying C1s concentrations by plotting $R_{eq}/C$ versus $R_{eq}$ (Fig. 9B) yielded a mean $K_D$ of 31.4 ± 4.2 nM, in agreement with the value estimated from monoexponential equations (Table II). Slightly higher $K_D$ values (48.5–56 nM) were determined for C1s when the unglycosylated CUB-EGF species was immobilized (Table II). Control experiments using C1s as the analyte and intact C1r as the immobilized ligand allowed us to determine a $K_D$ of 29.7 nM, close to the value obtained above for the interaction between C1s and the immobilized CUB-EGF fragment (Table II). Further experiments were aimed at measuring the ability of the soluble fragment C1sa to bind to immobilized C1r. Under this configuration, the $K_D$ of the interaction was estimated at 133–157 nM, indicating a 5-fold decrease of the affinity of C1sa compared with intact C1s (Table II), much smaller than the factor of 150–200 determined in the case of the C1r CUB-EGF fragment. Taken together, these data suggested that the large decrease of the affinity of CUB-EGF in solution was mainly due to a decreased stability and that covalent attachment of the fragment to the surface of the sensor chip stabilized it in a conformation appropriate for optimal binding affinity.

**DISCUSSION**

Baculovirus/insect cells systems have been used previously to express human complement proteases C1r (31) and C1s (32) and more recently to produce C1r deletion mutants (15) and modular fragments from the catalytic and interaction regions of C1s (14, 28). Baculovirus-mediated expression was used in the present study to produce the N-terminal CUB-EGF module pair of human C1r, and unexpectedly, this fragment was secreted under both glycosylated and unglycosylated forms. The coexistence of both forms in the culture medium as well as in the cell lysate indicates that glycosylation is not an absolute prerequisite for secretion of the CUB-EGF fragment by insect cells, in contrast to what has been observed for expression of human decorin (33). It should also be mentioned that a similar expression pattern, with a mixture of glycosylated and unglycosylated species, was obtained when the signal peptide of human C1r was replaced by that of honey bee melittin. Indeed, inefficient glycosylation of the CUB-EGF fragment does not appear to be inherent in the baculovirus/insect cells expression system, as the recombinant protein secreted by the *P. pastoris* yeast was totally unglycosylated (34), as was the recombinant CUB fragment produced in the present study. A likely explanation is therefore that the observed inefficiency of the glycosylation at Asn$^{108}$ mainly results from a lack of accessibility of this particular site, both in the isolated CUB fragment and in the CUB-EGF pair. However, the fact that the unglycosylated CUB-EGF fragment had the correct N-terminal sequence and that its functional properties were indistinguishable from those of its glycosylated counterpart indicates that the lack of carbohydrate at Asn$^{108}$ in the CUB module does not

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2 N. M. Thielens, unpublished observations.
significantly affect the folding of the fragment. In the same way, the presence of an oligosaccharide chain had no significant effect on the solubility of the CUB-EGF fragment. In this respect, it should be mentioned that the solubility limit of the CUB-EGF pair (about 0.1 mg/ml) was about 6 times lower than that of the larger tryptic fragment C1rα. Also, complete enzymatic deglycosylation of C1rα could be achieved (8), whereas the recombinant CUB-EGF fragment could only be partially deglycosylated using large amounts of peptide:N-glycosidase F.

The above differences suggest that the C-terminal part of C1rα, corresponding to the N-terminal end of the second CUB module (residues 176–208) and including another oligosaccharide linked to Asn204, has a stabilizing effect on the CUB-EGF module pair. Removal of the C-terminal region of C1rα could unmask hydrophobic areas and thereby decrease the solubility of the CUB-EGF fragment. This may possibly also reduce the accessibility to peptide:N-glycosidase F of the Asn 108-linked oligosaccharide in the first CUB module.

