NH_2-Terminal Calcium-Binding Domain of Human Complement C1s Mediates the Interaction of C1r with C1q†

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ABSTRACT: The assembly of C1, the first component of human complement, involves interactions between various domains of each of its three subcomponents, C1q, C1r, and C1s. The isolation, assignment of function, and structural characterization of the individual domains of C1r and C1s are critical for a thorough understanding of this complex assembly. The present study describes a 27-kDa plasmin-generated fragment derived from the NH_2-terminal half of the heavy A chain of C1s, the activated form of C1s. This fragment, C1s-α, was shown in the presence of Ca^{2+} to mimic the ability of whole C1s to self-associate, bind to C1r, and facilitate the binding of C1r to C1q. These results directly prove that the Ca^{2+}-binding sites of C1s as well as all of the determinants necessary for binding of C1s to C1r and C1q are located in the NH_2-terminal 27-kDa α region of the A chain.

C1s,[1] a multidomain serine protease, is an integral part of C1, the first component of complement (Valet & Cooper, 1974; Cooper, 1985). It is the enzymatic subcomponent of C1 which cleaves C2 and C4 during activation of the classical complement cascade. The activated form of C1s, designated C1s, is comprised of two polypeptides chains (see Figure 1A). The B chain contains the trypsin-like serine protease domain which is disulfide-linked to the C-terminal or γ region of the A chain, a region comprised of two homologous short consensus repeat units (SCRs) that are ubiquitous among complement proteins (Reid & Day, 1989). A fragment containing the complete catalytic B chain and the γ portion of the A chain has been isolated and shown to retain its proteolytic activity. However, this γB fragment is unable to bind Ca^{2+} or form any of the Ca^{2+}-dependent interactions with other subcomponents that are characteristic of the whole C1s molecule (Mori et al., 1980; Villiers et al., 1985; Busby & Ingham, 1988). Another fragment, C1s-A, containing the entire A chain but only a small portion of the B chain, was shown to undergo Ca^{2+}-induced dimerization and to form a tetramer with C1r which in turn binds to C1q to form a stable complex analogous to C1 (Busby & Ingham, 1988). Since both of these derivatives contain the γ region but only the latter exhibits Ca^{2+}-dependent interactions, the Ca^{2+}-binding and interaction sites were suggested to reside in the NH_2-terminal half of the A chain, in agreement with earlier predictions [see Arlaud et al. (1987b) and references cited therein].

The amino acid sequence of C1s exhibits 40% homology with and has a motif pattern similar to that of C1r (Leytus et al., 1986; Arlaud et al., 1987a; Mackinnon et al., 1987; Tosi et al., 1987). It has been shown conclusively that C1r interacts with C1s through its NH_2-terminal α region (Busby & Ingham, 1987), and it is assumed that this interaction involves the corresponding region of C1s. However, the α fragment of C1s has never been isolated. C1r alone, C1s alone, and the C1r-α/C1s complex do not associate with C1q (Busby & Ingham, 1988). Several models of the C1 complex have been proposed which differ primarily in the way the $C1r_2s_2$ tetramer interacts with C1q (Poon et al., 1983; Colomb et al., 1984a,b; Cooper, 1985; Weiss et al., 1986; Arlaud et al., 1987b; Schumaker et al., 1987; Perkins, 1985, 1989). Although it is known that the $C1r_2s_2$ tetramer binds to the collagenous portion of C1q (Reid et al., 1977; Siegel & Schumaker, 1983; Poon et al., 1983), it is not known which portion(s) of the C1r or C1s molecules make(s) this contact. To better understand these interactions and refine the model, it is necessary to examine each domain of the subcomponents to determine their function. The purpose of the present study was to isolate the NH_2-terminal α fragment of C1s (C1s-α) and determine whether the Ca^{2+}-dependent interactions so important for the functional integrity of C1 are contained within this fragment.

Materials and Methods

Unless specifically noted, all experiments were performed in 0.02 M Tris-HCl, pH 7.4, and 0.15 M NaCl (TBS). C1q, C1r, and C1s were purified as previously reported (Busby & Ingham, 1988). Nonreduced samples of C1r and C1s both yield a single band on SDS–PAGE with only a trace of a lower molecular weight contaminant. Reduced samples displayed two bands corresponding to the A and B chains with no evidence of single-chain zymogens. C1s was homogeneous by size-exclusion chromatography on Superose 12 (Pharmacia). It eluted as a monomer in TBS + 1 mM EDTA and completely dimerized when the column was equilibrated with TBS + 1 mM Ca^{2+} (see Figure 2). C1r consistently eluted as a dimer in buffers containing either Ca^{2+} or EDTA, with only a small amount of aggregated material eluting in the void volume of the column (Busby & Ingham, 1987). The nonreduced sample of C1q appeared as two bands on SDS–PAGE corresponding to the disulfide-linked A–B and C–C chains,

