# Domain Structure, Stability, and Interactions of Human Complement $C1\bar{s}$: Characterization of a Derivative Lacking Most of the B Chain

T. F. Busby and K. C. Ingham*

*Biochemistry Laboratory, American Red Cross Biomedical Research and Development, Rockville, Maryland 20855*

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ABSTRACT: A better understanding of the structure and function of C1 requires knowledge of the regions (domains) of the subcomponents that are responsible for $Ca^{2+}$-dependent assembly. Toward this end, $C1\bar{s}$ was digested with trypsin in the presence of $Ca^{2+}$, a treatment that rapidly degraded the B chain, leaving a 56-kDa fragment comprised of a complete A chain disulfide linked to a small (<4-kDa) residual piece of the B chain. The purified fragment, referred to as $C1\bar{s}$-A, was shown by fast exclusion chromatography to be similar to $C1\bar{s}$ in its ability to (1) reversibly dimerize in the presence of $Ca^{2+}$, (2) substitute for $C1\bar{s}$ in the formation of $C1\bar{r}_2\bar{s}_2$ tetramers, and (3) associate with $C1\bar{r}$ and C1q to form macromolecular C1. Although $C1\bar{s}$-A was itself catalytically and hemolytically inactive, it competitively inhibited the expression of the hemolytic activity of $C1\bar{s}$ in a reconstitution assay. When heated in the absence of $Ca^{2+}$, $C1\bar{s}$ exhibited a low-temperature transition (LTT) near 31 °C and a high-temperature transition (HTT) near 51 °C, similar to those previously observed in the homologous protein $C1\bar{r}$ [Busby, T. F., & Ingham, K. C. (1987) *Biochemistry 26*, 5564–5571]. The midpoint of the LTT was shifted to 58 °C in 5 mM $Ca^{2+}$ whereas the HTT was unaffected by $Ca^{2+}$. $C1\bar{s}$-A exhibited only a LTT whose midpoint and $Ca^{2+}$ dependence were similar to those of the LTT in $C1\bar{s}$. The HTT, which was accompanied by a loss of esterolytic activity, was reproduced in a plasmin-derived fragment representing the catalytic domain. These results provide strong support for the structural and functional independence of the catalytic and interaction domains of $C1\bar{s}$ and strengthen current models regarding the role of these domains in various interactions. They also provide direct proof for the occurrence of $Ca^{2+}$ binding sites on the A chain and demonstrate that all or most of the sites on $C1\bar{s}$ that are responsible for its interaction with $C1\bar{r}$ and C1q are located on the A chain.

$C1s$[1] is one of three subcomponents of C1, the first component of complement [reviewed by Cooper (1985), Schumaker et al. (1987), and Arlaud et al. (1987c)]. Like many serine proteases it is comprised of at least two independent domains: a catalytic domain responsible for the cleavage of C2 and C4 during classical activation of the complement cascade and one or more interaction domains responsible for the binding of C1s to the other subcomponents, C1q and C1r. The latter is also a serine protease whose amino acid sequence exhibits 40% homology with C1s (Leytus et al., 1986; Arlaud et al., 1987a; Tosi et al., 1987; Mackinnon et al., 1987). These two homologous proteins associate reversibly in the presence of $Ca^{2+}$ to form a stable tetramer, $C1r_2s_2$, which in turn binds to the third subcomponent, C1q. The 18 polypeptide chains of the latter define a hexavalent structure which mediates the binding of C1 to immune complexes and other activating substances, triggering the conversion of the associated zymogens, first C1r and then C1s, to their active two-chain forms, designated $C1\bar{r}$ and $C1\bar{s}$. The catalytic sites are located on the "light" B chains whereas the interaction domains are believed to reside on the "heavy" A chains. Treatment of either protein with plasmin removes the N-terminal two-thirds of the A chains and produces truncated forms in which the remaining C-terminal ($\gamma$) portion of the A chains are disulfide linked to the B chains. These catalytic $\gamma$-B domains retain their ability to cleave macromolecular substrates and to form stable complexes with C1-Inh but fail to bind $Ca^{2+}$ or participate in the $Ca^{2+}$-dependent associations characteristic of the parent proteins (Villiers et al., 1985). Less progress has been made in isolating the domains responsible for the latter properties.

We recently isolated an N-terminal fragment of the A chain of $C1\bar{r}$ that retained its ability to bind $C1\bar{s}$ in a $Ca^{2+}$-dependent manner (Busby & Ingham, 1987). The fragment was also shown to exhibit an irreversible thermal transition near 32 °C under physiological conditions in the absence of $Ca^{2+}$, similar to one seen with the intact protein. A second transition that occurred near 53 °C in whole $C1\bar{r}$, regardless of $Ca^{2+}$, was reproduced in the isolated $\gamma$-B fragment and thus assigned to the catalytic domain. An earlier study of $C1\bar{s}$ suggested that it also exhibits multiple thermal transitions analogous to those of $C1\bar{r}$ (Lennick et al., 1985). The objective of the present study was to isolate fragments of $C1\bar{s}$ that would aid in the assignment of the multiple transitions and allow a test of the structural and functional independence of the corresponding domains. In this way we have directly confirmed the location of the interaction domain of $C1\bar{s}$ on the A chain by isolating an essentially B chain free derivative that exhibits a low-temperature $Ca^{2+}$-sensitive transition and retains the ability to self-associate and heteroassociate in the presence of $Ca^{2+}$. Additionally, we show that the high-temperature $Ca^{2+}$-in-

* Author to whom correspondence should be addressed.

[1] Abbreviations: FITC, fluorescein isothiocyanate; ANS, 8-anilino-1-naphthalenesulfonate; EDTA, ethylenediaminetetraacetic acid; Z-Gly-Arg-sBzl, *N*-(benzyloxycarbonyl)-L-Gly-Arg thiobenzyl ester; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; kDa, kilodalton(s); SEC, size-exclusion chromatography; Tris, tris(hydroxymethyl)aminomethane; TBS, Tris-buffered saline (0.02 M Tris-HCl, 0.15 M NaCl, pH 7.4); LTT, low-temperature transition; HTT, high-temperature transition; C1, first component of complement; C1r and C1s, zymogen forms of C1 subcomponents; $C1\bar{r}$ and $C1\bar{s}$, activated two-chain forms of C1r and C1s; $C1\bar{s}$-A, large fragment of $C1\bar{s}$ containing the heavy A chain and a small (<4-kDa) portion of the light B chain; C1-Inh, C1 inhibitor.

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sensitive transition, like that in C1r̄, occurs in the catalytic domain. Finally, we present evidence that the binding site for heparin is located on the B chain of C1s̄.

## Materials and Methods

Unless otherwise stated, all experiments were performed in 0.02 M Tris-HCl buffer, pH 7.4, and 0.15 M NaCl (TBS). C1q, C1r̄, and C1s̄ were purified from Cohn fraction I of human plasma by affinity chromatography according to Bing et al. (1980), except that the immunoglobulin G–agarose was prepared according to Kolb et al. (1979). Nonreduced samples of C1r̄ and C1s̄ displayed a major band and a trace of a lower molecular weight contaminant on SDS–PAGE; reduced samples gave two bands corresponding to the A and B chains, with no detectable single-chain zymogen. C1s̄ was homogeneous by size-exclusion chromatography in the presence of EDTA, where it eluted as a monomer, and in the presence of $Ca^{2+}$, where it eluted as a dimer, with no evidence of a nonassociating population under the latter conditions. C1r̄ eluted as a dimer under both conditions, with no evidence of monomeric material. However, samples of C1r̄ that were homogeneous by SDS–PAGE consistently displayed small amounts of aggregated material in the void of the exclusion column, whether in EDTA or in $Ca^{2+}$.

