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Calcium-Sensitive Thermal Transitions and Domain Structure of Human Complement Subcomponent C1r̄†

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

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

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ABSTRACT: Fluorescent probes and other methods have been used to investigate the thermal stability of activated C1r and functionally intact fragments isolated from tryptic digests of the protein. This enzyme exhibits two irreversible transitions that differ with respect to their sensitivity to metal ions. The high-temperature transition occurs with a midpoint near 53 °C in 0.02 M tris(hydroxymethyl)aminomethane buffer and 0.15 M NaCl, pH 7.4. It is relatively insensitive to Ca^{2+} and ionic strength and is accompanied by a loss of catalytic activity. The low-temperature transition is most easily observed in the presence of ethylenediaminetetraacetic acid and is completely abolished by 100 μM Ca^{2+}. Its midpoint varies between 26 °C at low ionic strength and 40 °C in the presence of 0.5 M NaCl. The low-temperature transition results in extensive polymerization of the protein without loss of the esterolytic activity or the ability to react with C1 inhibitor; however, the ability to reconstitute hemolytically active C1̄ or even bind to C1s̄ in the presence of Ca^{2+} is destroyed. A highly purified N-terminal fragment generated by tryptic digestion of C1r̄ in the presence of Ca^{2+} retained its ability to interact with C1s̄, disrupting the formation of C1s̄ dimers in the presence of Ca^{2+}. In the absence of Ca^{2+}, this fragment displays only a low-temperature transition that is very similar to the one observed with the whole protein and that destroys its ability to bind to C1s̄. Addition of Ca^{2+} stabilizes this fragment, shifting the midpoint of its melting transition upward by more than 20 °C. Assignment of the high-temperature transition in C1r̄ to the catalytic domain was confirmed by observation of a similar transition in a catalytically active C-terminal fragment obtained from the same tryptic digest. The occurrence of independent thermal transitions in human C1r̄ and their assignment to specific regions of the molecule, together with the direct evidence that the N-terminal Ca^{2+}-sensitive domain is the one that interacts with C1s̄, provide strong support for current models of the domain structure of these proteins.

The first component of complement C1[1] is a large multi-protein complex comprised of three subcomponents designated C1q, C1r, and C1s [reviewed by Cooper (1985)]. Each of these contains multiple domains that can be visualized in the electron microscope and that appear to be responsible for various functions of the complex. Some domains are involved in the Ca^{2+}-dependent associations that hold the subcomponents together while others are responsible for the interaction of C1 with immune complexes or cellular receptors. Still other domains possess the catalytic sites responsible for the proteolytic action of C1̄ and for its interaction with C1 inhibitor.

The occurrence of multiple independent domains within a protein is often associated with the appearance of more than one thermal transition as the temperature of the protein is

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*Author to whom correspondence should be addressed at the Holland Laboratory: Biochemistry, American Red Cross, Rockville, MD 20855.

[1] Abbreviations: ANS, 8-anilino-1-naphthalenesulfonate; Z-Gly-Arg-sBzl, N-(benzyloxycarbonyl)-L-glycylarginine thiobenzyl ester; IgG, immunoglobulin G; EDTA, ethylenediaminetetraacetic acid; 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); C1-Inh, C1 inhibitor; C1, first component of complement; C1r and C1s, zymogen forms of C1 subcomponents; C1r̄ and C1s̄, activated forms of C1r and C1s, differing from the zymogens by the presence of single clip in the polypeptide chain; kDa, kilodalton(s).

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steadily increased while the change in some property is monitored (Privalov, 1979, 1982; Castellino et al., 1981; Davio & Low, 1982; Lennick et al., 1985). By examining the dependence of these transitions on solution conditions, one can learn more about the domain structure of the protein. In some cases it is possible to selectively manipulate certain transitions by addition of metal ions, ligands, or other macromolecules that bind to and stabilize a particular domain. Another more direct approach is to isolate individual domains and compare their behavior with that of the whole molecule. In this way it is often possible to identify which domains are responsible for a given transition and correlate the effects of heat with changes in specific functions to provide a better understanding of the structure/function relationships of the protein.

A previous report from this laboratory documented the occurrence of at least two independently unfolding domains in C1s̄ (Lennick et al., 1985). This study is concerned with the stability and domain structure of the homologous protein C1r̄. Preliminary experiments using the fluorescent probe 8-anilino-1-naphthalenesulfonate (ANS) revealed the presence of a thermal transition occurring near physiological temperature in the absence of Ca^{2+} (Busby et al., 1986). Several laboratories called early attention to the lability of this protein under various conditions, such as exposure to 37 °C in the presence of EDTA (de Bracco & Stroud, 1971; Ziccardi & Cooper, 1976a; Assimeh et al., 1978; Okamura & Fujii, 1978; Arlaud et al., 1980). Furthermore, temperature-dependent conformational transitions have been implicated in the mechanism of activation of C1 (Tschopp, 1982). These observations prompted us to further examine the heat stability and thermal transitions of C1r̄. It is shown that the enzyme exhibits two irreversible transitions which differ with respect to their dependence on Ca^{2+} concentration and ionic strength. The low-temperature transition, which occurs with a midpoint near 32 °C at physiological ionic strength in the absence of Ca^{2+}, results in polymerization of the protein without loss of esterolytic activity or the ability to react with C1 inhibitor. A second transition occurs above 50 °C and results in the destruction of the esterolytic activity. By examining the stability of functionally active fragments isolated from a tryptic digest of C1r̄, we have been able to assign the low-temperature transition to the N-terminal interaction domain and the high-temperature transition to the C-terminal catalytic domain. The results are discussed in terms of current models of the domain structure of C1r and C1s (Tschopp et al., 1980; Villiers et al., 1985; Arlaud et al., 1986; Weiss et al., 1986).