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[1] Abbreviations: C1, first component of complement; C1r and C1s, zymogen forms of complement subcomponents; C1r and C1s, activated forms of C1r and C1s, differing from the zymogens by the presence of a single clip in the polypeptide chain; C1s-A, 56-kDa fragment of C1s containing the heavy A chain and a small 4-kDa portion of the light B chain; C1s-γB, 54-kDa fragment of C1s containing the light B chain and the C-terminal or γ portion of the A chain; C1s-α, 27-kDa fragment of C1s composed of the interaction or α domain; DEGR, dansyl-L-glutamyl-glycyl-L-arginine chloromethyl ketone; EDTA, ethylenediaminetetraacetic acid; FITC, fluorescein isothiocyanate; kDa, kilodalton(s); SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane; TBS, Tris-buffered saline (0.02 M Tris-HCl/0.15 M NaCl, pH 7.4).

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while the reduced sample revealed the three separate A, B, and C chains as three bands (data not shown). C1q was also homogeneous by size-exclusion chromatography on Superose 6 (Pharmacia), with no evidence of aggregation.

C1$\bar{s}$-α was produced by incubating 50 mg of C1$\bar{s}$ (2 mg/mL) in 0.02 M Tris-HCl, pH 8.5, containing 1 M NaCl and 1 mM EDTA, with plasmin, kindly provided by Dr. Dudley Strickland (ARC, Rockville, MD) at a final C1$\bar{s}$:plasmin ratio of 100:1 (w/w) at 37 °C for 120 min. To terminate the plasmin digestion, *p*-nitrophenyl guanidinobenzoate (NPGB, in 80% dimethyl formamide/20% dimethyl sulfoxide) was added to the sample in a 1000-fold molar excess over plasmin. The digested material was then diluted 20-fold with water to reduce the concentration of NaCl and applied to a lysine–Sepharose column (1 × 30 cm) equilibrated in 0.02 M Tris-HCl, pH 7.4, and 0.05 M NaCl (Tris buffer) containing 1 mM EDTA to remove the plasmin. Efficient removal of plasmin is important to minimize further cleavage of C1$\bar{s}$-α. The unadsorbed material was then applied to a heparin–Sepharose column (1 × 10 cm) equilibrated in the same Tris buffer to remove any undigested C1$\bar{s}$ and C1$\bar{s}$-γB. The nonbinding material was collected, and 0.5 M $CaCl_2$ was added to bring the final Ca^{2+} concentration to 2 mM. This solution was applied to a C1$\bar{s}$–Sepharose column equilibrated in the Tris buffer containing 1 mM Ca^{2+}. The column was washed with the equilibrium buffer until the A_{280} returned to base line, and the bound protein was eluted with TBS containing 1 mM EDTA. Fractions containing protein were pooled, concentrated by ultrafiltration with an Amicon PM-10 membrane, and subjected to a final purification by size-exclusion chromatography on a Superose 12 column equilibrated in TBS containing 1 mM EDTA. Plastic tubes were used in all steps during preparation of C1$\bar{s}$-α to reduce adsorption of the fragment to the vessel wall.

Affinity adsorbents were prepared by attaching the ligands to CNBr-activated Sepharose (Pharmacia) by the procedure of March et al. (1974). Excess sites were blocked with ethanolamine. The lysine–Sepharose column was regenerated with 5 column volumes of 0.02 M Tris-HCl, pH 7.4, containing 0.05 M NaCl and 100 mM lysine to remove plasmin, followed by washing with the equilibration buffer. The heparin–Sepharose column was regenerated by eluting all bound protein with TBS containing 1 M NaCl and then equilibrating in the starting buffer.

The concentrations of C1$\bar{s}$ and C1$\bar{r}$ were determined by using the values of $E_{280,1\%}$ = 13.7 and 11.6 and M_r = 80 300 and 86 000, respectively (Busby & Ingham, 1988). Assuming that plasmin cleaves C1$\bar{s}$ at a site corresponding to the site at which C1$\bar{r}$ cleaves itself during autolytic digestion to produce the C1$\bar{r}$-α, β, and γB fragments (Gagnon & Arlaud, 1985), the $E_{280,1\%}$ value for the C1$\bar{s}$-α fragment was calculated to be 9.8. This was calculated by the method of Edelhoch (1967) from the number of Tyr, Trp, and disulfides in the C1$\bar{s}$-α fragment using the sequence data of Mackinnon et al. (1987) and Tosi et al. (1987). A molecular weight of 24 900 was calculated from the same sequence data, including 2700 kDa for the carbohydrate component.