The A chain containing fragment of C1s̄ (C1s̄-A) was produced by incubating 15 mL of C1s̄ (18 $\mu$M) with TPCK–trypsin (Sigma) at a final ratio of trypsin:C1s̄ of 1:400 (w/w) at 30 °C for 60 min in TBS and 1 mM $CaCl_2$. The digest was then passed over a Bio-Gel A-1.5M (Bio-Rad) size-exclusion column (2.5 × 175 cm) equilibrated at 4 °C in TBS, 0.02% $NaN_3$, and 1 mM EDTA. The $A_{280}$ of each 13.8-mL fraction was determined, and esterolytic assays were performed on all fractions containing protein. The fractions containing the C1s̄-A fragment were identified by SDS–PAGE.

The C1s̄ $\gamma$-B fragment was prepared by a modification of the method of Villiers et al. (1985). A sample of C1s̄ (35.5 $\mu$M in TBS and 1 mM EDTA) was incubated with plasmin (Sigma) at a final ratio of plasmin:C1s̄ of 1:100 (w/w) at 37 °C for 10 min. The digested material was then passed over a Superose 12 size-exclusion column (1 × 30 cm) equilibrated at room temperature with TBS and 1 mM $Ca^{2+}$. The fractions containing the $\gamma$-B fragments, determined by performing SDS–PAGE on a portion of each, were pooled, applied to a heparin–Sepharose column (0.9 × 2 cm) equilibrated at room temperature in 0.02 M Tris-HCl, pH 7.4, 0.05 M NaCl, 0.02% $NaN_3$, and 1 mM $Ca^{2+}$, and washed with the same buffer. The bound $\gamma$-B was eluted by increasing the NaCl to 1.0 M. The heparin–Sepharose column was prepared by attaching heparin (Sigma, hog mucosal) to CNBr-activated Sepharose according to the method of March et al. (1974).

The C1r̄$\alpha$ fragment was produced by trypsin digestion as previously described (Busby & Ingham, 1987) except the mixture of C1r̄ and trypsin was incubated for only 2 h at 30 °C instead of 20 h to minimize internal cleavage at additional sites.

The concentrations of C1s̄ and C1r̄ were determined by using values of $A_{280,1\%}$ = 13.7 and 11.6, respectively, calculated by the method of Edelhoch (1967) from the number of Tyr, Trp, and disulfides in C1s̄ (Mackinnon et al., 1987; Tosi et al., 1987) and C1r̄ (Leytus et al., 1986; Arlaud et al., 1987a). The molecular weights of C1s̄ (80 300) and C1r̄ (86 000) were calculated from the amino acid sequences plus 7.1% and 9.4%, respectively, for carbohydrate (Sim et al., 1977). Assuming that the proteolytic cleavage that generates the $\gamma$-B fragment of C1s̄ (Villiers et al., 1985) is in the same place as for C1r̄ (Arlaud et al., 1986), the $A_{280,1\%}$ value of C1s̄ $\gamma$-B was calculated to be 17.7, on the basis of a $M_r$ of 48 000 (amino acid + 50% of carbohydrate). The $A_{280,1\%}$ value for C1s̄-A was calculated to be 11.9, assuming a $M_r$ of 56 000 (amino acid + 100% of carbohydrate). The concentration of C1q was determined by using $A_{280,1\%}$ = 6.82 (Reid et al., 1972) and a $M_r$ of 459 000 (Reid, 1983).

Esterolytic activity for C1s̄ and C1r̄ were determined from the rate of hydrolysis of the synthetic thioester substrates Z-Lys-sBzl and Z-Gly-Arg-sBzl (Enzyme Systems Products), respectively, in the presence of the chromogenic thiol reagent dithiodipyridine (Sigma) as described in McRae et al. (1981) and Lennick et al. (1986).

SDS–PAGE was performed on 10–15% gradient acrylamide gels on a Pharmacia Phastgel system, and the protein bands were visualized with Coomassie blue.

Fluorescence measurements were made on a Perkin-Elmer fluorescence spectrophotometer as detailed in Busby and Ingham (1987). Melting curves were produced by heating samples at 1 °C/min, either in the presence of ANS (66 $\mu$M) while monitoring the fluorescence at 460 nm with excitation at 370 nm or in the absence of ANS while monitoring the intrinsic Trp fluorescence at 340 nm with excitation at 280 nm. Where possible, midpoints of the melting transitions were estimated by determining the temperature at which the measured fluorescence reached a value halfway between the extrapolated pre- and posttransition base lines.

C1s̄ was labeled with fluorescein isothiocyanate (FITC) by adding a small volume of FITC (30 mM in methanol) to a solution of C1s̄ to give a final molar ratio of FITC:C1s̄ of 15:1. After 70 min at 37 °C, the mixture was applied to a Sephadex G-50 column (0.8 × 22 cm) equilibrated with TBS, pH 7.4, and 1 mM $Ca^{2+}$ to remove the free dye. The degree of labeling was estimated spectrophotometrically to be between 0.7 and 1.0 mol of dye/mol of C1s̄ (Ingham & Brew, 1981). This FITC–C1s̄ retained its ability to dimerize, to complex with C1r̄, and to form a macromolecular C1̄ complex in the presence of $Ca^{2+}$. Higher degrees of labeling above 1 mol/mol progressively decreased the ability of C1s̄ to dimerize. Visual inspection of fluorescent bands on SDS–PAGE revealed incorporation of dye into both chains, with the light chain appearing to react faster than the heavy chain.

Size-exclusion chromatography (SEC) was performed on a Pharmacia FPLC system using a Superose 12 column at a flow rate of 1 mL/min. The proteins were eluted with TBS, pH 7.4, containing either 1 mM $Ca^{2+}$ or 1 mM EDTA while monitoring the adsorbance at 280 nm and/or the fluorescence. The fluorescence was monitored with a Shimadzu Model RF-530 fluorescence spectromonitor using excitation/emission wavelengths of 270/310 nm (tyrosine), 295/350 nm (tryptophan), or 495/525 nm (FITC). The elution position of each sample was determined from the output of the integration feature of the Pharmacia LCC-500 controller. Because of small variations in elution position of identical samples over the time period of this study, due to changes in the column bed volume, samples that were compared directly were injected on the same day, under identical elution conditions.