Materials and Methods

All experiments were performed in 0.02 M Tris-HCl, pH 7.4, and 0.15 M NaCl buffer (TBS) unless otherwise stated. Metal-free TBS was prepared by passing TBS through a column (2.5 × 25 cm) of Chelex 100 (Bio-Rad Laboratories) and adjusting the pH back to 7.4. The magnesium salt of 8-anilino-1-naphthalenesulfonate (ANS) was purchased from Molecular Probes (Eugene, OR). Bovine pancreatic trypsin (type III-S) was purchased from Sigma. Acrylamide agarose (ACA-44) was from LKB.

Activated C1r (C1r̄) and C1s (C1s̄) were prepared from Cohn fraction I of human plasma by affinity chromatography on IgG–Sepharose. The procedure used is similar to that of Bing et al. (1980), except that the IgG–Sepharose was prepared according to Kolb et al. (1979). Both proteins gave a single band on nonreduced and two bands on reduced SDS–PAGE with no zymogen detected. The zymogen form of C1r was purified by the method of Ziccardi and Copper (1976a) and gave a single band on nonreduced SDS–PAGE, with only a trace of the separate A and B chains of activated C1r̄ visible upon reduction. Concentrations of C1s were determined spectrophotometrically with A(280 nm, 1%) = 10.5 and M_r 87 000 (Cooper, 1985). Values for C1r were A(280 nm, 1%) = 11.6, calculated by the method of Edelhoch (1967) from the numbers of Tyr, Trp, and disulfides (Leytus et al., 1986; Arlaud et al., 1987) and M_r 86 000 [calculated from the amino acid sequence plus 9.4% carbohydrate (Sim et al., 1977)]. C1s̄–Sepharose was prepared by coupling the protein to CNBr-activated Sepharose following the procedure of Kolb et al. (1979).

Fragments were generated by incubation of C1r̄ (2 mg/mL) with 0.2 μM trypsin at 30 °C in TBS and 1 mM $CaCl_2$ for 20 h. The fragments were purified by size-exclusion chromatography on a Superose 12 column (Pharmacia) and, in the case of the α fragment, by affinity chromatography on a C1s̄–Sepharose column. The fluorescence spectrum of the purified α fragment showed a maximum near 305 nm, characteristic of tyrosine, with only a slight shoulder in the 330–350-nm region where tryptophan fluoresces. Since tryptophan absorbs 4–5-fold more strongly and emits with a much higher efficiency, this result signifies negligible contamination by Trp-containing fragments. Concentrations of the α and γ-B fragments were determined from the absorbance at 280 nm with molar extinctions coefficients of 20 100 and 76 840 M^{-1} cm^{-1}, respectively, calculated as above for whole C1r.

C1-Inh was purified as previously described (Lennick et al., 1985). Its concentration was based on an extinction coefficient of A(280, 1%) = 4.5 and M_r 104 000 (Haupt et al., 1970). The purified inhibitor produced a major band at 105 kDa and a minor band at 95 kDa on reduced SDS–polyacrylamide slab gels (Lennick et al., 1985).

C1r̄ was assayed by determining the rate of hydrolysis of the synthetic thio ester substrate Z-Gly-Arg-sBzl (Enzyme Systems Products) in the presence of the chromogenic thiol reagent dithiodipyridine (Sigma) as described by McRae et al. (1981). The assay buffer was 0.1 M Tris, pH 7.4, 0.1 M NaCl, 1 mM $CaCl_2$, and 9.2% dimethyl sulfoxide, and the assays were performed in a Cary 118 double-beam recording spectrophotometer with a cell holder thermostated at 30 °C. The rate of inhibition of C1r̄ by C1-Inh was determined spectrophotometrically by a procedure entirely analogous to that previously published for C1s̄ (Lennick et al., 1986). The reaction was initiated by adding C1r̄ at a final concentration of 10 nM to a solution containing Z-Gly-Arg-sBzl (0.1 mM), thiol reagent (0.3 mM), and C1-Inh (4.6 μM). The rate of inhibition was determined from the rate of decrease in the slope of the change in absorbance vs. time, corrected for a small amount of substrate depletion that occurred during the course of this reaction.

Fluorescence measurements were made on a Perkin-Elmer MPF-4 fluorescence spectrophotometer with the cell block temperature controlled by a Neslab Model RTE-5 circulating water bath connected to a DCR-4 digital controller/readout linked to a ETP-3 temperature programmer. Melting curves were determined by heating at a rate of 1 °C/min while monitoring the ANS fluorescence at excitation/emission wavelengths of 370/460 nm. The pH of the sample buffer varied from 7.5 at 4 °C to 6.9 at 60 °C. Light scattering experiments were performed on the same instrument with both the excitation and emission monochromators set at 350 nm.

Exclusion chromatography was performed with a Pharmacia fast-protein liquid chromatographic system with a Superose 12 column at a flow rate of 0.5 mL/min. The elution position of any given protein varied by as much as 0.5 mL during the

course of this work due to gradual decreases in the bed volume with continued use. Conclusions based on small differences in elution position of two samples were based only on comparisons made between samples injected the same day, under identical elution conditions. Elution of proteins was monitored by absorbance at 280 nm or by intrinsic fluorescence with a Shimadzu Model RF-530 fluorescence spectromonitor with excitation/emission wavelengths of 270/310 nm (tyrosine) or 295/350 nm (tryptophan).

