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ISOLATION AND CHARACTERIZATION OF THE PROENZYME FORM OF THE C1r SUBUNIT OF THE FIRST COMPLEMENT COMPONENT[1]

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The proenzyme form of C1r was isolated by sequential chromatography of the euglobulin fraction of human serum on DE-32 cellulose, TEAE cellulose, and Sephadex G-200. C1r was found to be a proteolytic enzyme with β-globulin mobility, a sedimentation rate of 7.5S, and a molecular weight of approximately 188,000 daltons. Isolated C1r preparations exhibited an affinity for C1q and C1s, forming a reversible complex with C1q and a firm complex with C1s in free solution.

Isolated C1r preparations did not activate proenzyme C1s, the natural substrate of the C1r enzyme, unless first activated by brief treatment with trypsin, a process accompanied by physicochemical changes in the molecule. It is not known whether the mechanism of conversion of C1r from a proenzyme into an active enzyme during C1 activation is also enzymatically mediated or, alternatively, whether conversion results from conformational changes in C1r induced by the attachment of C1, via C1q, to immunoglobulin molecules.

The first complement component, C1[4], is a calcium-dependent complex of three distinct proteins termed C1q, C1r, and C1s (1). Isolated C1q possesses combining sites for immunoglobulins (2), so C1q probably represents the site of attachment of C1 to immunoglobulin molecules. C1s, which normally exists within the C1 molecule in a proenzyme or precursor state, is activated or converted into a proteolytic enzyme after binding of C1 via C1q to immunoglobulin molecules. Once activated, the C1s subunit of C1 acts enzymatically on C2 and C4 and initiates the classical complement pathway (3). The function of C1r, which was recently isolated in a highly purified form (4), has not been clarified, although earlier studies by Naff and Ratnoff (5) with partially purified C1r strongly suggested that C1r is an enzymatic activator of C1s. The studies presented here are directed toward defining the role of C1r in the intramolecular process which leads to activation of the C1s enzyme. To this end, we here describe a method for isolation of the precursor or proenzyme form of C1r and present a physicochemical characterization of the isolated protein.

MATERIALS AND METHODS

Chemical and protein reagents, complement components, cellular intermediates and buffers. These reagents were obtained or isolated as previously described (6). All conductivity measurements were performed at 22°C.

Isolation of C1r. Preliminary studies indicated that C1r was most easily isolated from euglobulin prepared from serum at pH 6.0, although it could also be purified from euglobulin prepared at either pH 5.4 or 7.0. The first two steps in the isolation of C1r, i.e., preparation of euglobulin and DE-32 cellulose chromatography, were identical to those employed for the isolation of C1s. Approximately

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[4] Terminology conforms to the recommendations of the World Health Organization Committee on Complement Nomenclature (Bull. W. H. O., *39:* 939, 1968).

1200 ml of serum were cooled to 0°C, adjusted to pH 6.0, and dialyzed at 0°C for 14 hr against 10 liters of 0.013 M phosphate buffer (pH 6.0, conductivity 1.8 mmhos/cm). The precipitate was sedimented by centrifugation, washed twice with 250 ml of the dialyzing buffer, and dissolved in 250 ml of 0.047 sodium phosphate buffer containing 0.04 M NaCl and 0.001 M EDTA. This buffer, which was the starting buffer for DE-32 cellulose chromatography, had a pH of 7.5 and a conductance of 9.3 mmhos/cm. After overnight dialysis at 4°C against 2 liters of the same buffer, the dissolved precipitate was applied to a 3- x 40-cm column of DE-32 cellulose equilibrated with the same buffer. The column was washed with 2 liters of this buffer, and the adsorbed protein eluted with a linear NaCl gradient (1.8 liters of the same buffer containing 0.18 M NaCl). Fractions containing the C1r proenzyme peak were pooled and adjusted with NaOH and distilled water to a pH of 8.0 and a conductance of 8 mmhos/cm. The pool of active material was applied to a 2.5- x 35-cm TEAE cellulose column equilibrated with a 0.047 M sodium phosphate buffer (pH 8, containing 0.04 M NaCl and 0.001 M EDTA, 8 mmhos/cm). The column was washed with 1.5 liters of the same buffer and the adsorbed protein was eluted with a NaCl concentration gradient (1.5 liters of starting buffer and 1.5 liters of the same buffer containing 0.2 M NaCl). Fractions containing the C1r peak were pooled and concentrated by ultrafiltration (Amicon Corporation, Lexington, Mass.) to a volume of 1.5 ml. The pool was applied to a 2- x 100-cm column of Sephadex G-200 equilibrated with 0.047 M phosphate buffer, pH 7.5, containing 0.1 M NaCl and 0.001 M EDTA (15 mmhos/cm). C1r emerged as the central protein peak of this column (Fig. 1).