Hemolytic assays were performed with antibody-sensitized sheep erythrocytes (EA) and EAC4 by the method of Rapp and Borsos (1970). The sheep erythrocytes and antibody to sheep erythrocytes were purchased from Colorado Serum Co. (Denver, CO), and human C4 and guinea pig complement were obtained from Diamedix (Miami, FL). Various dilutions of C1̄ that had been reconstituted from purified C1q, C1r̄, and C1s̄ (and/or C1s̄-A) in $DGVB^{2+}$ buffer (3% dextrose, 1 mg/mL gelatin, 2 mM sodium barbital, 0.06 M NaCl, 1 mM

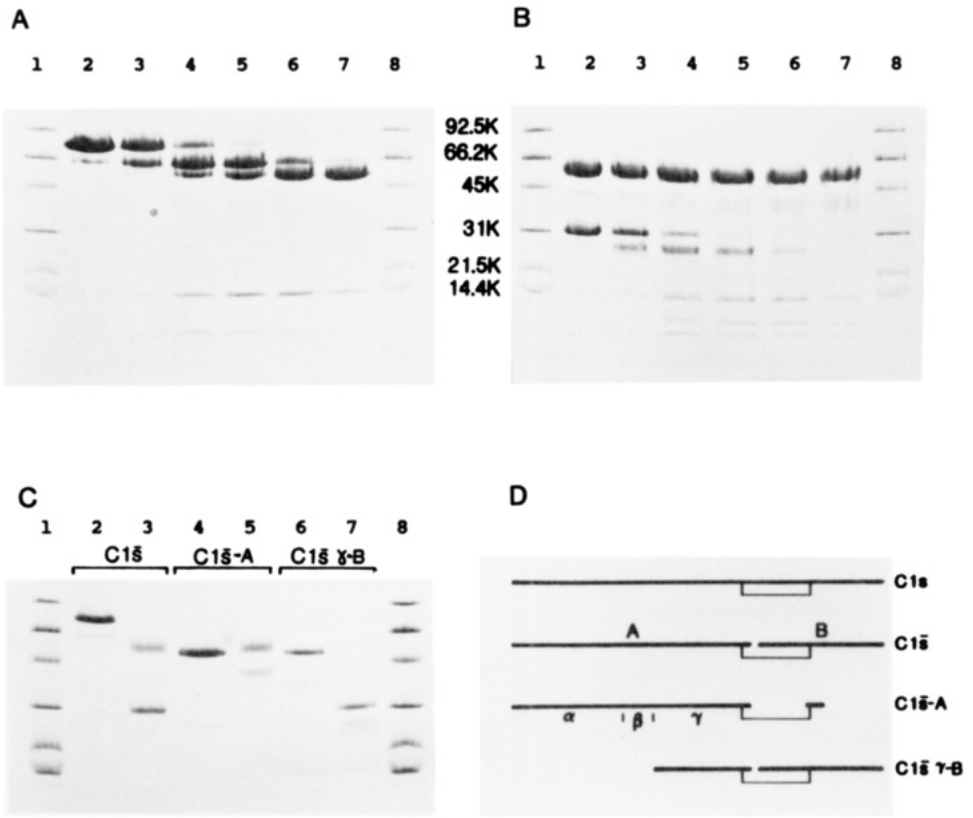



FIGURE 1: Electrophoretic analysis of C1s̄ and its fragments. (A and B) Time course of digestion of C1s̄ with trypsin. C1s̄ (18 μM in TBS, pH 7.4, and 1 mM $Ca^{2+}$) was incubated with trypsin at a final ratio of trypsin:C1s̄ of 1:400 (w/w) at 30 °C for increasing times and analyzed by SDS–PAGE before (A) and after (B) reduction. Lanes 1 and 8, low molecular weight standards; lane 2, C1s̄ alone; lane 3, 1 min; lane 4, 5 min; lane 5, 10 min; lane 6, 30 min; lane 7, 60 min. (C) SDS–PAGE of C1s̄ and its isolated fragments before (lanes 2, 4, and 6) and after (lanes 3, 5, and 7) reduction and alkylation. Lane 1 and 8, low molecular weight standards; lanes 2 and 3, C1s̄; lanes 4 and 5, C1s̄-A fragment; lanes 6 and 7, C1s̄ γ-B fragment. (D) Schematic representation of C1s̄ and its fragments.

$Mg^{2+}$, 0.15 mM $Ca^{2+}$, pH 7.35) were tested for their ability to hemolyze EAC4 cells. The C1q used in these assays was purified from fresh serum and was provided by Dr. Andrea Tenner.

N-Terminal protein sequence analysis of the C1s̄-A fragment was kindly performed by Dr. Fairwell Thomas (National Institutes of Health) on a Beckman System 891 sequencer.

## RESULTS

*Isolation and Characterization of C1s̄ Fragments.* Digestion of C1s̄ with trypsin in the presence of $Ca^{2+}$ rapidly degraded the B chain, leaving a fragment that comigrated with the A chain of C1s̄ on SDS–PAGE with an apparent $M_r$ of 56 000 (Figure 1A,B). The reduced gel (Figure 1B) shows that destruction of the B chain is complete within 30–60 min. Purification of the fragment as described under Materials and Methods produced material that was homogeneous by exclusion chromatography and by SDS–PAGE when nonreduced but showed varying amounts of a 38–40-kDa fragment upon reduction (Figure 1C, lanes 4 and 5). The purified fragment, referred to as C1s̄-A, had no detectable esterolytic activity toward Z-Lys-sBzl and failed to react with C1-Inh. However, as will be shown below, it retained all of the $Ca^{2+}$-dependent self-associating and heteroassociating properties of intact C1s̄.

N-Terminal sequence analysis of nonreduced C1s̄-A identified the amino acids shown in Chart I in ten successive cycles (the residues in parentheses were not detected but are included to facilitate interpretation). The first row corresponds exactly to the N-terminal sequence of the A chain of C1s̄ (Mackinnon et al., 1987; Tosi et al., 1987). The second row, obtained in approximately the same yield as the first, corresponds to a region of the B chain, which begins just after $Lys^{525}$ and

Chart I

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| | Glu | Pro | Thr | Met | Tyr | Gly | Glu | Ile | Leu | Ser |
| (Lys) | Met | Gly | Pro | Thr | Val | Ser | Pro | Ile | (Cys) | Leu |
| (Arg) | Val | (Gly) | Ala | (Thr) | (Ser) | Phe | Tyr | | | |

includes the interchain disulfide bond. Cysteine was not detected in cycle nine because the sample was not reduced before sequencing. The next possible position where trypsin might be expected to cleave the B chain is 32 amino acids later at $Arg^{557}$, and this is followed by six more trypsin-susceptible bonds within the next 14 amino acids. The third row represents a sequence within a disulfide loop in the γ region of the A chain beginning at $Arg^{316}$. A small amount of cleavage at this position could account for the low yields of Val, Ala, Phe, and Tyr detected in cycles 1, 3, 6, and 7 and would be consistent with the appearance of the 38–40-kDa band on reduced gels. C1s̄-A is therefore most likely composed of molecules containing the entire A chain of C1s̄, some of which are cleaved at $Arg^{316}$ and all of which are disulfide linked to a short segment of the B chain approximately 32 amino acids in length. This means that the nonreduced C1s̄-A fragment (Figure 1C, lane 4) is several kilodaltons larger than the A chain alone, even though it appears slightly smaller when compared to reduced A chain of C1s̄ (Figure 1C, lane 3), presumably due to the more compact nature of the nonreduced material. A similar effect occurs upon reduction of the single-chain zymogen form of C1s (Arlaud et al., 1977). The major band in the reduced form of C1s̄-A (lane 5) is indistinguishable from the A chain of C1s̄.