For the hemolytic assays, the antibody-sensitized sheep erythrocytes (EA) and EAC4 were prepared according to the procedure of Rapp and Borsos (1970). The sheep erythrocytes and antibody to sheep erythrocytes were obtained from Accurate Chemical and Scientific (Westbury, NY). Human C4 and guinea pig complement were purchased from Cordis (Miami, FL). C$\bar{1}$ was reconstituted with purified C1q (Bing et al., 1980), C1$\bar{s}$, and C1$\bar{r}$ and tested in various dilutions for its ability to hemolyze EAC4 cells.

Results

Thermal Transitions Detected by ANS Fluorescence. ANS is a hydrophobic fluorescent probe that has proven extremely useful for detecting thermal denaturation and other conformational changes in plasma proteins (Gally & Edelman, 1965; Busby et al., 1981; Isenman, 1983; Lennick et al., 1985). It has the convenient property of being almost nonfluorescent in aqueous solution while fluorescing strongly when bound to hydrophobic sites on certain proteins. Most plasma proteins have little affinity for ANS in their native conformation but acquire a strong affinity upon thermal denaturation, presumably because of the exposure of previously buried hydrophobic residues. Thus, the presence of a small amount of the dye in solution with a protein whose temperature is steadily raised in a fluorometer provides a sensitive method for detecting such transitions.

Figure 1A shows the effect of ionic strength and Ca^{2+} on the melting curve for C1$\bar{r}$ as determined by ANS fluorescence. Two transitions are apparent in the absence of Ca^{2+}. The first occurs with a midpoint near 26 °C in the absence of NaCl, 32 °C in physiological saline, and 40 °C in 0.5 M NaCl (latter not shown). It is completely inhibited by 0.1 mM $CaCl_2$. The second transition has a midpoint near 53 °C, is relatively insensitive to ionic strength and Ca^{2+} ions, and usually has about twice the amplitude of the low-temperature transition. Note that the high-temperature transition occurs in the same position whether or not the low-temperature transition has occurred, reflecting the independence of the two transitions and suggesting that they occur on different regions of the C1$\bar{r}$ molecule. $MgCl_2$ at a concentration of 1 mM did not prevent the low-temperature transition but did stabilize it, shifting the midpoint to about 39 °C in TBS (not shown). The amount of magnesium (33 μM) present as a result of using the Mg^{2+} salt of ANS was not enough to affect the midpoints.

Figure 1B illustrates the irreversible nature of both thermal transitions with respect to their effects of ANS fluorescence. Heating C1$\bar{r}$ to 37 °C in the presence of EDTA (arrow a), followed by cooling (arrow b), returns the fluorescence to a much higher value than the original. A second heating of the same sample (arrow c) produces no transition in the 37 °C region, but the main transition above 50 °C appears as usual, although with a slightly higher midpoint and slightly lower amplitude in this experiment than in most experiments. Cooling from this point (arrow d) produces a fluorescence intensity much higher than any previous value. These elevated values of ANS fluorescence cannot be reversed by addition of Ca^{2+}.

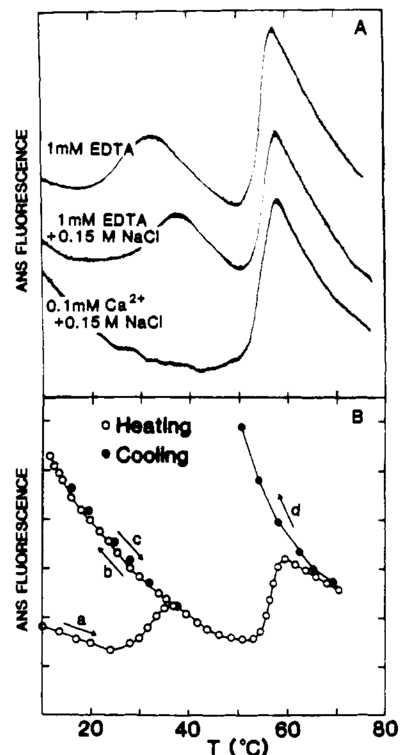

FIGURE 1: (A) Effects of Ca^{2+} and ionic strength on thermal transitions of C1$\bar{r}$ as detected by ANS fluorescence. Samples of C1$\bar{r}$ (0.38 mg/mL) in 0.02 M Tris-HCl, pH 7.4, were heated under the indicated conditions at 1 °C/min in the presence of 66 μM ANS. The fluorescence was monitored at 460 nm with excitation at 370 nm. The curves have been arbitrarily shifted along the vertical axis to facilitate comparison. (B) Irreversibility of the thermal transitions of C1$\bar{r}$. A solution of C1$\bar{r}$ (0.38 mg/mL) in 0.02 M Tris-HCl and 5 mM EDTA, pH 7.5, was heated at 1 °C/min in the presence of 66 μM ANS from 10 to 37 °C (a), then cooled to 10 °C (b), reheated to 77 °C (c), and again cooled (d). The fluorescence was monitored as in (A) (O, heating; ●, cooling).

C1$\bar{r}$ that was treated with 1 mM EDTA and then passed over a Sephadex G-50 column equilibrated with metal-free TBS gave a melting profile indistinguishable from those obtained in the presence of EDTA. This suggests that the chelating agent need not be present to induce the low-temperature transition. Experiments with [^{14}C]EDTA excluded the possibility that some EDTA could remain associated with the protein during chromatography on Sephadex G-50. We also examined the effect of heating rate. When C1$\bar{r}$ was heated in the presence of EDTA at 0.2 °C/min instead of the usual 1.0 °C/min, the midpoint of the low-temperature transition shifted downward by about 2.5 °C with no significant effect on the high-temperature transition. This is consistent with the fact that EDTA-containing samples which have been kept for several hours at room temperature exhibit either no transition or a transition of diminished amplitude when subsequently heated at 1 °C/min in the presence of ANS. Thus, the transition will occur at lower temperatures if given sufficient time. However, the protein can withstand at least 2 days at 4 °C in the presence of EDTA since those conditions were encountered during its purification. The ability of a given sample to exhibit a low-temperature transition can now be taken as an additional measure of its integrity.