C1$\bar{s}$ was labeled with fluorescein isothiocyanate (FITC) as in Busby and Ingham (1988), and the degree of labeling was determined to be 0.9 mol of dye/mol of C1$\bar{s}$ by the method of Ingham and Brew (1981). Labeling of C1$\bar{r}$ with dansyl-L-glutamyl-glycyl-L-arginine chloromethyl ketone (DEGR, Calbiochem) was done according to the method of Nesheim et al. (1981). The degree of labeling was determined to be 1.1 mol/mol.

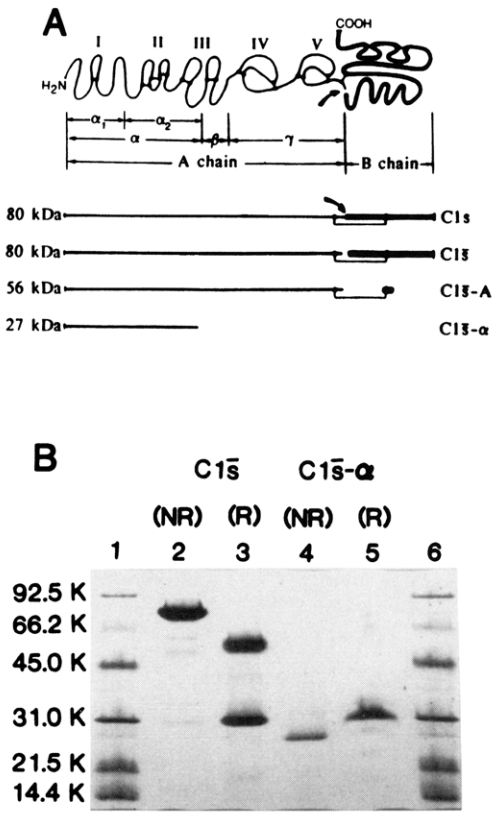

FIGURE 1: (A) Schematic illustration of the modular structure of C1$\bar{s}$ showing the presence of five sequence motifs (Mackinnon et al., 1987; Tosi et al., 1987) and the definition of some of the fragments referred to in the text. Motifs I and III are unique to C1r and C1s. Module II is homologous to epidermal growth factor. Motifs IV and V are the short consensus repeat units. The bold line represents the serine protease domain. (B) Analysis of C1$\bar{s}$ and C1$\bar{s}$-α fragment by SDS–PAGE. Lanes 1 and 6 contain standard proteins of designated molecular weight. Lane 2 contains C1$\bar{s}$ nonreduced; lane 3, C1$\bar{s}$ reduced; lane 4, C1$\bar{s}$-α nonreduced; lane 5, C1$\bar{s}$-α reduced. The bands were visualized with Coomassie blue.

Size-exclusion chromatography was performed on Superose 12 columns using a Pharmacia FPLC system. The proteins were eluted at 1 mL/min with TBS containing either 1 mM EDTA or 1 mM Ca^{2+} while monitoring the A_{280} and/or fluorescence. A Shimadzu Model RF-535 fluorescence spectromonitor was used to monitor the fluorescence of the eluted samples at excitation/emission wavelengths of 280/350 (tryptophan), 495/525 (FITC), and 340/520 nm (DEGR). The integration feature of the Pharmacia LCC-500 controller was used to determine the elution position of the protein samples. Due to variations in the elution position of identical samples over the course of this study, only samples injected on the same day under identical conditions were directly compared.

The NH_2-terminal sequence analysis of the C1$\bar{s}$-α fragment was performed on a Beckman 890M sequencer.

SDS–PAGE were performed on the Pharmacia Phastgel system using 8–25% gradient acrylamide gels, and the proteins were visualized by staining with Coomassie blue.

Results

Isolation and Characterization of C1$\bar{s}$-α. The isolation procedure is described under Materials and Methods. The main difficulty arises from the presence of proteolytically sensitive bonds between motifs I and II whose cleavage leads to functionally inactive α_1 and α_2 fragments (see Figure 1A) (Villiers et al., 1985; Thielens et al., 1989; our unpublished observations). This was not a problem with C1$\bar{r}$-α (Busby &

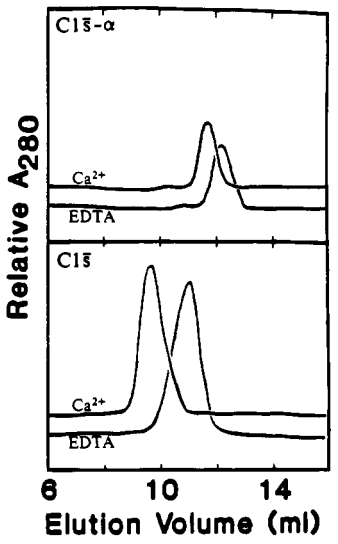

FIGURE 2: Self-association of the C1s̄-α fragment and whole C1s̄ in the presence of Ca^{2+} as shown by size-exclusion chromatography. The purified C1s̄-α fragment (0.2 mL, 0.02 mg/mL) and whole C1s̄ (0.05 mL, 2 mg/mL) were injected onto the Superose 12 column equilibrated in TBS containing either 1 mM Ca^{2+} or 1 mM EDTA and eluted at 1 mL/min while monitoring the absorbance at 280 nm. The absorbance units full-scale for C1s̄-α and C1s̄ were 0.010 and 0.100, respectively.