The purified C1s̄ γ-B fragment, derived from a plasmin digest (Villiers et al., 1985), is shown in Figure 1C, lanes 6

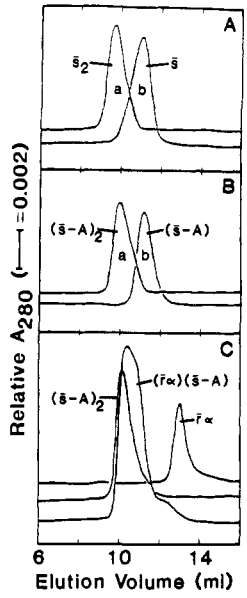



FIGURE 2: Demonstration by size-exclusion chromatography that C1$\bar{s}$-A, like C1$\bar{s}$, can form $Ca^{2+}$-dependent dimers with itself and that these dimers can be disrupted by the $\alpha$ domain of C1$\bar{r}$. (A) C1$\bar{s}$ (10 $\mu$L of a 25 $\mu$M solution) and (B) C1$\bar{s}$-A (30 $\mu$L of a 10 $\mu$M solution) were injected onto a Superose 12 size-exclusion chromatography column equilibrated in TBS containing either 1 mM $Ca^{2+}$ (curves a) or 1 mM EDTA (curves b). (C) C1$\bar{s}$-A and C1$\bar{r}\alpha$ were injected alone, or mixed, onto the Superose 12 column equilibrated in TBS and 1 mM $Ca^{2+}$. The samples were eluted at a flow rate of 1 mL/min and monitored by $A_{280}$.

and 7. Upon reduction, the B chain migrates as a sharp band, suggesting that it is predominantly uncleaved and of uniform size. The $\gamma$ fragment migrates as a diffuse 24–27-kDa band, suggesting that it is heterogeneous with substantial but variable portions of the $\beta$ region attached. This would be consistent with the presence of several potential cleavage sites in the $\beta$ region and with N-terminal sequence analyses that detected three or more residues in each cycle, in addition to the major ones corresponding to the N-terminus of the B chain. In agreement with Villiers et al. (1985), the isolated fragment retained its catalytic activity against the synthetic substrate Z-Lys-sBzl and was totally inactivated with C1-Inh but failed to dimerize or interact with C1$\bar{r}$ in the presence of $Ca^{2+}$ (data not shown).

*Interactions of C1$\bar{s}$-A with Other Subcomponents.* Size-exclusion chromatography in EDTA and $Ca^{2+}$ was used to characterize the self-associating properties of C1$\bar{s}$-A (Figure 2B) in comparison to C1$\bar{s}$ (Figure 2A). C1$\bar{s}$-A consistently eluted a little later than C1$\bar{s}$. Both proteins exhibited a large shift toward the void when chromatographed in the presence of $Ca^{2+}$ (curves a), where C1$\bar{s}$ is known to exist as a dimer (Valet & Cooper, 1974a; Tschopp et al., 1980a). Thus C1$\bar{s}$-A, like the parent protein, undergoes reversible dimerization in the presence of $Ca^{2+}$.

In a previous report from this laboratory (Busby & Ingham, 1987), it was shown that $Ca^{2+}$-dependent dimerization of C1$\bar{s}$ could be disrupted by addition of a purified 35-kDa fragment representing the N-terminal ($\alpha$) interaction domain of C1$\bar{r}$. Figure 2C shows that dimers of C1$\bar{s}$-A can be disrupted in a similar fashion. The C1$\bar{r}\alpha$ fragment eluted at 13.0 mL, the C1$\bar{s}$-A fragment at 10.1 mL, and the mixture of the two at 10.3 mL. In the elution profile of the mixture, the peak corresponding to monomeric C1$\bar{r}\alpha$ was depleted. The peak of the mixture was much broader than the C1$\bar{s}$-A peak with a pronounced shoulder near 10.8 mL. This is most likely due to a rapid equilibration between C1$\bar{s}$-A dimers ($M_r$ ~121 000)

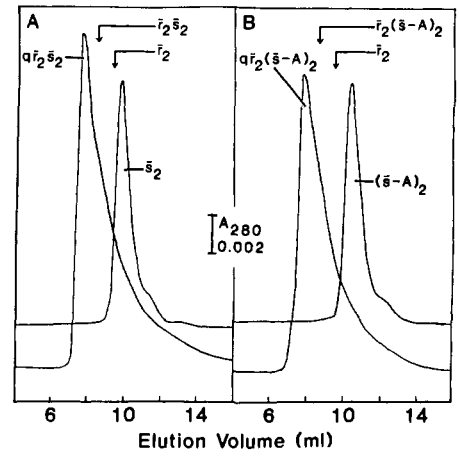



FIGURE 3: Demonstration by size-exclusion chromatography that both C1$\bar{s}$ (A) and C1$\bar{s}$-A (B) are capable of interacting with C1$\bar{r}$ and C1q to form macromolecular C1. (A) C1$\bar{s}$ alone (10 $\mu$L of a 25 $\mu$M solution) or mixed with C1$\bar{r}$ (12 $\mu$L of a 21 $\mu$M solution) and C1q (10 $\mu$L of a 12 $\mu$M solution) to yield C1q$\bar{r}_2\bar{s}_2$. (B) C1$\bar{s}$-A (25 $\mu$L of a 10.5 $\mu$M solution) alone and mixed with C1$\bar{r}$ and C1q were injected onto a Superose 12 column equilibrated in TBS and 1 mM $Ca^{2+}$. The proteins were eluted at a flow rate of 1 mL/min and monitored by $A_{280}$. The elution positions of C1$\bar{r}_2$ alone and mixed with C1$\bar{s}$ ($\bar{r}_2\bar{s}_2$) and C1$\bar{s}$-A [$\bar{r}_2(\bar{s}\text{-A})_2$] are shown by the arrows.

and some C1$\bar{s}$-A/C1$\bar{r}\alpha$ complexes ($M_r$ ~95 000). When the mixture was chromatographed in the presence of 1 mM EDTA, where no interaction would be expected, the C1$\bar{s}$-A monomers eluted at 11.5 mL (as in Figure 2B, curve b) and the elution of C1$\bar{r}\alpha$ was unaffected (data not shown). This pattern is almost identical with that seen with whole C1$\bar{s}$ (Busby & Ingham, 1987) and indicates that the mode of association of C1$\bar{s}$-A with itself and with C1$\bar{r}$ is similar to that of intact C1$\bar{s}$, involving the same domains.

Exclusion chromatography experiments also revealed striking similarities between C1$\bar{s}$ and C1$\bar{s}$-A with respect to their ability to associate with C1$\bar{r}$ in the presence of $Ca^{2+}$ to form complexes which in turn associate with C1q to form macromolecular C$\bar{1}$ (Figure 3). Preincubation of either protein with approximately stoichiometric amounts of C1$\bar{r}$, which by itself elutes as a dimer at the position indicated by the arrows, produced complexes (presumably tetrameric) that eluted as single peaks, also indicated by arrows, at a position earlier than that of any of the individual components (the profiles indicated by the arrows have been omitted for clarity). Addition of C1q to either of these mixtures caused a further shift; although there is some trailing into the region of the individual components, most of the material is carried along with C1q, eluting in the void. In control experiments, C1q had no effect on the elution of C1$\bar{s}$-A alone, C1$\bar{r}$ alone, C1$\bar{s}$ alone, or C1$\bar{s}$ complexed with C1$\bar{r}\alpha$. The trailing of the reconstituted C$\bar{1}$ samples may be due to the use of protein concentrations and conditions of ionic strength that are near the limits required to maintain the association between C1q and C1$\bar{r}_2\bar{s}_2$ (Siegel & Schumaker, 1983). Although the initial protein concentrations were 10-fold above the reported dissociation constants, there is, depending on the volume of the mixtures injected, a considerable dilution on the column. Attempts to increase the affinity by lowering the ionic strength (Colten et al., 1968) appeared to be complicated by interactions of C1q with the column.