The zymogen form of C1r also gave two ANS-detected transitions having midpoints within 1.5 °C of those obtained for the activated form under identical conditions in spite of their different methods of purification from different sources (see Materials and Methods). In addition, C1r showed the previously reported increase in tryptophan fluorescence when

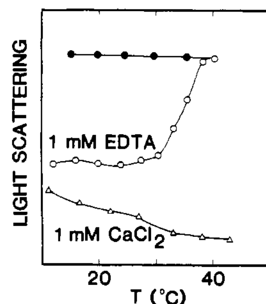

FIGURE 2: The low-temperature transition of C1r̄ as detected by light scattering. C1r̄ (0.38 mg/mL) in TBS, pH 7.4, in the presence of 1 mM EDTA was heated (O) from 10 to 40 °C at 1 °C/min and then cooled (●) at about the same rate while the intensity of light scattered at 350 nm was monitored. The presence of 1 mM $CaCl_2$ instead of EDTA in an identical sample (Δ) prevented the increase in light scattering.

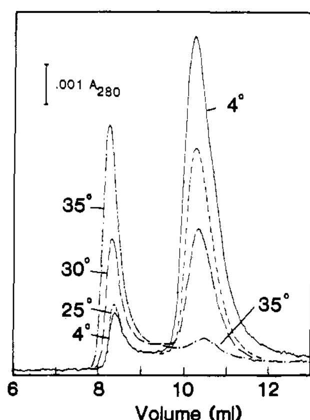

FIGURE 3: Effect of heating on elution of C1r̄ during size-exclusion chromatography. C1r̄ (0.36 mg/mL) in TBS was heated at 1 °C/min. Samples were withdrawn at the indicated temperatures and kept at 4 °C until chromatographed on the Superose 12 column equilibrated in TBS and 1 mM $CaCl_2$ at room temperature. The protein was eluted at 0.5 mL/min and monitored by absorbance at 280 nm at a sensitivity of 0.01 full scale.

exposed to 37 °C in the presence of EDTA whereas C1r̄ did not (Villiers et al., 1983).

Evidence for Aggregation. The low-temperature transition of C1r̄ can also be monitored by light scattering in the fluorometer. As shown in Figure 2, the scattering intensity increased between 30 and 40 °C, with a midpoint near 34 °C, and remained at the elevated level upon cooling to the starting temperature. This further illustrates that the transition is irreversible. The sample containing Ca^{2+} showed no evidence of a transition. Since neither sample was visibly turbid when examined at the end of the experiment, the increase in light scattering by the EDTA-containing sample suggests that soluble aggregates are formed as a result of the low-temperature transition.

Further evidence for this conclusion is provided in Figure 3. In this experiment, samples of C1r̄ were successively removed at various points along the heating curve in the region of the low-temperature transition and subjected to exclusion chromatography. Prior to heating, C1r̄ eluted in a symmetrical peak centered near 10.2 mL with a small amount of material in the void. Above 25 °C the amount of material in the void began to increase with a corresponding decrease in the original peak. By 35 °C the original peak had virtually disappeared; however, the area under the void peak was consistently less than that under the original peak, suggesting that some of the protein had bound to the column. Similar results were obtained on an acrylamide agarose column equilibrated in TBS

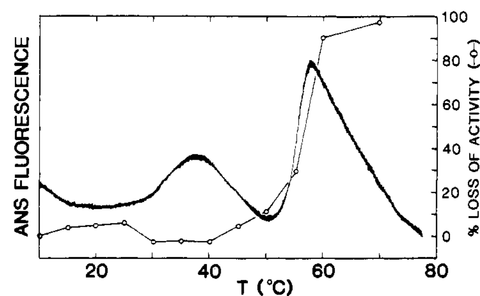

FIGURE 4: Effects of heating in the presence of EDTA on esterolytic activity of C1r̄. The enzyme (1 mg/mL in TBS and 1 mM EDTA) was heated at 1 °C/min from 10 to 77 °C. Aliquots were removed at 5-deg intervals and assayed for ability to hydrolyze Z-Gly-Arg-sBzl as determined with a chromogenic thiol reagent (see Materials and Methods). An identical sample containing 66 μM ANS was heated at the same time while the ANS fluorescence was monitored.

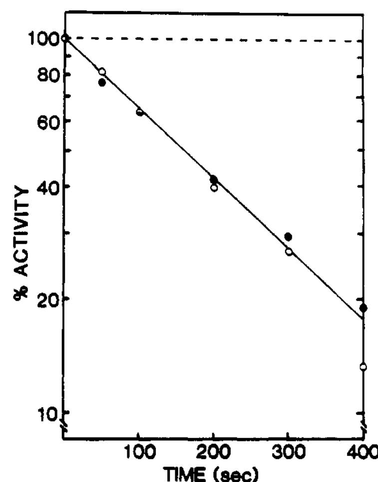

FIGURE 5: Effect of the low-temperature transition of C1r̄ on the rate of its reaction with C1 inhibitor. Half of a sample of C1r̄ (1 mg/mL in TBS and 1 mM EDTA) was incubated at 37 °C for 30 min (●), while the second half was kept at 4 °C (O). At $t = 0$, C1r̄ at a final concentration of 10 nM was added to the assay solution containing C1-Inh (4.6 μM in TBS and 1 mM $CaCl_2$), and the loss of esterolytic activity was continuously monitored with Z-Gly-Arg-sBzl coupled to a chromogenic thiol reagent (see Materials and Methods). The dashed line shows the effect of omitting the inhibitor.

and 1 mM EDTA at 4 °C, except that, in this case, the heated protein was completely recovered in the void.