Surface plasmon resonance spectroscopy has been used successfully to study various Ca2+-dependent interactions, such as the binding of recoverin to phospholipids (35), of calpain to calpastatin (36, 37), and of tissue factor to coagulation factor VIIa (38–40). This technique was used in the present work to study Ca2+-dependent binding of C1r and its fragments to C1s. The data obtained with intact C1r and C1s indicate that the proteins associate in the presence of Ca2+ with high affinity, with a KD ranging from 10.9 (immobilized C1s) to 29.7 nM (immobilized C1r), close to the value (32 nM) determined previously by tracer sedimentation equilibrium (41). Based on the KD values determined in the present study, and on the physiological serum concentrations of C1r and C1s (about 0.2 and 0.4 μM, respectively (2)), it may be anticipated that virtually all of the C1r and C1s molecules are associated within the Ca2+-dependent C1s-C1r-C1r-C1s tetrameric complex in normal sera.

Both size-exclusion chromatography and surface plasmon resonance spectroscopy studies show that, as previously found for the larger proteolytic fragment C1rα (8), the CUB-EGF module pair itself essentially retains the ability of intact C1r to bind to the α region of C1s in the presence of Ca2+ ions. However, the latter technique clearly shows that the affinity of both fragments C1rα and CUB-EGF for immobilized C1s is 150–200 times lower than that of intact C1r. It is clear from the koff values of the interactions that most of the difference arises from the dissociation phase, which proceeds very slowly in the case of C1r (koff = 3.7 × 10^{-4} s^{-1}) and about 30 times faster in the case of the fragments (koff = 0.9–1.4 × 10^{-2} s^{-1}) (see Table II). It may be argued that this difference arises from the fact that intact C1r is a dimer, whereas both its α and CUB-EGF fragments are monomers. However, the possibility that a single C1r-C1r dimer could simultaneously bind two C1s molecules appears unlikely because of the low density of immobilized C1s used, and considering that our data of the interaction between C1r and C1s satisfactorily fitted a single-site binding model when either protein was immobilized. Nevertheless, it appears likely that the koff value for the C1r/C1s interaction was slightly underestimated when C1r was used as the soluble analyte, considering that a 3-fold increase was observed when C1r was immobilized (see Table II). In any case, as discussed above, the KD values determined in the present study for the interaction between intact C1r and C1s are similar to that obtained previously by a different method (41), and a value in the nanomolar range is fully consistent with formation of a tight C1s-C1r-C1r-C1s tetramer in serum.

It appears clear that although the soluble CUB-EGF module pair does bind immobilized C1s in the presence of Ca2+, its binding affinity shows an important decrease compared with
intact C1r, mainly because of an increased tendency of the CUB-EGF-C1s complex to dissociate. A possible explanation is that the CUB-EGF pair lacks accessory ligand(s) that would provide further stabilization of the interaction within intact C1r. As C1rα shows a similar decreased affinity for immobilized C1s, such ligands could be located within the second CUB module, in the region C-terminal to the cleavage site by trypsin (see Fig. 1). Alternatively, the missing part of the second CUB module may not be directly involved in the interaction per se but could simply stabilize the structure of the preceding CUB-EGF pair and hence tighten the interaction. As discussed above, C1rα itself shows a significantly increased solubility compared with CUB-EGF, suggesting a stabilizing effect of the N-terminal segment of the second CUB module, but further stabilization of the module pair may require the remainder of the second CUB module. Indeed, our binding studies using CUB-EGF as the immobilized ligand clearly show that, under these conditions, this fragment recovers a binding affinity for C1s that is very close to that of the intact C1r molecule. A likely hypothesis is therefore that the isolated CUB-EGF fragment of C1r contains all of the ligands required for efficient binding to C1s but exhibits a decreased stability due to the lack of contacts normally occurring with the remainder of the protein.

Analysis of the interaction between soluble C1sα and immobi-

lized C1r shows that C1sα also exhibits a decreased affinity compared with intact C1s. However, this decrease is 30–40 times less than that observed in the case of C1r CUB-EGF and is contributed by both a decrease in $k_{on}$ and an increase in $k_{off}$ (see Table II). In this respect, it should be pointed out that the C1s CUB-EGF fragment was found to be soluble at a concentration of 10 mg/ml (14), i.e. a value that is 100 times higher than the estimated solubility limit of C1r CUB-EGF (see “Results”). In view of this large difference, it appears likely that the postulated instability of the C1r CUB-EGF fragment in solution thought to be responsible for its decreased binding affinity is related to its poor solubility and to its observed tendency to form aggregates at high concentration. This observation appears consistent with the above hypothesis that the CUB-EGF moiety of C1r makes contacts with other parts of the protein.