Another approach to characterizing the interactions of these proteins involved the use of a fluorescent-labeled derivative of C1$\bar{s}$ (FITC–C1$\bar{s}$), whose elution behavior could be monitored independently of any other proteins in the system. As shown in Figure 4, FITC–C1$\bar{s}$ ($\bar{s}$*) retained the ability to

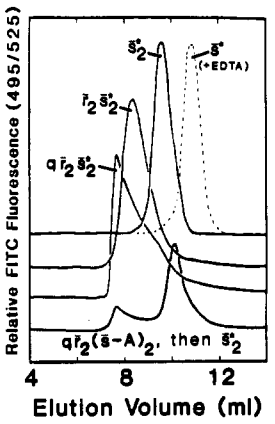



FIGURE 4: Competition of C1s̄-A with FITC–C1s̄ for complexation with C1r̄ and C1q to form C1̄. FITC–C1s̄ (s̄*) was injected alone (40 μL of an 8.7 μM solution), mixed with C1r̄ (65 μL of a 5.6 μM solution), or mixed with C1r̄ and C1q (110 μL of a 1.7 μM solution) onto a Superose 12 column equilibrated in TBS and 1 mM $Ca^{2+}$. The lower profile shows the effect of mixing C1s̄-A (80 μL of a 4.5 μM solution) with C1r̄ and C1q before adding FITC–C1s̄, incubating 30 min at 30 °C, and injecting onto the column. The dashed profile represents the elution position of FITC–C1s̄ monomer in EDTA. Elution was monitored by fluorescence at excitation/emission wavelengths of 495/525 nm.

dimerize in the presence of $Ca^{2+}$ and to associate with C1r̄ to form a tetramer which could associate with C1q to form C1. These assembly reactions were complete within the time required to mix the components and inject them onto the column. However, as reported earlier by Bartholomew and Esser (1977), once C1s̄ or its derivatives had been incorporated into C1, their subsequent displacement or exchange was relatively slow. For example, the lower profile in Figure 4 shows that when C1s̄-A was added to a mixture of C1q and C1r̄ prior to the addition of FITC–C1s̄, only 17% of the fluorescence was incorporated even after 30 min at 30 °C. A similar experiment with C1s̄ led to incorporation of only 7% of the FITC–C1s̄ (not shown). The converse experiment in which C1s̄-A and C1s̄ were added after reconstituting C1 with FITC–C1s̄ resulted in the displacement of 70 and 80%, respectively, of the fluorescence after 30 min. These observations suggest that C1s̄ is slightly more effective than C1s̄-A in terms of its ability to compete with FITC–C1s̄ and that C1 formed with the latter derivative is less stable than C1 formed with C1s̄ or C1s̄-A.

Additional evidence that C1s̄-A is capable of forming a macromolecular complex with C1r̄ and C1q was obtained from hemolytic assays in which C1s̄-A, which was itself hemolytically inactive, was allowed to compete with whole C1s̄. The results of three different experiments are presented in Table I. When a mixture of C1q and C1r̄ was exposed to C1s̄-A before adding C1s̄ (samples 3–6), there was a marked inhibition of the activity relative to that obtained when C1s̄-A was omitted (sample 2); this inhibition could be partially overcome by addition of larger amounts of C1s̄ (samples 3–6 of Table I). When C1s̄ was added to C1r̄ before addition of C1s̄-A, the C1s̄ appeared to express its full activity even in the presence of a 4-fold excess of C1s̄-A (samples 7–10). When the C1s̄-A fragment and whole C1s̄ were premixed and added simultaneously to C1q and C1r̄ (samples 11–15, experiment 3), there was a good correlation between the amount of lysis and the proportion of active C1s̄ in the mixture. These results indicate that C1s̄ and C1s̄-A combine with the other subcomponents at similar rates and that the resulting reconstituted C1 molecules are stable and slow to exchange during subsequent incubations and dilutions.

Table I: Competitive Inhibition of Hemolytic Activity of C1s̄ by C1s̄-A

| sample[a] | no. | concn (μM) C1s̄-A | concn (μM) C1s̄ | % lysis in expt 1 | % lysis in expt 2 | % lysis in expt 3 |
|---|---|---|---|---|---|---|
| controls | 1 | 0.4 | 0 | 11.1 | 7.9 | −1.5 |
| | 2 | 0 | 0.4 | 100.7 | 84.8 | 23.4 |
| C1s̄-A added first | 3 | 0.8 | 0.4 | 40.5 | 29.5 | −0.3 |
| | 4 | 0.4 | 0.4 | 68.7 | 40.0 | 0.2 |
| | 5 | 0.4 | 0.8 | 70.6 | 41.5 | 1.6 |
| | 6 | 0.4 | 1.6 | 79.5 | 57.2 | 5.5 |
| C1s̄ added first | 7 | 0.4 | 0.8 | 100.9 | 90.1 | 32.0 |
| | 8 | 0.4 | 0.4 | 101.7 | 87.7 | 26.4 |
| | 9 | 0.8 | 0.4 | 102.1 | 88.7 | 30.9 |
| | 10 | 1.6 | 0.4 | 102.2 | 87.5 | 34 |
| C1s̄ and C1s̄-A premixed | 11 | 0.4 | 1.6 | | | 22.8 |
| | 12 | 0.4 | 0.8 | | | 18.7 |
| | 13 | 0.4 | 0.4 | | | 10.9 |
| | 14 | 0.8 | 0.4 | | | 10.3 |
| | 15 | 1.6 | 0.4 | | | 8.1 |

[a] C1 was reconstituted by mixing C1q (final concentration 0.2 μM) and C1r̄ (final concentration 0.4 μM) with C1s̄ and/or C1s̄-A at the final concentration shown. The controls (samples 1 and 2) and the premixed samples (sample 11–15) were incubated 5 min at 30 °C, followed by 1:20000 dilution and assay. When C1s̄ and C1s̄-A were added at different times, each component was incubated at 30 °C for 5 min with C1r̄ and C1q before the second component was added and incubated an additional 5 min. Percent lysis is shown relative to that obtained by suspension of the cells in water.

*Interactions with Heparin.* Heparin has been shown to bind to C1s̄ and to accelerate the reaction of C1s̄ with C1-Inh 15–30-fold (Sim et al., 1980; McKay et al., 1981; Lennick et al., 1986). However, nothing is known about the location of the heparin binding site on C1s̄. Therefore, the purified C1s̄-A and γ-B fragments were tested for their ability to bind to heparin–Sepharose. The C1s̄-A fragment failed to bind to immobilized heparin, while the C1s̄ γ-B fragment bound readily and could be eluted with increased ionic strength, an observation that proved useful in its purification. The concentration of NaCl required for peak elution in a gradient was approximately 0.45 M, similar to that for intact C1s̄ (Lennick et al., 1986). Since both fragments contain the γ portion of the A chain, it would appear that the heparin binding site is located on the B chain. Alternatively, the heparin binding site could be comprised of residues from both the γ fragment and the B chain such that removal of the latter would disrupt this composite site.

*Thermal Transitions of the Domains of C1s̄.* Lennick et al. (1985) observed that upon heating C1s̄ in phosphate-buffered saline, multiple transition could be detected by differential scanning calorimetry and by the fluorescent probe 8-anilino-1-naphthalenesulfonate (ANS). The latter method was used here to examine the dependence of these transtions on $Ca^{2+}$ (Figure 5A). Heating C1s̄ at 1 °C/min in the presence of 1 mM EDTA produced a low-temperature transition (LTT) with a midpoint near 31 °C and a high-temperature transition (HTT) with a midpoint near 52 °C. In 1 mM $Ca^{2+}$, only the HTT was seen. At still higher concentration of $Ca^{2+}$ (5 mM), the HTT transition again occurred in the same position, but a second transition at higher temperature was discernible. This same pattern has been observed by differential scanning calorimetry (not shown).