Effects on Activity. Figure 4 shows the effect of heating C1r̄ on its ability to catalyze the hydrolysis of a synthetic substrate, Z-Gly-Arg-sBzl. Heating through the low-temperature transition had no significant effect on this property. Continued heating caused a definite loss of esterolytic activity, coincident with the high-temperature transition. Thus, the latter transition probably involves structural damage to the catalytic domain. This result indicates that the aggregation which accompanies the low-temperature transition occurs in such a way as to preserve the integrity of the active site. Even stronger evidence for this conclusion comes from the observation that the low-temperature transition had no effect on the rate at which C1-Inh reacted with the enzyme to abolish its activity (Figure 5). Analysis by SDS-PAGE confirmed that C1r̄ which had undergone the low-temperature transition was indistinguishable from native C1r̄ in terms of its ability to combine with the inhibitor to form a stable complex (not shown). Thus, the accessibility of the active site of C1r̄, even toward a macromolecular "substrate" such as C1-Inh, is not affected by the aggregation that accompanies the low-temperature transition.

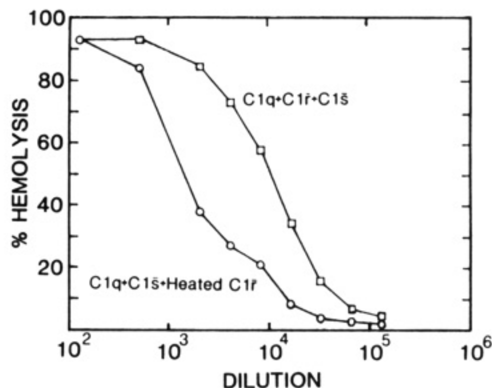

FIGURE 6: Effect of the low-temperature transition of C1r̄ on its ability to form hemolytically active C1̄. Half of a sample of C1r̄ (1 mg/mL in TBS and 1 mM EDTA) was incubated at 37 °C for 30 min (○). The second half of the sample was kept at 4 °C (□). Various dilutions of each sample were mixed with excess C1s̄ and C1q and assayed for hemolytic activity.

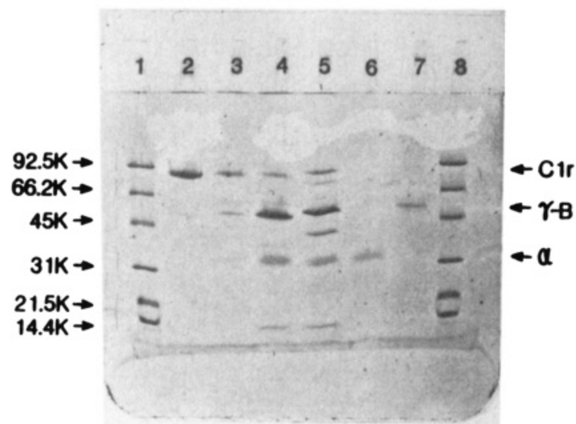

FIGURE 7: SDS–PAGE of various nonreduced samples of C1r̄ and its fragments. Lanes 1 and 8 contain standard proteins of designated M_r. Other lanes contain (2) C1r̄, (3) C1r̄ after 30 min at 37 °C in EDTA, (4) C1r̄ after 5 h at 37 °C in EDTA, (5) C1r̄ after 20 h at 30 °C in the presence of Ca^{2+} and trypsin, (6) purified α domain, and (7) purified γ-B domain.

We also investigated the effects of heating on the ability of C1r̄ to recombine with excess C1s̄ and C1q to reconstitute hemolytic activity. As shown in Figure 6, under conditions where C1r̄ is limiting, the amount of C1r̄ required for 50% lysis of antibody-coated erythrocytes increased by an order of magnitude after exposure to 37 °C in the presence of EDTA. This was consistent with exclusion chromatography experiments showing that heated C1r̄ was no longer able to form a Ca^{2+}-dependent complex with C1s (data not shown).

Preparation of C1r̄ Fragments. C1r̄ is known to undergo slow autolytic cleavage at 37 °C in the absence of Ca^{2+} (Assimeh et al., 1978; Okamura & Fujii, 1978; Arlaud et al., 1980). After about 5 h the A (heavy) chain is cleaved into three dominant fragments designated α (35 kDa), β (7 kDa), and γ (19.5 kDa) (see Figure 11 below). Careful analysis by Arlaud and colleagues revealed that the α fragment corresponds to the N-terminal Ca^{2+}-binding interaction domain (Arlaud et al., 1980, 1986; Villiers et al., 1985). Since the low-temperature transition of C1r̄ is prevented by Ca^{2+} and has no apparent effect on the catalytic properties, it seemed reasonable to hypothesize that this transition was localized on the N-terminal interaction domain. Proof of this assignment would require isolation of that domain and demonstration that it exhibits a low-temperature transition similar to that seen with whole C1r̄. Given the conditions required for autolysis, any α fragments generated in this manner would be expected to have already undergone the low-temperature transition. This could explain the observation by Arlaud et al. (1986) that the only α fragments recovered by exclusion chromatography of an autolytic digest of C1r̄ were as high molecular weight aggregates (M_r ~300 000) eluting close to the void.