Electrophoretic analyses. A modification of the method of Davis (7) was employed for electrophoretic studies in polyacrylamide gels. Six per cent gels were prepared and pre-electrophoresed in 0.01 M Tris-HCl, pH 8.3, containing sufficient NaCl to achieve a conductance of 8 mmhos/cm for 4 hr at 3 mÅ per gel. The buffer was then replaced with glycine-Tris buffer (7) (0.2 mmhos/cm) (7) and the gels were overlayered with 0.01 M Tris-HCl containing NaCl, as described above, and 1% sucrose. The samples, after addition of NaCl to raise the conductance to 8 mmhos/cm and sucrose to 4% concentration, were layered under the buffer directly on top of the running gel. The gels were then electrophoresed at 3 mÅ per gel until the marker dye reached the bottom of the gel tube. Preliminary studies showed these precautions to be necessary to prevent precipitation of C1r under the low ionic strength conditions prevailing in Davis' method and to prevent inactivation by unknown substances present in freshly polymerized gels.

Sucrose gradient ultracentrifugation and molecular weight determinations. These methods were as previously described (6).

Detection of C1r by C1s activation. The proenzyme form of C1r was detected by a four-step hemolytic test system. First, C1r present in column fractions was activated by brief treatment with trypsin, and activated $\overline{C1r}$ so formed was quantitated by its ability to activate C1s, its natural substrate. Activated $\overline{C1s}$, in turn, was detected by its ability to cleave added C4. Generally, 10 μl of column fractions were incubated with 10 ng of trypsin for 5 min at 37°C in the presence of 0.001 M EDTA. Trypsin was then neutralized by the addition of 20 ng of soybean trypsin inhibitor (SBTI).[5] Next, 0.15 μg of C1s was added and the mixtures were incubated for 45 min at 37°C to allow the newly formed $\overline{C1r}$ to activate C1s. After incubation, 1.5 μg of C4 were added and the mixtures were incubated further for 30 min at 37°C to allow the newly formed $\overline{C1s}$ to inactivate C4. Inactivation of C4 was then measured by hemolytic methods (8). When isolated C1r was thus tested, 2% trypsin (w/w) was found to be optimal for activation (smaller amounts did not fully activate C1r and larger amounts led to loss of C1r activity).

Quantitation of C1r activation and C1r activity. In some studies activation of C1r was assayed and C1r activity was quantitated spectrophotometrically. The assay system was based upon ability of $\overline{C1r}$ to activate C1s; newly activated $\overline{C1s}$, in turn, was detected by its ability to cleave *N*-acetyl-L-tyrosine ethyl ester (ALTEE) (6). In this test system 20 to 50 μg of C1r were incubated with 2% trypsin (w/w) in

[5] Abbreviations used in this paper: SBTI, soybean trypsin inhibitor; ALTEE, *N*-acetyl-L-tyrosine ethyl ester; At, acetyl-L-tyrosine; SGVB, sucrose gelatin Veronal buffer.