Ingham, 1987) which lacks the relevant Lys and Arg residues in the corresponding region of its sequence (Leytus, 1986; Arlaud et al., 1987a). Both C1r̄ and C1s̄ are known to undergo low-temperature unfolding transitions near 37 °C that have been assigned to the α regions of the two proteins (Busby & Ingham, 1987, 1988; Medved et al., 1989). These transitions are stabilized by low concentrations of Ca^{2+} and, to a lesser extent, by high concentrations of NaCl. Conditions which increase the thermal stability of proteins also tend to protect them from proteolysis. Ca^{2+} prevented not only the deleterious cleavage between motifs I and II of C1s̄ but also the desired cleavage that generates the α fragment. NaCl appeared to partially and selectively inhibit the undesired cleavage leading to the transient appearance of intact C1s̄-α. After rapid removal of plasmin with lysine–Sepharose in the presence of the inhibitor NPGB, the fragment was recovered in yields 1–3% of theoretical, which was sufficient for the experiments described below.

SDS–PAGE of purified C1s̄-α revealed a single band with a molecular mass of 27 kDa for the nonreduced and 31 kDa for the reduced fragment (Figure 1B). There was no evidence of smaller fragments upon reduction indicating the absence of additional cleavage within disulfide loops. The larger molecular weight of the reduced fragment can be attributed to the more compact nature of the nonreduced protein. Calculation of the molecular masses of C1s̄-α and C1s̄-αβ from the published sequence (Mackinnon et al., 1987; Tosi et al., 1987) yielded values of 24.9 and 32.6 kDa, respectively. Comparison of these calculated values to the values obtained by SDS–PAGE suggests that the "α fragment" may contain a portion of the β region.

NH_2-terminal sequence analysis of this C1s̄-α preparation through seven cycles revealed a single sequence of E-P-T-M-Y-G-E- (-Glu-Pro-Thr-Met-Tyr-Gly-Glu-). This corresponds to the first seven amino acid residues of the C1s̄ A chain (Mackinnon et al., 1987; Tosi et al., 1987) and confirms that the isolated fragment is in fact the NH_2-terminal α portion of the C1s̄ A chain. Interestingly, a region of the C1s̄ B chain (residues 467–471) has the same -E-P-T-M-Y- sequence; however, the next two residues are -Y-G-, so the analyzed fragment could not be from the B chain.

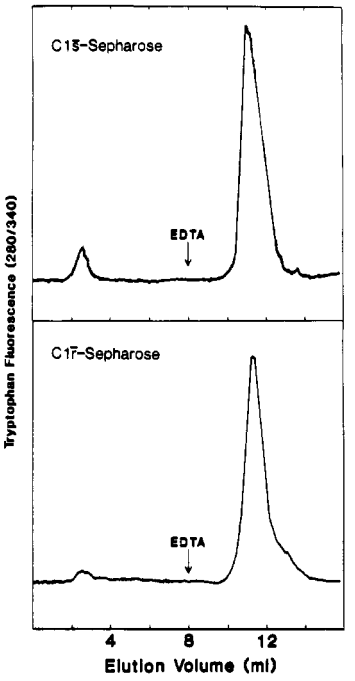

FIGURE 3: Demonstration of the ability of C1s̄-α to bind to C1s̄ and C1r̄ by affinity chromatography. A sample of C1s̄-α (0.5 mL, 0.02 mg/mL) was applied to a C1s̄–Sepharose (top) and a C1r̄–Sepharose (bottom) column (7-mL packed volume, 1 mg of protein/mL of gel) equilibrated in TBS containing 1 mM Ca^{2+}. Bound protein was eluted with TBS containing 1 mM EDTA and monitored by tryptophan fluorescence.

Homologous Interactions of the C1s̄-α Fragment. Size-exclusion chromatography was used to assess the ability of C1s̄-α to self-associate in the presence of Ca^{2+}. As shown in Figure 2 (top), the fragment eluted 0.6 mL earlier when the column was equilibrated in 1 mM Ca^{2+} as compared to 1 mM EDTA. Figure 2 (bottom) illustrates the results of a similar experiment with whole C1s̄, which is known to form a dimer in the presence of Ca^{2+} (Tschopp et al., 1980). Although the shift in the elution of C1s-α was smaller than the shift for C1s̄, the molar concentration of C1s-α was approximately 30-fold lower than that of C1s̄ when applied to the column. We conclude that the fragment, like intact C1s̄, is capable of undergoing a Ca^{2+}-dependent self-association.