In order to prevent the transition from occurring during the generation and isolation of fragments, we sought a method that could be conducted in the presence of Ca^{2+}. Autolysis of C1r̄ does not occur in the presence of Ca^{2+}. However, it was found that digestion with trypsin for 20 h at 30 °C in the presence of 1 mM Ca^{2+} produced fragmentation patterns on SDS–PAGE (Figure 7, lane 5) that closely resembled those which occur during autolysis (Figure 7, lane 4). Although additional minor bands are apparent in the tryptic digest, each of the four major bands in the autolytic digest is accompanied by a corresponding band in the tryptic digest. Application of such a digest to the size-exclusion column in a Ca^{2+}-containing buffer produced the elution profiles shown in Figure 8, monitored by either Trp or Tyr fluorescence. Comparison of the two profiles allowed peak D to be tentatively identified as the α fragment, which, according to the reported amino acid sequence (Leytus et al., 1986; Arlaud et al., 1987), contains no tryptophan. This assignment was confirmed by SDS–PAGE, where peak D was found to migrate to the same position as the 35-kDa fragment produced by autolysis (Figure 7, lane 6) and by functional studies presented below. Similarly, peak C appeared to contain primarily the γ-B fragment (Figure 7, lane 7), which should exist as a stable dimer (C1r̄ II) under the elution conditions of Figure 8 (Okamura & Fujii, 1978; Assimeh et al., 1978; Arlaud et al., 1980). This material was as active as C1r̄ in terms of its ability to cleave Z-Gly-Arg-sBzl, whereas peak D had no detectable activity. Peak A represents undigested C1r̄ (dimer) while peak B contains a mixture of partially digested species intermediate in size between intact C1r̄ dimer and γ-B dimer. The connecting β fragment, also devoid of Trp (Arlaud et al., 1987; Leytus et al., 1986), has a molecular weight of only 7000 and could be located in peak F, which has a high ratio of Tyr to Trp fluorescence. Peak E was not identified.

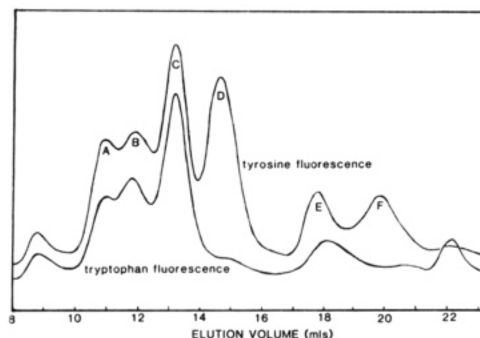

FIGURE 8: Size-exclusion chromatography of trypsin-digested C1r̄. The protein (0.32 mg/mL) was digested with trypsin (0.2 μM) for 20 h at 30 °C in TBS containing 1 mM $CaCl_2$, and 0.25-mL portions were applied to the Superose 12 column equilibrated in the same solvent. The eluate was continuously monitored by fluorescence with excitation/emission wavelengths of 270/310 (tyrosine) or 295/350 (tryptophan) at a flow rate of 0.5 mL/min while 0.5-mL fractions were collected. The various peaks were identified by SDS–PAGE and other methods (see text). The α fragment, which contains no tryptophan, is located in peak D.

Interaction of the α Fragment with C1s̄. According to current models, the N-terminal (α) domain of C1r is the one that interacts with the corresponding (homologous) domain of C1s to form the $C1r_2C1s_2$ tetramer in the presence of Ca^{2+} (see Figure 11). If C1r is absent, C1s self-associates through its own N-terminal domain to form a dimer. If this picture

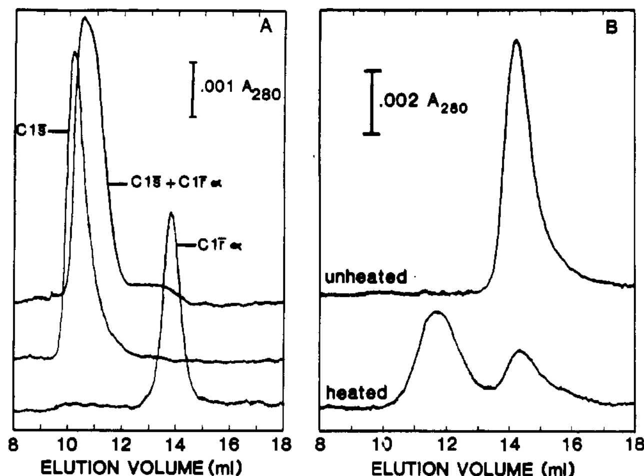

FIGURE 9: (A) Disruption of C1s̄ dimers by isolated α fragment of C1r̄ as determined by size-exclusion chromatography. C1s̄, C1r̄α, and a mixture (2:1 molar ratio) of the two were loaded onto the Superose 12 column equilibrated in TBS and 1 mM $CaCl_2$. Elution was monitored by the absorbance at 280 nm. The concentrations and volumes of the injected samples were C1s̄ (50 μL, 0.19 mg/mL) and C1r̄α (100 μL, 0.019 mg/mL). (B) Size-exclusion chromatography of purified C1r̄α showing self-association after heating at 37 °C in the presence of EDTA. Samples of the purified fragment (0.05 mg/mL) unheated or heated for 30 min in TBS containing 1 mM EDTA were injected into the Superose 12 column equilibrated in TBS and 1 mM $CaCl_2$ at room temperature, and their elution was monitored by absorbance at 280 nm.