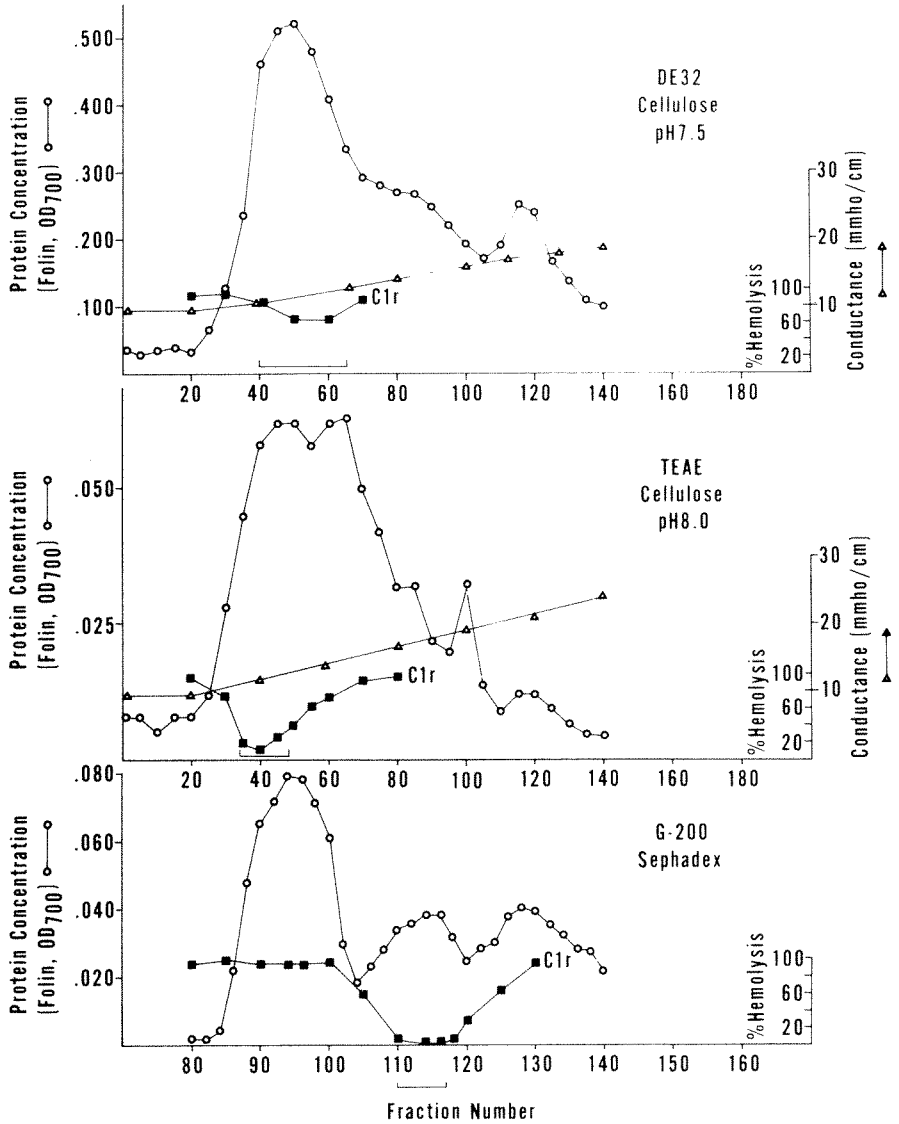

Figure 1. Chromatographic steps in the isolation of C1r proenzyme. Chromatography of human serum euglobulin on DE-32 cellulose at pH 7.5 (*upper panel*). C1r eluted at a conductance of 12 mmhos/cm as shown. Fractions containing the peak of C1r proenzyme were pooled, adjusted to pH 8.0 at a conductance of 8 mmhos/cm, and applied to a TEAE cellulose column at pH 8.0 (*middle panel*). C1r proenzyme eluted prior to the main protein peak, as shown, at a conductance of 12 mmhos/cm, as indicated. Fractions were pooled, concentrated, and applied to a Sephadex G-200 column (*lower panel*) at pH 7.5. C1r emerged as the central protein peak.

0.15 ml of Tris-NaCl EDTA buffer (6) for 5 min at 37°C, after which a 2-fold excess (w/w) of SBTI was added in 0.1 ml to inactivate the trypsin. Next, 10 to 20 μg of C1s and 0.75 ml of 0.001 M ALTEE were added. Cleavage was assessed by comparing the absorbance (at 238 nm) of the cuvettes containing ALTEE with that of a control cuvette containing the cleavage product, acetyl-L-tyrosine (AT), during a 70 min period at 30°C. A Beckman DU spectrophotometer with a Gilford recording attachment was employed for these studies. Controls of non-trypsin-treated C1r with C1s, neutralized trypsin with C1s and C1s alone were incubated with ALTEE in all studies.

Reconstitution of C1 from C1q, C1r and C1s as a measure of C1r activity. One microgram of C1q, 0.2 μg C1s and varying amounts of C1r in a total volume of 30 μl were incubated with 10 μl of 0.02 M $CaCl_2$ for 15 min at 30°C. Dilutions of the reaction mixture were then made and an aliquot incubated with 3×10^7 EAC4 in sucrose gelatin Veronal buffer (SGVB) for 15 min at 30°C. Subsequently, the cells in each tube were washed with SGVB at room temperature and the formed EAC14 were detected by lysis after

sequential addition of oxidized C2 (20 effective molecules/cell) and EDTA serum (9). A-1n (1-y) value of unity was obtained with an input of 19 molecules of isolated C1r per cell.

Analysis of interactions of C1r with C1q and C1s in free solution. Varying amounts of C1q were incubated with C1r or C1s for 15 min at 30°C in the presence of 0.001 M calcium. Radiolabeled ^{125}I-C1q was generally employed to facilitate subsequent detection of this protein. The mixtures were then sedimented in sucrose gradients. C1r and C1s were localized in gradient fractions by ability to reconstitute macromolecular C1. For these tests two of the components were supplied while the gradient fractions served as the missing subcomponent. The mixtures of C1q, C1r, and C1s were incubated with EAC4, after which the cells were washed and incubated with C2 and EDTA serum as described above. Appropriate combinations of 1 μg of C1q, 0.4 μg of C1r, and 0.2 μg of C1s were employed. C1r was also tested in gradient fractions by ability to activate C1s as described above.