Both transitions of C1s̄ appear to be irreversible. When samples that had been heated in EDTA to 42 °C, through the LTT, were cooled, the ANS fluorescence returned to a value severalfold higher than the original and this elevated fluorescence could not be reversed by addition of $Ca^{2+}$. When samples that had been heated in $Ca^{2+}$ or EDTA, through the HTT, were cooled, the final intensity was 30–50 times higher

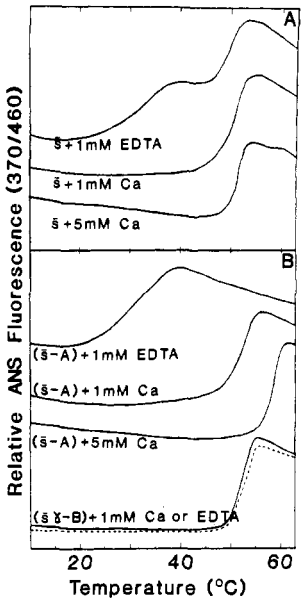



FIGURE 5: Thermal transitions of C1s̄ and its fragments detected by ANS fluorescence. Samples of (A) C1s̄ (1.9–3.8 μM) and (B) C1s̄-A (4.5–26 μM) and C1s̄ γ-B (3.3 μM) containing 66 μM ANS were heated at 1 °C/min in the presence of either 1 mM EDTA, 1 mM $Ca^{2+}$, or 5 mM $Ca^{2+}$ while monitoring the change in ANS fluorescence (370/460 nm). Curves have been arbitrarily displaced for comparison.

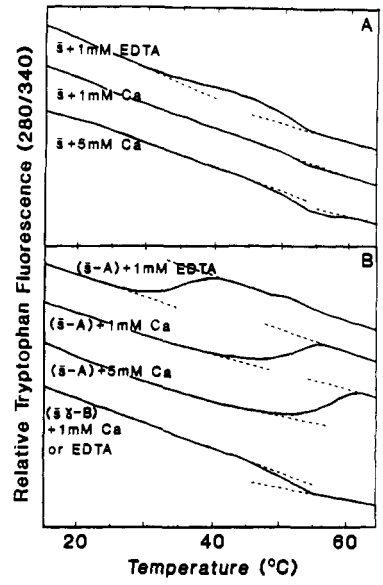



FIGURE 6: Thermal transitions of C1s̄ and its fragments detected by intrinsic Trp fluorescence. Samples of (A) C1s̄ (1.49 μM) and (B) C1s̄-A (2.4 μM) and C1s̄ γ-B (1.5 μM) were heated at 1 °C/min in the presence of either 1 mM EDTA, 1 mM $Ca^{2+}$, or 5 mM $Ca^{2+}$ while monitoring the change in Trp fluorescence (280/340 nm). Curves have been arbitrarily displaced for comparison.

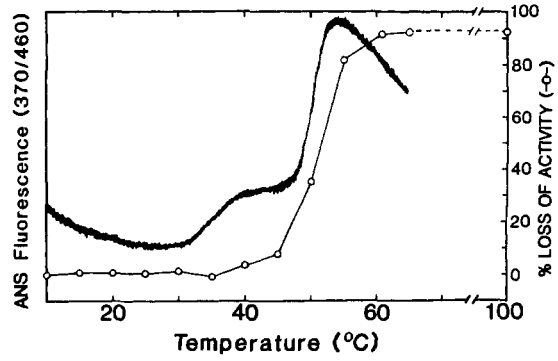



FIGURE 7: Change in the esterolytic activity of C1s̄ during thermal transitions. A sample of C1s̄ (2.7 μM in TBS and 1 mM EDTA) was heated at 1 °C/min, and an aliquot was removed every 5 °C and placed in an ice bath. At 65 °C an aliquot was removed and the remaining sample was heated to 100 °C and boiled for 10 min. An identical sample containing 66 μM ANS was heated at the same time to 65 °C while monitoring the ANS fluorescence (370/460 nm). The remaining esterolytic activity (O) of the heated samples was determined with the synthetic substrate Z-Lys-sBzl as described under Materials and Methods.

than the original. This pattern is similar to that seen previously with C1r̄ (Busby & Ingham, 1987). However, the increase in light scattering that accompanied the LTT of C1r̄ was not seen with C1s̄, and size-exclusion chromatography showed little evidence of aggregation as a result of the LTT.

When the fragments of C1s̄ were heated (Figure 5B), the isolated C1s̄-A fragment displayed *only* a LTT with a midpoint at 31.0 °C in 1 mM EDTA. The midpoint of this single transition shifted to 51.5 °C in the presence of 1 mM $Ca^{2+}$ and 58.0 °C in 5 mM $Ca^{2+}$, similar to the response of the LTT in whole C1s̄. The C1s̄ γ-B fragment showed a single transition near 52 °C in either 1 mM $Ca^{2+}$, 5 mM $Ca^{2+}$ (not shown), or 1 mM EDTA, similar to the HTT of C1s̄.

Thermal transitions in C1s̄ and its fragments could also be detected by monitoring the fluorescence of the intrinsic probe, tryptophan. As shown in Figure 6A, when C1s̄ was heated in the presence of 1 mM EDTA, there was a steady decrease in the intensity interrupted by a small increase above 30 °C, corresponding to the LTT, followed by a small decrease in the region of the HTT. This is in contrast to C1r̄ whose Trp fluorescence under the same conditions decreased smoothly with increasing temperature and showed no inflections (not shown). The same was true for C1s̄ when heated in the presence of 1 mM $Ca^{2+}$. However, in 5 mM $Ca^{2+}$, a slight decrease of the intensity in the region of the HTT, followed by an increase around 57 °C, was seen. Upon heating the isolated fragments of C1s̄ (Figure 6B), the C1s̄-A fragment showed an increase in the Trp fluorescence intensity centered near 30 °C in 1 mM EDTA. This transition was also irreversible; the fluorescence returned to a value 35% above the original on cooling to the original temperature. Attempts to reverse the transition by incubating with $Ca^{2+}$ were unsuccessful; subsequent heating in EDTA produced no detectable transition. The midpoint of this LTT shifted to 52 °C in 1 mM $Ca^{2+}$ and 58 °C in 5 mM $Ca^{2+}$, similar to the pattern in whole C1s̄. The γ-B fragment in either 1 mM $Ca^{2+}$ or EDTA displayed only a decrease in the intensity around 52 °C. The transition temperatures and responses to $Ca^{2+}$ correlated well with those seen by ANS fluorescence, diminishing concern that the presence of the ANS probe might have affected the results. It appears that the melting behavior of whole C1s̄ can be accounted for by the behavior of individual independent domains which, when isolated, retain a structure whose stability is similar to that which they had in the parent protein. The larger amplitude of the Trp fluorescence transition in C1s̄-A relative to whole C1s̄ is expected since 7 out of 12 Trps are located in the B chain. On the other hand, C1s̄ γ-B contains 11 of the 12 Trps, and its transition has an amplitude similar to that in whole C1s̄.