is correct, the isolated α fragment of C1r̄ might be expected to interfere with Ca^{2+}-induced dimerization of C1s̄. Figure 9A presents the results of an experiment designed to test this possibility by size-exclusion chromatography. Material from peak D in Figure 8 that was further purified on C1s̄–agarose eluted as a single peak at 13.8 mL when rechromatographed in the presence (or absence) of Ca^{2+}. Purified C1s̄ also eluted as a single peak at 10.2 mL, corresponding to the dimer. When C1r̄α was mixed with a 2-fold excess of C1s̄ and the two were applied together, most of the α peak disappeared, indicating that it had formed a complex with C1s̄. The C1s̄ peak became broader and eluted slightly later at 10.6 mL, suggesting that the C1s̄ dimers were at least partially disrupted through interaction with the α fragment of C1r̄. The resulting complexes would have a molecular weight of approximately 110 000, intermediate between C1s̄ dimer (170 000) and C1s̄ monomer (85 000). When this experiment was repeated with a buffer containing EDTA, the elution of the α fragment was not affected and the elution position of the C1s̄ in the mixture did not vary from that of C1s̄ alone (data not shown). Also, the C1r̄ α fragment bound to a column of C1s̄–Sepharose in the presence of Ca^{2+} and was readily eluted with EDTA (data not shown). This property of the fragment was lost after 30 min at 37 °C in EDTA. These observations confirm the identity and functional integrity of the α domain as isolated.

Thermal Transitions of the Fragments. Figure 10 compares the melting behavior of the purified fragments to that of the parent molecule. The α fragment in the presence of EDTA and ANS displayed only a low-temperature transition almost identical with that seen with whole C1r̄. In the presence of 1 mM $CaCl_2$, this transition shifted more than 20 °C to a position close to that of the high-temperature transition in whole C1r̄. The purified γ-B domain showed only a high-temperature transition, regardless of Ca^{2+}, and this occurred at about the same temperature as the corresponding transition in whole C1r̄. Thus, the melting behavior of C1r̄ as monitored by ANS fluorescence is entirely accounted for by the independent behavior of these two domains.

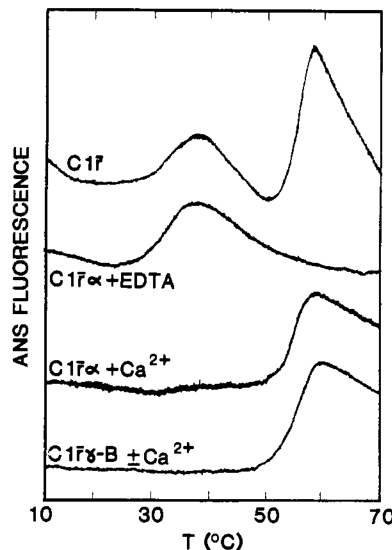

FIGURE 10: Comparison of ANS-detected melting transitions of C1r̄α and C1r̄γ-B fragments in the presence and absence of EDTA with transitions of whole C1r̄ in the presence of EDTA. Each of the proteins (0.2–0.4 mg/mL in TBS) was heated from 4 to 65 °C at 1 °C/min in the presence of 66 μM ANS and either 1 mM EDTA or 1 mM $CaCl_2$. The fluorescence was monitored as in Figure 1. The curves have been arbitrarily displaced along the vertical axis to facilitate comparison.

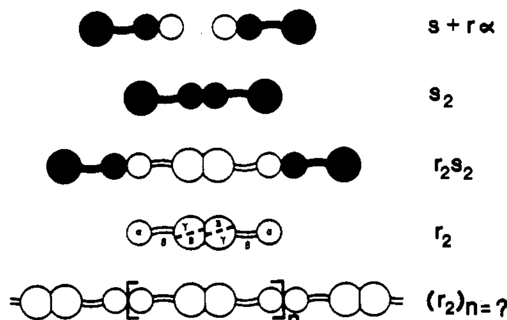

FIGURE 11: Schematic illustrations of the domain structure of $C1r_2$, $C1s_2$, and the $C1r_2C1s_2$ ($C1r_2s_2$) complex taken from Villiers et al. (1985) and Arlaud et al. (1986). Catalytic domains are represented by large circles and interaction domains by small circles. The fragments generated by autolysis or digestion with trypsin are designated α, β, and γ. B represents the light chain of C1r̄ which, together with γ, comprises the catalytic domain. The top line illustrates disruption of C1s dimers by the interaction domain of C1r. The bottom line illustrates one possible mode of polymerization of C1r that would preserve access to the catalytic domain.

We next examined the effect of heating the α fragment on its elution from the size-exclusion column. As shown in Figure 9B, exposure to 37 °C in the presence of EDTA caused most of the material to elute well ahead of the unheated control. Thus the isolated fragment, like whole C1r̄, undergoes irreversible self-association under these conditions. However, its degree of association appears to be limited to relatively small oligomers, no larger than a tetramer.

Discussion

A schematic representation of the multidomain structures of C1r̄ and C1s̄ taken from Villiers et al. (1985) is presented in Figure 11. This picture is based on a variety of electron microscopic, proteolytic fragmentation, chemical modification, and other data from several laboratories (Arlaud et al., 1986; Weiss et al., 1986; Schumaker et al., 1986, and references cited therein). In both of these homologous proteins, the large circles represent the catalytic domains comprised of the B chain and

a portion of the A chain referred to as γ. The small circles represent the so-called interaction domains containing the N-terminal portion of the A-chain designated α. Connecting these two major domains is a short segment of the A chain termed β. C1r̄ self-associates through its catalytic domains to form a stable dimer in both the presence and absence of Ca^{2+}. C1s̄ also forms dimers but only in the presence of Ca^{2+} and not through its catalytic domain but through its N-terminal interaction domain. If both C1r̄ and Ca^{2+} are present, the interaction domains of C1s̄ associate not with themselves but with the corresponding domains of C1r̄ to form a tetramer as shown. The central position of the C1r̄ dimer within the tetramer was recently confirmed in the electron microscope by observing the terminal location of avidin-conjugated ferritin bound to tetramers prepared with biotin-labeled C1s (Weiss et al., 1986).