Although C1s̄–Sepharose affinity chromatography was used to purify the α fragment, its ability to rebind to immobilized C1s̄ in a Ca^{2+}-dependent manner was evaluated. As shown in Figure 3 (top panel), when applied to a C1s̄-Sepharose column equilibrated in Ca^{2+}, the majority of C1s̄-α bound and was easily eluted with a buffer containing EDTA. The ability of this fragment to self-associate and bind to whole C1s̄ in Ca^{2+} proves that it binds Ca^{2+} and that it contains all or most of the determinants responsible for the self-association of whole C1s̄ and the C1s̄-A fragment.

Interaction with C1r̄. In previous reports from this laboratory, it was shown that an isolated C1r̄-α fragment was able to bind to immobilized C1s̄ and to disrupt the self-association of C1s̄ and C1s̄-A in the fluid phase (Busby & Ingham, 1987, 1988). This confirmed that the NH_2-terminal region of the C1r̄ A chain was responsible for the heterologous association with C1s̄ but did not identify the corresponding region of C1s̄. The results presented in Figure 3 (bottom panel) show that when the C1s̄-α fragment was applied to a C1r̄–Sepharose column equilibrated in TBS + 1 mM Ca^{2+}, it bound quantitatively and was easily eluted with EDTA. Thus, it can now be concluded that the interaction of native C1r̄ with C1s̄ occurs

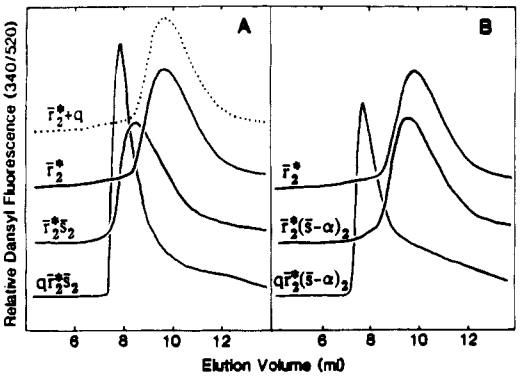

FIGURE 4: Binding of fluorescent-labeled C1$\bar{r}$ to C1q requires the presence of C1$\bar{s}$ (panel A) or C1$\bar{s}$-α (panel B). To the Superose 12 column equilibrated in TBS + 1 mM Ca^{2+} was applied 0.073 mL of 2.7 μM DEGR-C1$\bar{r}$ alone (profile $\bar{r}^*_2$), premixed with a 10-fold molar excess of C1q (profile $\bar{r}^*_2$+q) or with an equimolar amount of either C1$\bar{s}$ (profile $\bar{r}^*_2\bar{s}_2$) or C1$\bar{s}$-α [profile $\bar{r}^*_2(\bar{s}$-$\alpha)_2$]. The lower curves were obtained by adding a 10-fold molar excess of C1q to the latter two mixtures.

through their respective NH_2-terminal α regions.

Interaction with C1r and C1q. It was previously demonstrated that the A chain of C1$\bar{s}$ contained all of the determinants required to mediate the binding of C1$\bar{r}$ to C1q (Busby & Ingham, 1988). To determine if the isolated C1$\bar{s}$-α fragment was capable of eliciting the same interactions, C1$\bar{r}$ was labeled at the active-site serine with a fluorescent reagent, dansyl-L-glutamyl-glycyl-L-arginine chloromethyl ketone (DEGR). This allowed the elution position of the dansyl fluorescence during size-exclusion chromatography to be monitored independently of any other proteins in the system. As shown in Figure 4, the elution of DEGR-C1$\bar{r}$ was unaffected by the presence of a 10-fold excess of C1q. However, if DEGR-C1$\bar{r}$ was first mixed with a stoichiometric amount of either C1$\bar{s}$ or C1$\bar{s}$-α, addition of C1q shifted the fluorescence to the void volume of the column, consistent with the formation of a C1-like complex in both cases. Addition of C1$\bar{s}$ alone caused the DEGR-C1$\bar{r}$ to elute at an intermediate position corresponding to the tetramer. Addition of C1$\bar{s}$-α alone caused only a slight shift, consistent with the fact that the molecular weight of the (DEGR-C1$\bar{r}$)$_2$(C1$\bar{s}$-α)$_2$ complex would be only 25% greater than the DEGR-C1$\bar{r}$ dimer.