*What Are the Consequences of the Low- and High-Temperature Transitions*? The effect of heating C1s̄ on its ability to hydrolyze the synthetic substrate Z-Lys-sBzl was examined. A sample was heated at 1 °C/min, and an aliquot was removed every 5 °C and placed on ice for subsequent assay. An identical sample containing ANS was heated at the same time while monitoring the ANS fluorescence. As shown in Figure 7, there was no significant change in the catalytic activity of

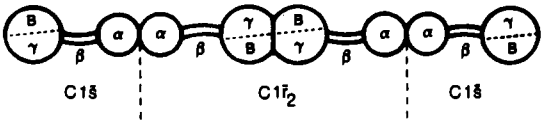
C1s̄ during the LTT. Above 45 °C there was an abrupt decrease in activity coincident with the rise in ANS fluorescence due to the HTT. However, a small level of activity, about 8% of the original, remained even after continued heating up to 100 °C. This residual activity could be abolished by reaction of the heated material with C1-Inh at room temperature prior to assay.

The rate of reaction of C1s̄ with C1-Inh was not affected by the LTT. The pseudo-first-order rate constant for inhibition, measured as previously described (Lennick et al., 1986), changed insignificantly after incubation of C1s̄ at 45 °C in EDTA for 30 min.

Competition experiments of the type shown in Figure 4 showed no significant differences between native C1s̄ and C1s̄ that had been heated through the LTT. However, C1s̄-A that had undergone the LTT appeared to be slightly less effective in terms of its ability to displace FITC–C1s̄ from C1̄ (data not shown). Similarly, measurements of the kind in Table I suggested that the ability of C1s̄-A to block reconstitution of hemolytically active C1̄ from C1s̄, C1r̄, and C1q was slightly diminished after heating at 42 °C for 30 min in the presence of EDTA, whereas the hemolytic activity of C1s̄ itself was unaffected by the LTT. The basis of the apparent small difference between whole C1s̄ and C1s̄-A was not explored further.

## Discussion

C1s̄ is a multidomain serine protease that is involved in many interactions. The simplest of these is its $Ca^{2+}$-dependent dimerization with itself (Valet & Cooper, 1974a; Tschopp et al., 1980a). In the presence of C1r̄, which forms a stable dimer with or without $Ca^{2+}$, a tetrameric complex is formed having the schematic structure (Villiers et al., 1985; Arlaud et al., 1986, 1987c):




This model, which places the C1r̄ dimer in a central position with a single C1s̄ molecule attached through its α domain to each α domain of the C1r̄ dimer is supported, but not proven, by a variety of electron microscopic, proteolytic fragmentation, and chemical modification data (Tschopp et al., 1980a; Villiers et al., 1985; Weiss et al., 1986; Arlaud et al., 1986, 1987c). The isolated C1r̄α domain has been shown to bind C1s̄ in the presence of $Ca^{2+}$ and to interfere with $Ca^{2+}$-induced dimerization of C1s̄ (Busby & Ingham, 1987), providing direct evidence that C1r̄α is responsible for the interaction with C1s̄. We now provide direct evidence that the determinants on C1s̄ that are required for binding to C1r̄ are located on the A chain, most likely in the N-terminal interaction domain. Thus, in the presence of $Ca^{2+}$, an isolated C1s̄ fragment, almost totally devoid of the B chain, dimerizes, forms a tetrameric complex with C1r̄ and a bimolecular complex with the isolated α domain of C1r̄. It is worth emphasizing that these reactions of C1s̄-A were quantitative, in spite of the fact that a variable but significant fraction of the C1s̄-A molecules appeared to be cleaved at an Arg–Gly bond within a disulfide loop in the γ region. These observations strengthen the proposed model for the self-association and heteroassociation of C1s̄ with C1r̄ through the N-terminal interaction domain of both proteins.

Although the zymogen forms of C1r and C1s may interact separately with C1q (Valet & Cooper, 1974a,b; Lakatos, 1987), the activated forms do not appear to do so with sufficient strength to be detected by our methods. However, the $Ca^{2+}$-dependent tetramer of both forms binds readily (Hughes-Jones & Gorick, 1982; Kilchherr et al., 1982; Siegel & Schumaker, 1983; Ziccardi, 1984;, this work) with a dissociation constant of $10^{-7}$–$10^{-9}$ M (Ziccardi & Tschopp, 1982; Siegel & Schumaker, 1983; Lakatos, 1987). The interaction of $C1\bar{r}_2\bar{s}_2$ with C1q is known to involve the collagenous domain of C1q, most likely the arms (Reid et al., 1977; Siegel & Schumaker, 1983; Poon et al., 1983). However, the regions of the C1r̄ and C1s̄ molecules that are responsible for binding to C1q have not been identified. Our results suggest that all of the determinants on C1s̄ that are required for that binding are located within the A chain. This conclusion is based on the observation that C1s̄-A mediated the binding of C1r̄ to C1q in a manner similar to that of C1s̄ and was an effective inhibitor of FITC–C1s̄ in a chromatographic assay and of unlabeled C1s̄ in a hemolytic assay. The exact part of the C1s̄ A chain that is responsible for this binding to C1q cannot be determined from these results, but future studies with smaller fragments might provide this information.

The small C1r̄α fragment was able to form a complex with C1s̄ and C1s̄-A but these complexes failed to bind to C1q. This observation is relevant to considerations of the mechanism by which the tetrameric complexes bind with high affinity to C1q while the individual dimers bind weakly or not at all. One possibility is that C1r̄ and C1s̄ each have affinities for C1q that are too weak to detect independently but whose free energies add up to produce a strong interaction of the tetramer. This form of multivalent attachment has been shown to account for the high-affinity binding of C1q to oligomers of IgG [Tschopp et al. (1980b) and references cited therein]. Alternatively, or in addition, the interaction between C1r̄ and C1s̄ may induce new conformations in one or both molecules that now recognize C1q. Such conformations would presumably be confined to the interaction (α) domains and could conceivably be present in C1r̄α/C1s̄ and C1r̄α/C1s̄-A complexes. However, because of the absence of an intact C1r̄ dimer, these complexes would still possess fewer potential interaction sites than a complete tetramer.

The thermal denaturation results provide strong support for the structural independence of the catalytic and interaction domains of C1s̄, whose melting behavior is remarkably similar to that of the previously studied homologous C1r̄ (Busby & Ingham, 1987). Both proteins exhibit a LTT that occurs near physiological temperature in a metal-free environment and is strongly stabilized by $Ca^{2+}$, as well as a HTT above 50 °C that is insensitive to $Ca^{2+}$. In both cases, these transitions, including their dependence on $Ca^{2+}$, can be qualitatively reproduced by fragments isolated from proteolytic digests of the parent proteins. With C1s̄-A and C1s̄, the LTT was actually shifted above the HTT in 5 mM $Ca^{2+}$. In the case of C1r̄, the LTT was unequivocally assigned to the N-terminal α domain. Although we have not yet isolated the corresponding domain of C1s̄, its presence in C1s̄-A and the striking parallel between the behavior of the two systems make it almost certain that the LTT in C1s̄ and C1s̄-A occurs in the α domain. The fact that both transitions in both proteins occur at very similar temperatures in the isolated fragments as in the parent protein further documents the structural integrity of the isolated domains and suggests that they do not interact significantly with each other intramolecularly in the intact proteins. Stabilization of the LTT in C1s̄-A by $Ca^{2+}$ proves that a binding site (or sites) for this metal ion is (are) located on the A chain, most likely in the α domain, as deduced by Villiers et al. (1985) on the basis of indirect evidence, namely, the loss of $Ca^{2+}$-dependent associations in the γ-B domain and the inhibition

by $Ca^{2+}$ of the production of that domain with plasmin.