The results presented here provide additional evidence for the structural and functional independence of the two major domains of C1r̄. In the absence of Ca^{2+}, each domain unfolds independently producing two well-resolved thermal transitions as detected by the fluorescence probe, ANS. The low-temperature transition arises from the N-terminal interaction domain. It is observed only in the absence of Ca^{2+} ions with a midpoint that lies between 32 and 34 °C at physiological ionic strength but varies as much as 15 °C between 0.0 and 0.5 M NaCl. It can also be detected by light scattering and by the appearance of aggregates in the size-exclusion column. The high-temperature transition has a midpoint near 53 °C and is relatively insensitive to ionic strength and Ca^{2+}. This transition is associated with a complete loss of esterolytic activity and is assigned to the catalytic (γ-B) domain.

The assignment of the low-temperature transition to the N-terminal interaction domain was first made on the basis of its lack of effect on catalytic activity and its stabilization by Ca^{2+}. The exact location of the Ca^{2+} binding site(s) on C1r̄ has (have) not been determined. Since the interaction with C1s̄ requires Ca^{2+}, it is reasonable to assume the presence of Ca^{2+} binding sites on the interaction domain. Furthermore, Villiers et al. (1980) report that C1r̄-II, a γ-B dimer isolated from autolytic digests, failed to bind Ca^{2+}, further implicating the N-terminal region of the molecule. However, the best evidence for our assignment came from the successful isolation of the N-terminal α fragment and the observation that it exhibited a low-temperature transition identical with the one in whole C1r̄. This fragment was strongly stabilized by Ca^{2+}, proving that the metal ion binds to the isolated domain and further strengthening the concept that this domain is responsible for Ca^{2+} binding in whole C1r̄. In the presence of Ca^{2+}, the transition of the isolated α domain occurred at a temperature very close to that of the catalytic domain. This would explain the failure to resolve two transitions in whole C1r̄ in the presence of Ca^{2+}, even though both probably occur. Although variations in the amplitude of the ANS fluorescence-detected transitions preclude a quantitative analysis, the amplitude of the high-temperature transition in Ca^{2+} was consistently greater than in EDTA, suggesting that both domains contribute in the former case.

The isolated α fragment of C1r̄ bound reversibly in a Ca^{2+}-dependent manner to C1s̄–Sepharose and could be eluted with EDTA. When mixed with C1s̄, it appeared to interfere with the ability of the latter to form dimers as monitored by exclusion chromatography. These observations provide strong support for the mode of heteroassociation depicted in Figure 11. The Ca^{2+}-mediated heteroassociation between α domains is apparently much stronger than the self-association of C1s̄. The α domain of C1r̄ did not self-associate in Ca^{2+}; its elution position on the size-exclusion column was unaffected by the metal ion.

It is quite remarkable that, in spite of the extensive aggregation that accompanies the low-temperature transition of C1r̄, there was no significant loss in esterolytic activity or in the ability to react with C1-Inh. The data indicate that aggregation occurs in a relatively specific way such as to preserve access to the catalytic site. In fact, aggregation is probably the wrong word to describe this phenomenon; self-association or polymerization would be more appropriate. The fact that the isolated α fragment exhibits limited association provides a plausible mechanism for the more extensive process in whole C1r̄ which is dimeric and therefore bifunctional. The lower portion of Figure 11 depicts one mode of self-association that would preserve access to the catalytic domain. The actual extent of polymerization is not known; two or three dimers linked together would be sufficient to shift the elution to the void of the exclusion column. It is also possible that cyclic structures could be formed, analogous to those seen in negatively stained specimens of whole C1 by electron microscopy (Tschopp et al., 1980).

de Bracco and Stroud (1971) were the first to document the instability of C1r in the absence of metal ions, especially above room temperature. They found that the hemolytic activity of a euglobulin preparation of C1 was lost in 10 mM EDTA at 30 °C but could be recovered by addition of purified C1r provided the latter had not itself been exposed to similar conditions. Interestingly, this loss in ability to reconstitute hemolytic activity, since confirmed by others (Ziccardi & Cooper, 1976a,b; Bauer & Valet, 1982; this work), was compounded at low ionic strength, conditions that we found to lower the midpoint of the low-temperature transition. A diminished ability to reconstitute C1 can now be explained in terms of the irreversible polymerization and loss of C1s-binding ability which are triggered by exposure to slightly elevated temperature in the absence of Ca^{2+}, conditions not normally considered to be denaturing.

Villiers et al. (1982) reported that heat activation of C1r zymogen (37 °C; EDTA) prior to labeling by the lactoperoxidase method (at 4 °C) caused a large decrease in the proportion of ^{125}I distributing in the A chain. In contrast, C1r̄ that was activated during purification by adsorption of C1 on solid-phase immune complexes gave the same distribution as the zymogen. Analogous results were reported earlier by Bauer and Valet (1981). Since heat activation involves conditions expected to produce the low-temperature transition, we would interpret this difference in terms of masking of tyrosine residues, either directly at the interface between the associated N-terminal domains or indirectly via conformational changes. We would predict that most of this difference should occur in the α fragments where the transition occurs and where 42% of the tyrosines are found (Leytus et al., 1986; Arlaud et al., 1987).

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Registry No. C1r̄, 80295-69-8; Ca, 7440-70-2; complement C1 inhibitor, 80295-38-1.

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