Human complement protein C9 is shown to be a metalloprotein that binds 1 mol of Ca\(^{2+}\)/mol of C9 with a dissociation constant of 3 \(\mu\)M as measured by equilibrium dialysis. Incubation with EDTA removes the bound calcium, resulting in a apoprotein with decreased thermal stability. This loss in stability leads to aggregation and, therefore, to loss of hemolytic activity upon heating to a few degrees above the physiological temperature. Heat-induced aggregation of apoC9 can be prevented by salts that stabilize proteins according to the Hofmeister series of lyotropic ions, suggesting that the ion in native C9 may ligand with more than one structural element or domain of the protein. Ligand blotting indicates that the calcium binding site is located in the amino-terminal half of the protein. Removal of calcium by inclusion of EDTA in assay mixtures has no effect on the hemolytic activity of C9, and its capacity to bind to C8 in solution, or to small unilamellar lipid vesicles at temperatures at or below the physiological range. Although we do not know yet the precise structural and functional role of the bound calcium, it is clear that it provides thermal stability to C9 and it may have a function in regulation of membrane insertion.

The necessity for divalent metal ions in complement activation and expression of function has been known for a long time (1). Lepow and colleagues (2) have demonstrated that Ca\(^{2+}\) is required for C1\(^{2}\) function, and Mg\(^{2+}\) was shown to be an obligatory constituent of the C3 convertase of the alternative pathway of complement (3). Involvement of such metal ions in the assembly and the functional activity of the C5b-9 complex, also called membrane attack complex, was thought to be negligible since it was known that the complex can be assembled and is functional in the presence of 10 mM EDTA (4). However, other reports indicated that the terminal complement protein C9 could be easily inactivated by heavy metal ions (4–9) and that such ions caused polymerization of C9 into a tubular structure called poly(C9) (8–11). In addition, Kitamura et al. (12) reported that divalent metal ions were required for guinea pig C9-mediated lysis of erythrocytes carrying human C5b-8.

Previous results from this laboratory (11) demonstrated that EDTA induced aggregation and inactivation of human C9 at elevated temperatures. We have now investigated the inhibitory effect of EDTA in more detail and found that human C9 is a calcium binding protein. Parts of this report have been published in preliminary form (13).

**MATERIALS AND METHODS**

**Chemicals**—EDTA, CaCl\(_2\), NaCl, LiCl, NH\(_4\)Cl, Tris-HCl, choline chloride, Na\(_2\)SO\(_4\), and sodium citrate were purchased from Fisher, Mops from Research Organics, Quin2 from Molecular Probes, and sodium dodecyl sulfate (SDS)\(^{2}\) and acrylamide from Serva Fine Biochemicals Inc. \(^{3}\)CaCl\(_2\) (specific activity 27 mCi/mg) and Na\(^{25}\) (specific activity 18.9 Ci/mg) in aqueous solution were purchased from Amersham Corp. Egg lecithin was purchased from Avanti Polar Lipids, Inc., and all other chemicals were from Sigma.

**Solutions**—Calcium-free buffers were prepared from deionized water (WATER-I, Gelman Sciences, Inc.) and then further purified by ion-exchange chromatography on Chelex 100.

**Proteins**—Human \(\alpha\)-thrombin was the gift of Dr. John Fenton (Albany, NY). Human C8 and C9 were purified from Cohn Fraction III (Cutter Laboratories) as described by Esser and Sodetz (14), except that the final gel filtration step was conducted on Sephacryl S-200 equilibrated with 10 mM Mops, 150 mM NaCl, pH 7.2 (Mops-buffered saline). C9 to be analyzed by plasma emission spectroscopy was extensively dialyzed against Ca\(^{2+}\)-free Mops-buffered saline. ApoC9 was prepared by chromatography of C9 on a 7 × 0.9-cm column packed with Triazine(carboxymethyl)ethylendiamine-agarose (Pierce Chemical Co.) and equilibrated in Ca\(^{2+}\)-free Mops-buffered saline.