Another approach to demonstrating the interaction of C1$\bar{s}$-α with C1$\bar{r}$ and C1q was to compete the unlabeled C1$\bar{s}$-α fragment with fluorescein-labeled C1$\bar{s}$ (FITC-C1$\bar{s}$). The elution position of FITC-C1$\bar{s}$ was monitored when injected alone and in various combinations with C1$\bar{r}$ and C1q. As shown in Figure 5, the FITC-C1$\bar{s}$ behaved like native C1$\bar{s}$, retaining its ability to form dimers (s^*_2), combine with C1$\bar{r}$ to form the tetramer ($r_2s^*_2$), and to form a C1 complex ($qr_2s^*_2$) which eluted in the void of the column. However, if unlabeled C1$\bar{s}$-α was first mixed with C1q and C1$\bar{r}$, prior to the addition of an equimolar amount of FITC-C1$\bar{s}$, only a fraction of the fluorescence shifted to the void (Figure 5, bottom curve). This shows that C1$\bar{s}$-α is able to compete with labeled-C1$\bar{s}$ in the formation of a C1 complex. If C1$\bar{s}$-α and FITC-C1$\bar{s}$ have similar affinities for the other components, one might expect that, at equilibrium, half of each would be complexed in C1 while the remainder would elute as a mixture of (C1$\bar{s}$-α)$_2$ dimers, (FITC–C1$\bar{s}$)$_2$ dimers, and C1$\bar{s}$-α/FITC-C1$\bar{s}$ complexes. As can be seen in Figure 5 (lower curve), less than half of the FITC-C1$\bar{s}$ was incorporated into the C1 complex, indicating that the affinity of C1$\bar{s}$-α for the other components is comparable to that of whole C1$\bar{s}$. These experiments demonstrate that the C1$\bar{s}$-α fragment is able to form a multiprotein

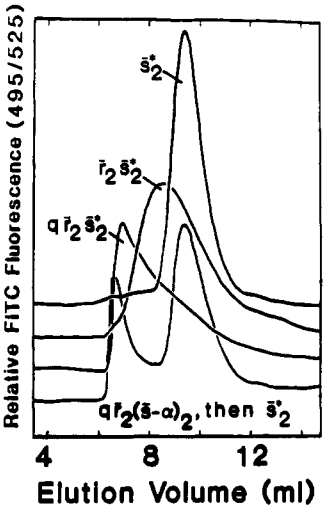

FIGURE 5: Competition between C1$\bar{s}$-α and fluorescent-labeled C1$\bar{s}$ for the C1$\bar{s}$-binding site on C1. C1$\bar{s}$ was labeled with fluorescein isothiocyanate and injected onto a Superose 12 size-exclusion column equilibrated in TBS + 1 mM Ca^{2+}. The top three profiles show that the labeled C1$\bar{s}$ retained the ability to dimerize (profile $\bar{s}^*_2$), form a tetramer with C1$\bar{r}$ (profile $\bar{r}_2\bar{s}^*_2$), and associate with C1$\bar{r}$ and C1q to form a C1 complex (profile $q\bar{r}_2\bar{s}^*_2$). In the bottom profile, C1$\bar{s}$-α was mixed with C1q and C1$\bar{r}$ and incubated for 5 min at 37 °C prior to addition of FITC-C1$\bar{s}$; after an additional 5-min incubation, the mixture was injected into the column. Note that C1$\bar{s}$-α effectively blocks incorporation of FITC-C1$\bar{s}$ into the C1 complex. Protein volumes and concentrations in the applied samples were 22 μL, 14.4 μM for FITC-C1$\bar{s}$; 20 μL, 16 μM for C1$\bar{r}$; 250 μL, 6.3 μM for C1q; and 155 μL, 2.1 μM for C1$\bar{s}$-α.

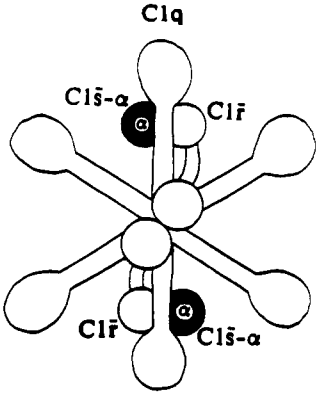

FIGURE 6: Schematic diagram showing the formation of a modified C1 with two molecules of C1$\bar{s}$-α, two molecules of C1$\bar{r}$, and one molecule of C1q. As this model illustrates, the C1$\bar{s}$-α fragment or domain possesses all of the interaction sites of C1$\bar{s}$ necessary to induce a C1 complex when mixed with C1$\bar{r}$ and C1q. This diagram is adapted from the model of Colomb et al. (1984a,b).

complex with C1$\bar{r}$ and C1q and therefore possesses all of the interaction sites required for native C1$\bar{s}$ to form C1.