Our study of the Ca$^{2+}$ dependence of the interaction between C1r or its fragments and immobilized C1s shows that half-maximal binding occurs at Ca$^{2+}$ concentrations ranging from 5 μM in the case of C1r to 10–16 μM for the various fragments. These values are very close to one another and are in good agreement with the $K_D$ values (32–38 μM) determined previously by equilibrium dialysis for Ca$^{2+}$-binding by fragments C1rα and C1sα (8). Thus, whereas the C1r EGF module alone binds Ca$^{2+}$ very poorly ($K_D = 10$ mM) (12, 18), both C1rα and CUB-EGF bind Ca$^{2+}$ with an affinity comparable to that of intact C1r. It may be concluded therefore that the C1r CUB-EGF module pair, alone, contains all of the ligands that participate in Ca$^{2+}$ binding and that the observed decreased affinity of C1rα and CUB-EGF for C1s is not a consequence of a decreased affinity for Ca$^{2+}$ ions.

The size-exclusion chromatography and fluorescence measurements performed in this work both strongly support the hypothesis that binding of Ca$^{2+}$ to the CUB-EGF fragment induces a more compact conformation of this module pair, a process that is also observed in the C1rα fragment. This hypothesis is fully consistent with previous data indicating that Ca$^{2+}$ ions stabilize the C1rα fragment, shifting upwards the midpoint of its melting transition by more than 20 °C (7). Formation of a compact Ca$^{2+}$-dependent CUB-EGF assembly is also reminiscent of data obtained with the N-terminal Gla-EGF-GlA and Gla-EGF modular fragments from blood coagulation factors IX (42) and X (43), respectively. In the former case, size-exclusion chromatography and spectroscopic measurements indicated a more compact conformation of the Gla-EGF fragment in the presence of Ca$^{2+}$, and a Ca$^{2+}$-dependent complex between the isolated Gla module and Gla-EGF-EFGG fragment pair could be reconstituted from a mixture of these fragments (42). This ability to assemble spontaneously in the presence of Ca$^{2+}$ is not shared by the isolated C1r CUB and EGF modules, as judged by both size-exclusion chromatography and fluorescence spectroscopy. In the same way, a CUB + EGF mixture was unable to mediate Ca$^{2+}$-dependent C1s binding or even to compete for C1s binding. In the case of factor X, small angle x-ray scattering studies showed a contraction of the Gla-EGF module pair upon addition of Ca$^{2+}$, and NMR spectroscopy indicated that the two modules move toward each other using the Ca$^{2+}$-binding site as a hinge (43). The authors proposed that a general property of EGF modules exhibiting the consensus sequence for Ca$^{2+}$ binding may be the ability to induce an intermodule orientation compatible with biological activity, an hypothesis that is supported by recent studies on coagulation factor VII suggesting that the Ca$^{2+}$-binding site in the N-terminal EGF module may stabilize the orientation of the Gla module relative to the EGF module in order to facilitate interaction with tissue factor (39). Our present data are con-
sistent with the occurrence of a similar mechanism in the N-terminal interaction region of C1r, as schematized in the model presented in Fig. 10. In this model, Ca\(^{2+}\) binding through ligands in the EGF module of C1r (18) would allow the CUB and EGF modules to move toward each other, inducing formation of a more compact conformation with concomitant shielding of one or more tyrosine residues. This Ca\(^{2+}\)-dependent CUB-EGF assembly would provide the appropriate conformation as well as the ligands required for interaction with the homologous CUB-EGF pair of C1s.

Detailed knowledge of the three-dimensional structure of the CUB-EGF module pair of C1r is now required to test this model at the atomic level. Among other unsolved and intriguing questions are the structure of the CUB module and the nature of its contribution to Ca\(^{2+}\) binding, as this module may either stabilize the Ca\(^{2+}\)-binding site of the EGF module in the appropriate conformation or possibly provide an additional ligand for the Ca\(^{2+}\) ion. Expression of the CUB module and CUB-EGF pair was initially undertaken in part with a view to solve their structure by NMR spectroscopy, but the low solubility of both fragments clearly precludes such a study. Given the possible stabilizing effect of the neighboring second CUB module on the CUB-EGF-CUB array is being considered.

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