Additional evidence for the assignment of the LTT in $C1\bar{s}$ to the $\alpha$ domain comes from a comparison of the temperature dependence of its Trp fluorescence to that of $C1\bar{r}$. With $C1\bar{s}$ there was a small rise corresponding to the LTT and a small decrease corresponding to the HTT. Although the changes were small, they correlated well with those seen by ANS fluorescence, including the dependence on $Ca^{2+}$. More importantly, they were reproduced in the fragments with even greater amplitude, providing additional evidence for the independence of the individual domains within $C1\bar{s}$. By contrast, with $C1\bar{r}$, no inflections were seen in the plots of Trp fluorescence versus temperature (not shown). Examination of the primary structures reveals the absence of Trp in the $\alpha$ domain of $C1\bar{r}$ (Leytus et al., 1986; Arlaud et al., 1987a), while $C1\bar{s}$ contains a single Trp at position 25 (Mackinnon et al., 1987; Tosi et al., 1987) which could account for the change in fluorescence during the LTT. The next Trp in the sequence of either protein does not occur until the $\gamma$ region. The fluorescence changes during the HTT are more difficult to assign since the $\gamma$-B domains of $C1\bar{r}$ and $C1\bar{s}$ contain 9 and 11 Trps, respectively, only 7 of which align in the sequence.

Another important difference between $C1\bar{r}$ and $C1\bar{s}$ was the curious lack of any significant consequence of the LTT on the measured functions of the latter. The transition in $C1\bar{s}$ was clearly irreversible in terms of both ANS and Trp fluorescence, the values of which remained elevated after cooling and were not reversed by $Ca^{2+}$. However, in contrast to $C1\bar{r}$, there was no significant aggregation, no tendency to bind to the Superose 12 column, no loss of protein–protein interactions, and no loss of hemolytic activity. The increased ability to enhance ANS fluorescence represents an increase in the affinity for this hydrophobic probe through the generation of binding sites on the surface of the protein, sites whose functional significance, if any, remains to be determined. In $C1\bar{r}$, such sites could play a role in the aggregation that occurs during the LTT. The lack of aggregation in $C1\bar{s}$ could reflect the much higher net charge of its $\alpha$ fragment (−11 to −15) relative to that in C1r (0 to +4) (Tosi et al., 1987). Finally, the changes induced by the LTT in this domain could alter functions other than those that we have measured, such as the ability to support zymogen activation.

The $\alpha$ domains of C1r and C1s are comprised of two distinct regions of primary structure, regions I and II (Arlaud et al., 1987c; Tosi et al., 1987). Regions I are unique to these proteins and on that basis are proposed to be involved in binding to C1q (Arlaud et al., 1987c). Regions II resemble epidermal growth factor (EGF) like structures that are found, often in multiple copies, in several other complement proteins as well as in several coagulation factors where they often contain a $\beta$-hydroxylated Asp or Asn residue that is implicated in $Ca^{2+}$ binding (Stenflo et al., 1987). By analogy, Arlaud et al. (1987a–c) have proposed a role for $\beta$-OH-$Asn^{150}$ in the binding of $Ca^{2+}$ by C1r. Tosi et al. (1987) find an Asn in the homologous region of the cDNA-derived sequence of C1s and suggest that it also will prove to be hydroxylated. The above-mentioned Trp in C1s$\alpha$ is well separated from the proposed $Ca^{2+}$ binding site, more than 90 residues away, in region I. Yet, it signals an *irreversible* thermal transition that is dramatically stabilized by $Ca^{2+}$ and has little or no effect on binding to C1q. This implies that region I either binds $Ca^{2+}$ in its own right or is stabilized indirectly through strong interaction with a $Ca^{2+}$-bound form of the EGF-like region. Ohlin and Stenflo (1987) provided evidence that binding of $Ca^{2+}$ to the EGF homology region of protein C could produce conformational changes in other parts of the protein. It should be pointed out that EGF itself is an extremely stable structure, requiring high concentrations of GdmCl to observe its thermal unfolding (Holladay et al., 1976). If Arlaud et al. (1987c) and Tosi et al. (1987) are correct in their prediction that C1s–C1s and C1s–C1r interactions are mediated by the EGF-like structures in those molecules, and if our observation of a Trp fluorescence change in $C1\bar{s}$-A during the LTT reflects an irreversible change in the tertiary structure of region I, then it would appear that regions I and II in $C1\bar{s}$ are strongly associated in the presence of $Ca^{2+}$. Removal of $Ca^{2+}$ might disrupt that association and allow region I to unfold. Efforts are in progress to isolate smaller fragments to test this possibility and further localize the protein–protein interaction sites of C1s.

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**Registry No.** $C1\bar{s}$, 80295-70-1.

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# Three-Dimensional Structure of Acyl Carrier Protein Determined by NMR Pseudoenergy and Distance Geometry Calculations†

T. A. Holak,*,‡ S. K. Kearsley, Y. Kim, and J. H. Prestegard*
*Department of Chemistry, Yale University, New Haven, Connecticut 06511*
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ABSTRACT: Distance constraints from two-dimensional NMR cross-relaxation data are used to derive a three-dimensional structure for acyl carrier protein from *Escherichia coli*. Several approaches to structure determination are explored. The most successful proves to be an approach that combines the early stages of a distance geometry program with energy minimization in the presence of NMR constraints represented as pseudopotentials. Approximately 450 proton to proton distance constraints including 50 long-range constraints were included in these programs. Starting structures were generated at random by the distance geometry program and energies minimized by a molecular mechanics module to give final structures. Seven of the structures were deemed acceptable on the basis of agreement with experimentally determined distances. Root-mean-square deviations from the mean of these structures for backbone atoms range from 2 to 3 Å. All structures show three roughly parallel helices with hydrophobic residues facing inward and hydrophilic residues facing outward. A hydrophobic cleft is recognizable and is identified as a likely site for acyl chain binding.

Acyl carrier protein (ACP)[1] is a small protein of interest because of its role in fatty acid synthesis. In *Escherichia coli* the protein is a cytosolic component of 8847 daltons that carries fatty acid chains via a thioester linkage to a phosphopantetheine prosthetic group as the chains are elongated by the fatty acid synthetase system (Ohlrogge, 1987; Thompson, 1980). Carriers are normally viewed as rather passive participants in biochemical events. However, in the case of ACP there is evidence to suggest that the presence of the protein can influence activities of enzymes associated with fatty acid synthesis. Consider, for example, thioesterases that cleave the fatty acid from the prosthetic group near the end of the elongation process. The phosphopantetheine prosthetic group is common to both ACP and coenzyme A, making acyl-CoAs homologous with acyl-ACPs for regions well displaced in both directions from the site of chain attachment. Yet, there appear to be significant differences in abilities of

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‡ Present address: Max-Planck-Institut für Biochemie, 8033 Martinsried, München, West Germany.

[1] Abbreviations: ACP, acyl carrier protein; 2D-NMR, two-dimensional nuclear magnetic resonance; NOESY, nuclear Overhauser effect spectroscopy; AMBER, assisted model building with energy refinement; DISGEO, distance geometry; CoA, coenzyme A; RMS, root mean square.

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