Discussion

The observations presented here provide the first direct proof that all of the Ca^{2+}-dependent interactions of human C1$\bar{s}$ with itself and with other subcomponents of C1 are mediated by the NH_2-terminal 27–31-kDa α region of its heavy chain. This assignment was made possible by isolating a fragment of 27–31 kDa molecular mass which had the same NH_2-terminal sequence as C1$\bar{s}$ and showing that it retained the ability of the parent molecule to self-associate, bind to C1$\bar{r}$, and, most dramatic, mediate the binding of C1$\bar{r}$ to C1q. The latter observation is embodied in the schematic diagram of Figure 6 fashioned after the model of C1 proposed by Colomb, Arlaud, and co-workers (Colomb et al., 1984a,b; Arlaud et al.,

1987b; Villiers et al., 1985). Our findings should provide useful constraints to those attempting to refine models of the structure of the C1 complex [reviewed by Perkins (1989)]. It would appear that neither the catalytic B chain domain nor the two short-consensus repeat units contained within the γ region of C1s̄ contribute significantly to the interaction of $C1\bar{r}_2\bar{s}_2$ with the collagenous domains of C1q.

Although Figure 6 suggests a direct contact between C1q and C1s̄-α, there is no proof for this. It is possible that C1s̄-α induces a conformational change in C1r̄ that enhances its affinity for C1q. We reported earlier that the isolated C1r̄-α fragment was unable to mediate the binding of C1s̄ to C1q, even though it was shown to bind to C1s̄ in a Ca^{2+}-dependent manner and disrupt the formation of C1s̄ dimers. The resulting C1s/C1r-α complexes might also be expected to have undergone any conformational changes arising from heterologous α–α interactions. Their failure to bind to C1q may simply reflect the fact that they would be monovalent with respect to the number of C1q-binding sites whereas the complex between C1s̄-α and the stable C1r̄ dimer would be divalent, as depicted in Figure 6. This would allow twice as many contacts, accounting for the greater affinity of C1q for (C1s̄-α)-$(C1\bar{r})_2$(C1s̄-α) versus C1s̄/C1r̄-α. The competition experiment of Figure 5 suggests that this affinity is of the same order as that for the tetramer containing whole C1s̄.

It must be emphasized that all of our data were obtained with the activated two-chain forms of C1r̄ and C1s̄ or fragments derived therefrom. It is possible that the single-chain zymogen forms would behave differently. Indeed, the zymogen form of C1 is known to be significantly more stable than the activated form with respect to dissociation of the r_2s_2 tetramer from C1q (Siegel & Schumaker, 1983). This additional stability could arise from activation-sensitive modulating effects of the γB regions of C1r and/or C1s on the respective α regions (domain–domain interactions) or from direct contacts between C1q and residues in the γB regions of one or both proteins, residues whose conformation or accessibility might be affected by activation. In this context, it is interesting to speculate about a possible role for the EPTMY sequence found at the NH_2 terminus of the C1s̄ A chain and again about seven residues beyond the active-site histidine in the catalytic B chain. We predict that C1s-α will also mediate the binding of zymogen C1r to C1q. If so, it will be of great interest to determine whether the C1r in such complexes becomes activated by antibody-containing or nonimmune activators.

The primary structure of C1r and C1s contains a series of "motifs" or "modules" designated (beginning from the NH_2 terminus) I, II, III, IV, V, and B (Leytus et al., 1986; see Figure 1A). The first and third of these are unique to C1r̄ and C1s̄ and have thus been postulated to be involved in the binding to C1q (Arlaud et al., 1987b). The present findings certainly diminish the potential importance of modules IV, V, and B in C1q binding but do not reveal the relative importance of modules I, II, and III. At least the first disulfide loop of the latter module is contained in the fragment described here and could thus be involved. Module II, the so-called EGF-like domain, is found with varying degrees of homology in numerous other proteins (Rees et al., 1988). This module has been shown to contain postranslationally hydroxylated Asp or Asn residues in several proteins including both C1r̄ and C1s̄ (Przysiecki et al., 1987; Arlaud et al., 1987c) and has been implicated in Ca^{2+} binding (Stenflo et al., 1987; Persson et al., 1989). The Ca^{2+}-dependent self-association exhibited by C1s̄-α is virtual proof that this fragment binds Ca^{2+} but does not localize that binding to the EGF-like module. In fact, subfragments of C1s̄-α that contained modules I or II failed in our hands to dimerize or bind to immobilized C1s̄ (data not shown). It appears from this work and that of Thielens et al. (1989) that modules I and II are both required to confer these functions. Additional work will be required to define the nature of the Ca^{2+}-binding site(s) and the mechanism of Ca^{2+}-induced interactions.