**Plasma Emission Spectroscopy**—Calcium analysis was performed at 280 nm using extinction coefficients of \(\varepsilon = 13.9 \text{ cm}^{-1} \text{M}^{-1}\) for Ca\(^{2+}\) and \(\varepsilon = 9.6 \text{ cm}^{-1} \text{M}^{-1}\) for Ca\(^{2+}\) in solutions at 0.1% (w/w). The plasma samples were processed for calcium analysis as described by Esser and Sodetz (14), except that the final gel filtration step was conducted on Sephacryl S-200 equilibrated with 10 mM Mops, 150 mM NaCl, pH 7.2 (Mops-buffered saline). C9 to be analyzed by plasma emission spectroscopy was extensively dialyzed against Ca\(^{2+}\)-free Mops-buffered saline. ApoC9 was prepared by chromatography of C9 on a 7 × 0.9-cm column packed with Triazine(carboxymethyl)ethylendiamine-agarose (Pierce Chemical Co.) and equilibrated in Ca\(^{2+}\)-free Mops-buffered saline.

**Hemolytic Activity Assays**—The hemolytic activity of C9 was assayed using EAC1-8 as described (14) except that in the case of apoC9 10 mM EDTA was present in the assay buffer.

**Plasma Emission Spectroscopy**—Calcium analysis was performed at 280 nm using extinction coefficients of \(\varepsilon = 13.9 \text{ cm}^{-1} \text{M}^{-1}\) for Ca\(^{2+}\) and \(\varepsilon = 9.6 \text{ cm}^{-1} \text{M}^{-1}\) for Ca\(^{2+}\) in solutions at 0.1% (w/w). The plasma samples were processed for calcium analysis as described by Esser and Sodetz (14), except that the final gel filtration step was conducted on Sephacryl S-200 equilibrated with 10 mM Mops, 150 mM NaCl, pH 7.2 (Mops-buffered saline). C9 to be analyzed by plasma emission spectroscopy was extensively dialyzed against Ca\(^{2+}\)-free Mops-buffered saline. ApoC9 was prepared by chromatography of C9 on a 7 × 0.9-cm column packed with Triazine(carboxymethyl)ethylendiamine-agarose (Pierce Chemical Co.) and equilibrated in Ca\(^{2+}\)-free Mops-buffered saline.

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we chose an incubation temperature of 48 °C and 2 h of incubation time. Under these conditions, about 5, 10, or 50 μM EDTA, respectively, are required to reduce the hemolytic activity of 0.5, 1, or 5 μM of C9 by 50% (Fig. 2). Surprisingly, however, a further increase in molar excess of EDTA over C9 leads to loss of inhibition, and at a total concentration of 100 mM EDTA the protein was completely protected against heat inactivation. Inhibition of C9 hemolytic activity at low molar excess of EDTA was directly related to the chelating capacity of this agent since Ca<sup>2+</sup>EDTA had no effect, and an EDTA analog, Quin2, caused inactivation at similar concentrations (Fig. 2).

**Effect of Solute Conditions on C9 Inactivation by EDTA** —To investigate whether maintenance of full hemolytic activity of C9 at high concentration of EDTA could be caused by EDTA binding or is a general ionic effect, we tested a series of solutes. Increasing amounts of different ionic solutes were added to an incubation mixture containing 1 μM C9 in Mops-buffered saline and inhibitory concentrations of EDTA (0.1 mM). As shown in Fig. 3, adding additional NaCl caused progressive recovery of the hemolytic activity of C9 and after addition of 0.85 M NaCl (corresponding to a total concentration of 1 M) complete protection of C9 against EDTA inactivation could be achieved (Fig. 3).

Among the sodium salts tested, citrate, phosphate, sulfate,


**Calcium Binding by Human C9**

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**FIG. 3. Effect of solutes on C9 inactivation by EDTA.** C9 (1 μM) was incubated in EDTA buffer (10 mM Mops, 0.15 M NaCl, 0.1 mM EDTA, pH 7.2) for 2 h at 48 °C in the presence of additional varying concentrations of NaCl (•), or the sodium salts of the indicated anions (open symbols), or the chloride salts of the indicated cations (closed symbols) and assayed for hemolytic activity. Lysis of 1 μM C9 incubated under the same conditions but without EDTA was set to 100% for each experimental point.

**FIG. 4. Inactivation of C9 by chaotropic salts.** C9 (1 μM) in Mops-buffered saline was incubated for 2 h at 48 °C in the presence of additional varying concentrations of NaCl (•), or the sodium salts of the indicated anions (open symbols), or the chloride salts of the indicated cations (closed symbols) and assayed for hemolytic activity. Lysis of 1 μM C9 incubated under the same conditions but without EDTA was set to 100% for each experimental point.

and chloride were most protective and in the following order: citrate > sulfate = phosphate > chloride. The concentrations of these salts necessary to preserve full hemolytic activity were 75, 200, and 550 mM, respectively. Incubation of C9 with these salt concentrations without EDTA and without calcium ions also abrogated the basal loss of ≈10% activity at 48 °C after 2 h incubation.

None of the other monovalent chloride salts tested, NH₄Cl, LiCl, choline chloride, or Tris-Cl, had a protective effect against EDTA-induced inactivation (Fig. 3). On the contrary, they inactivated C9 at concentrations higher than 400 mM even in the absence of EDTA. Other salts, such as NaNO₃, NaC1O₄, and NaSCN, were especially potent in destroying C9 hemolytic activity (Fig. 4), and inclusion of 5 mM CaCl₂ required about 1.5-fold higher concentration of the chaotropic salt to achieve the same level of inactivation.

**Calcium Binding Properties of C9**—Because the accumulated evidence indicated strongly that the inactivation of C9 was related to the chelating action of EDTA we tested directly for binding of Ca²⁺ ions to C9. Using the ligand blotting technique of Maruyama et al. (19) we observed strong binding of ⁴⁰Ca²⁺ to C9 fixed on nitrocellulose sheets. The calcium binding site is located in the C9α fragment of thrombin-nicked C9 (C9α) as indicated by uptake of ⁴⁰Ca by C9α but not by C9b (Fig. 5). Probing for calcium binding to C8 we noticed that the C8α portion, but not the C8β chain, also contains a calcium binding site (Fig. 5, lane 1). Reduction and carboxymethylation of C9, C9α, and C9 resulted in no labeling (data not shown). From the densitometer scans of the autoradiograms we verified that the amount of ⁴⁰Ca²⁺ bound to C9 or C9α was directly proportional to the amount of protein applied to the gel. Although these results demonstrated the binding of Ca²⁺ to C9, they did not prove that native C9, as isolated, contains this metal. Therefore, we used plasma emission spectroscopy to analyze the metal content of C9. For these experiments the protein was freed of weakly bound metal ions by extensive dialysis against calcium-free Mops-buffered saline. In two experiments, using 2.19 or 5.17 μM C9, respectively, the calcium content was found to be 0.94 or 0.91 atoms per C9 molecule. The Ca/C9 stoichiometry was also determined in two different ways by measuring incorporation of

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**TABLE I**

| Experiment | Method* | Incubation Time | Incubation Temp. | Calcium (μM) | C9 (μM) | Ca/C9 molar ratio |
|------------|---------|-----------------|-----------------|--------------|---------|------------------|
| 1          | A       | 10 min          | 48 °C           | 2.06         | 2.19    | 0.94             |
| 2          | A       | 4.73            | 5.17            | 5.78         | 5.81    | 1.02             |
| 3          | B       | 10 min          | 48 °C           | 3.00         | 3.09    | 0.94             |
| 4          | B       | 10 min          | 48 °C           | 3.00         | 3.09    | 0.94             |
| 5          | B       | 30 min          | 22 °C           | 3.00         | 3.09    | 0.94             |
| 6          | B       | 30 min          | 22 °C           | 3.00         | 3.09    | 0.94             |
| 7          | B       | 30 min          | 22 °C           | 3.00         | 3.09    | 0.94             |
| 8          | B       | 30 min          | 22 °C           | 3.00         | 3.09    | 0.94             |
| 9          | C       | 960 min         | 22 °C           | 3.50         | 3.95    | 0.88             |

*Method A, plasma emission spectroscopy; method B, ⁴⁰Ca loading of apoC9 and gel permeation/centrifugation; method C, equilibrium dialysis with ⁴⁰Ca²⁺.*
Ca²⁺ Binding by Human C9

FIG. 6. Scatchard analysis of calcium binding to apoC9. Binding of ⁴⁴CaCl₂ (from 5 to 50 μM) to apoC9 (3.5 μM) was measured by equilibrium dialysis as described under "Materials and Methods." Data are plotted according to Scatchard (45), and the straight line was derived by linear regression analysis; r = mol of calcium bound per mol of C9.