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The Small Molecular Mass Ubiquinone-Binding Protein (QPc-9.5 kDa) in Mitochondrial Ubiquinol-Cytochrome *c* Reductase: Isolation, Ubiquinone-Binding Domain, and Immunoinhibition†

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ABSTRACT: The small molecular mass ubiquinone-binding protein (QPc-9.5 kDa) was purified to homogeneity from 3-azido-2-methyl-5-methoxy-6-(3,7-dimethyl[^{3}H]octyl)-1,4-benzoquinol-labeled bovine heart mitochondrial ubiquinol-cytochrome *c* reductase. The N-terminal amino acid sequence of the isolated protein is Gly-Arg-Gln-Phe-Gly-His-Leu-Thr-Arg-Val-Arg-His-, which is identical with that of a M_r = 9500 protein in the reductase [Borchart et al. (1986) *FEBS Lett. 200*, 81-86]. A ubiquinone-binding peptide was prepared from [^{3}H]azidoubiquinol-labeled QPc-9.5 kDa protein by trypsin digestion followed by HPLC separation. The partial N-terminal amino acid sequence of this peptide, Val-Ala-Pro-Pro-Phe-Val-Ala-Phe-Tyr-Leu-, corresponds to amino acid residues 48-57 in the reported M_r = 9500 protein. According to the proposed structural model for the M_r = 9500 protein, the azido-Q-labeled peptide is located in the membrane on the matrix side. These results confirm our previous assessment that the M_r = 13 400 subunit is not the small molecular weight Q-binding protein. Purified antibodies against QPc-9.5 kDa have a high titer with isolated QPc-9.5 kDa protein and complexes that contain it. Although antibodies against QPc-9.5 kDa do not inhibit intact succinate- and ubiquinol-cytochrome *c* reductases, a decrease of 85% and 20% in restoration of succinate- and ubiquinol-cytochrome *c* reductases, respectively, is observed when delipidated succinate- or ubiquinol-cytochrome reductases are incubated with antibodies prior to reconstitution with ubiquinone and phospholipid, indicating that epitopes at the catalytic site of QPc-9.5 kDa are buried in the phospholipid environment. Antibodies against QPc-9.5 kDa cause an increase of the apparent K_m for ubiquinol 2 in ubiquinol-cytochrome *c* reductase, suggesting that the low level of inhibition of the reductase by these antibodies may be due to the use of excess ubiquinol 2 in the assay mixture. Since antibodies against QPc-9.5 kDa inhibit 75% of the antimycin-sensitive plastoquinone reduction activity in the reconstituted succinate-cytochrome *c* reductase, QPc-9.5 kDa may be involved in the Q_i site. The topological arrangement of QPc-9.5 kDa in the mitochondrial membrane was examined immunologically with an anti-QPc-9.5 kDa Fab' fragment-horseradish peroxidase conjugate. When intact mitochondria (mitoplasts) or electron-transport particles (ETP) are exposed to this conjugate, peroxidase activity is found in both preparations, with ETP having the higher activity. This suggests that QPc-9.5 kDa is transmembranous, possibly with more mass on the matrix side of the membrane.

Bovine heart mitochondrial ubiquinol-cytochrome *c* reductase, known as complex III, catalyzes electron transfer from ubiquinol to cytochrome *c*. It contains five redox-active centers: two *b*-type cytochromes (cytochrome *b*-565 and cytochrome *b*-562), one *c*-type cytochrome (cytochrome c_1), one high-potential iron-sulfur center (2Fe-2S Rieske's center), and one ubiquinone (Q).[1] Purified reductase shows seven protein bands in SDS-PAGE using the Weber-Osborn gel system. The two smaller molecular weight protein bands are resolved into six bands when subjected to a high-resolution gel system, thus giving a total of 11 protein subunits for ubiquinol-cytochrome *c* reductase (Schägger et al., 1986; von Jagow et al., 1986; Gonzalez-Halphen et al., 1988). While cytochrome *b*, cytochrome c_1, and the iron-sulfur protein, identified as subunits III-V, respectively, in both gel systems, have been purified to homogeneity and their amino acid sequences reported (Wakabayashi et al., 1980; Anderson et al., 1982; Shimomura et al., 1984; Schägger et al., 1987), information on the protein structure of the Q-associated proteins is rather limited.

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[1] Abbreviations: [^{3}H]azido-QH_2, 3-azido-2-methyl-5-methoxy-6-(3,7-dimethyl[^{3}H]octyl)-1,4-benzoquinol; ELISA, enzyme-linked immunosorbent assay; ETP, electron-transport particles; HRP, horseradish peroxidase; HPLC, high-performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; PITC, phenyl isothiocyanate; PTC, phenylthiocarbamoyl; PTH, phenylthiohydantoin; PBS, phosphate-buffered saline; Q, ubiquinone; Q_2, 2,3-dimethoxy-5-methyl-6-geranyl-1,4-benzoquinone; QPc-9.5 kDa, the small molecular mass ubiquinone-binding protein in ubiquinol-cytochrome *c* reductase; QPs, a protein complex that converts succinate dehydrogenase into succinate-ubiquinone reductase; [^{3}H]Q_0C_{10}NAPA, 2,3-dimethoxy-5-methyl-6-[10-[3-(4-azido-2-nitroanilino)[^{3}H]propionoxy]decyl]-1,4-benzoquinone; SDS, sodium dodecyl sulfate; TUT, 50 mM Tris-acetate buffer, pH 7.8, containing 2 M urea and 1.5% Triton X-100.

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