4⁴Ca²⁺ into apoC9. ApoC9 was prepared by passage of C9 through a solid-phase EDTA column and the eluting calcium-free protein was incubated with ⁴⁴Ca²⁺. Free and bound ligand was separated by centrifugation through a small column packed with Sephadex G-50 (fine) as described under "Materials and Methods." From several such experiments an average calcium binding stoichiometry of 0.91 ± 0.06 ⁴⁴Ca ions per C9 molecule was calculated. Incorporation of ⁴⁴Ca²⁺ did not require elevated temperatures. The results from the equilibrium dialysis experiments indicated that apoC9 bound 0.89 atom of ⁴⁴Ca/mol of C9 in excellent agreement with the filtration assays (Table I). From the equilibrium dialysis measurements a dissociation constant (Kₐ) of 3.1 μM was calculated (Fig. 6).

Structural and Functional Properties of ApoC9—To investigate further the role of the bound calcium in C9 we prepared apoC9 as described above. The kinetics of temperature-induced apoC9 inactivation were comparable to the kinetics of EDTA-induced inhibition of native C9 (Fig. 1B). Such a decrease in thermal stability can also be demonstrated by differential scanning calorimetry (24). Importantly, no difference in hemolytic activity between apoC9 and native C9 could be demonstrated (Fig. 7). Since C₈α-γ also bound ⁴⁴Ca²⁺ (Fig. 5), and since it is known that the C₈α chain provides the binding site for C9 in the membrane attack complex (25) we tested for the ability of C9 to bind to C8 at reduced ionic strength in the presence and absence of EDTA and Ca²⁺, respectively (Fig. 8, A and C). Again, no effect of EDTA on the association of C8 and C9 could be detected. It should be noticed that C9 and C8 were incubated with EDTA for only short times to prevent secondary structural changes in the proteins that might interfere with binding but that the sucrose density gradients contained the chelator. In an earlier publication (23) we described the binding of C9 to SUV prepared from either dipalmitoyl lecithin or egg lecithin. Since several...
proteins have been discovered recently that bind to lipids only in the presence of calcium ions (26) we repeated these studies again, but paying attention to the effect of free calcium ions in the binding process. As shown in Fig. 1D, binding of C9 to SUV was not affected by EDTA.

**DISCUSSION**

Our results demonstrate a hitherto unrecognized feature of human complement protein C9: it is a serum protein that binds calcium with high affinity. Three different and independent types of assay indicate that one calcium ion is bound per molecule of C9 with a dissociation constant of 3 μM at physiological pH and ionic strength. Incubation of C9 at elevated temperatures with a 10-15-fold molar excess of EDTA over C9 causes removal of the metal ion and protein aggregation.

The apoprotein can be stabilized against heat-induced aggregation by salts according to the Hofmeister series of lyotropic ions (27); kosmotropes protect C9 and chaotropes cause denaturation. Thus, the surprising finding that high concentrations of EDTA do not cause loss of C9 hemolytic activity but low concentrations do can be explained by the "salting out" effect of EDTA. This anion acts just like other kosmotropes, such as sulfate or citrate, at high concentrations. The bound calcium can be removed at room temperature by chromatography on a solid-phase EDTA column, and the ion can be incorporated again at even lower temperatures. Thus, it is evident from these results that aggregation of C9 follows removal of the bound calcium ion and that it can be prevented by stabilizing the protein. Thus it appears that the bound calcium promotes protein stability perhaps by liganding to different structural elements or domains of the folded peptide chain. Removal of the metal would facilitate thermal unfolding which can be counterbalanced by addition of kosmotropes. However, despite this loss in stability we could not detect functional differences between apoC9 and native C9 as long as the apo form is not aggregated. The hemolytic activities of both forms, as well as the binding of C9 to C8 at reduced ionic strength, were similar. Furthermore, no difference in lipid binding between apoC9 and native C9 could be detected.

The inability of EDTA to affect the binding of C9 to lipid bilayers indicates that C9 is not a member of a recently discovered class of calcium-dependent lipid-binding proteins (26). This conclusion is corroborated by the fact that the consensus sequence of one Gly separated by 12 residues from an Arg and a second Gly separated by 29 residues from another Arg (28) is not present in either of the human C9 sequences published by DiScipio et al. (29) or by Stanley et al. (30). Recent evidence indicates that the cysteine-rich domain found in epidermal growth factor factor and present in many other proteins can be involved in calcium binding. An asparagine in this sequence is posttranslationally modified to yield β-hydroxyaspartic acid and this amine acid side chain is thought to provide a calcium binding ligand in proteins that contain this region, such as C1r, protein C, protein Z, and factors IX and X (for review see Ref. 31). We do not believe, however, that the cysteine-rich epidermal growth factor domain is responsible for calcium binding in C9. First, the domain is located in the C9b fragment (30) which does not bind calcium. Second, the same domain is also located in C8α (32) but this chain also does not bind calcium. The most likely candidate for a calcium binding site appears to be the highly negatively charged cysteine-rich domain that is found in the low density lipoprotein receptor (30). This conclusion is based on similarities between C9 and α-lactalbumin. Just like C9, α-lactalbumin binds calcium very strongly (33) and inserts spontaneously into SUV lipid bilayers (34). The structure of the calcium binding site in α-lactalbumin has been solved recently at high resolution (35). This binding site shows strong similarity to the cysteine-rich low density lipoprotein receptor domain. The fact that C8δ does not bind calcium but C8α-γ does, although both contain this domain (32, 36), can be explained by loss of a critical calcium binding ligand in C8δ.

The observed stoichiometry of one calcium ion per C9 molecule is also in accord with the presence of one cysteine-rich low density lipoprotein receptor domain in C9, and this sequence is located in the C9a fragment.

The fact that apoC9, or C9 in EDTA, at physiological temperatures is functionally as competent as native C9 raises the question: why should C9 be a calcium binding protein? Although we do not have a clear answer to this question, several possibilities come to mind. C9 is a secreted protein that must pass one or several membranes during and after its synthesis. Current hypotheses predict that precursor proteins cross the endoplasmic reticulum in a differently folded state (for pertinent reviews see Refs. 37 and 38). Once they achieve their final conformation, either by folding around a prosthetic group (39) or after formation of disulfide bonds (40), they cannot spontaneously enter a membrane again. To function as a lytic protein C9, however, must again insert into a target membrane but only after association with its receptor, the C5b-8 complex. Thus, one can suggest two reasons for the presence of calcium in C9. First, after synthesis and translocation across the endoplasmic reticulum membrane and incorporation into transport or secretory vesicles, C9 could fold in a calcium-dependent manner into its Snal form and thereby assure that it will not interact spontaneously with other host membranes when it arrives in the blood. Second, after binding to its receptor, the affinity for calcium could be lowered and the protein could now switch back to a form that is capable of inserting into membranes. Such a mechanism is suggested by our observations that the thermal stability of C9 is decreased by choline ions (24) and since the choline group is a constituent of lecithins, it is conceivable that the high local concentration of this lipid on cell membranes could facilitate such a conformational change. Both suggestions can be tested experimentally. For example, acquisition of calcium binding activity could be visualized by ligand blotting of the protein along the secretory pathway. And although we did not detect overt differences in hemolytic activity between native and calcium-free C9, we may be able to detect such differences, if they exist, for example by carefully measuring the activation energies required for lysis by native C9 and apoC9. In any case, it is intriguing that among the terminal complement proteins only C9 and C8α are calcium binding proteins, and these two proteins are the only ones within the assembled membrane attack complex that enter the target membrane (41-43).

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