RESULTS

Isolation and yield of C1r. Early studies indicated that C1r could be precipitated from serum in a non-activated state at pH 5.4, 6.0, or 7.0, provided that the temperature during precipitation was maintained at 0°C. Strict temperature control was not necessary for subsequent steps. The average yield of C1r of three consecutive preparations was 1.3 mg (range 0.5 mg to 2.6 mg). Since C1r activity cannot be accurately measured in serum, the yield on a protein basis could not be calculated, nor could the recovery in terms of specific activity be determined.

Electrophoretic homogeneity of isolated C1r. Isolated C1r exhibited one band on electrophoresis on polyacrylamide gels (Fig. 2); proenzyme C1r activity eluted from sectioned gels correlated perfectly with the position of the stained protein band. Isolated C1r was not reactive in Ouchterlony analyses with antiserum to whole human serum or to IgG, IgA, IgM, albumin, C1q, C1s, C3, C4, C5, C6, C8, and C9.

Physical properties and molecular weight of C1r. A sedimentation rate of 7.5S was observed on ultracentrifugation of isolated C1r in sucrose gradients in the presence of 0.001 M EDTA or calcium. The elution position of C1r from a calibrated Sephadex G-200 column indicated an apparent molecular weight of 185,000 daltons (Fig. 3). By calculation from the sedimentation rate (obtained by sucrose gradient ultracentrifugal analysis) and the diffusion coefficient (calculated by elution position from Sephadex columns) a molecular weight of 188,000 was obtained (Table I).

C1r activated by treatment with trypsin sedimented in sucrose gradients with a rate of 6S and on electrophoresis in polyacrylamide gels migrated faster than proenzyme C1r. In addition, trypsin-activated C1r, although fully able to activate C1s, was unable to interact with C1q and C1s to reconstitute C1. These studies indicate that trypsin activation of C1r is accompanied by physicochemical changes in the C1r molecule, probably secondary to proteolytic cleavage.

Activation state of C1r. Isolated C1r preparations did not activate proenzyme C1s unless first activated with trypsin. Activation of C1s was assessed by determining its ability to cleave the synthetic C1s substrate ALTEE. In the typical experiment shown in Figure 4, 50 μg of C1r were unable to activate 12.5 μg of C1s during 70 min of incubation at 30°C. However, after incubation with 2% trypsin (1 μg) for 5 min at 30°C and neutralization of trypsin with excess SBTI before addition of C1s, C1r was able to activate C1s. The synthetic substrate ALTEE was not cleaved by neutralized trypsin alone or by trypsin-treated C1r. C1r was also

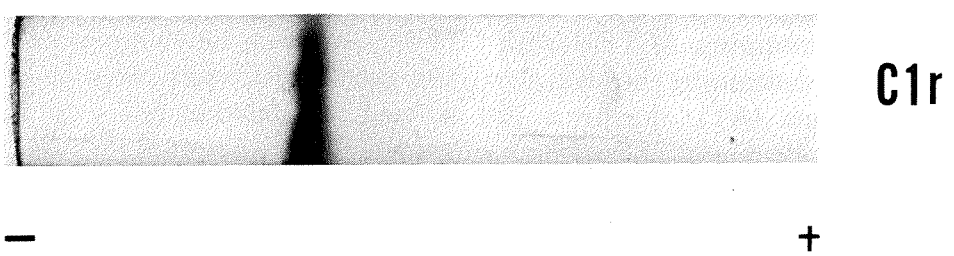

Figure 2. Mobility of C1r on electrophoresis in a 6% polyacrylamide gel. The anode is to the right.

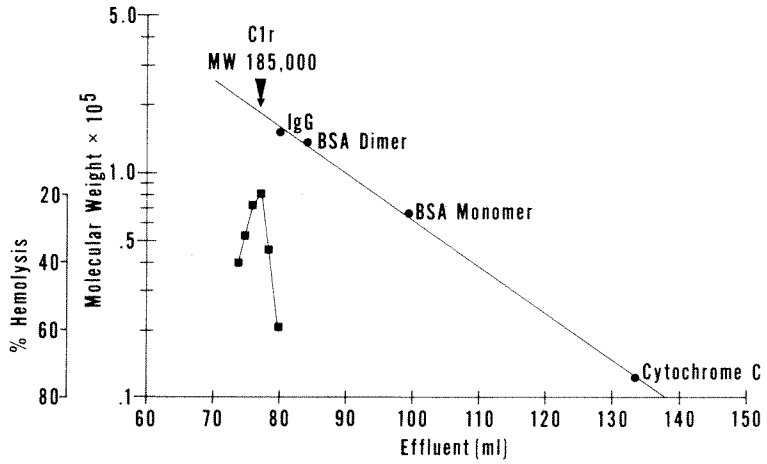

Figure 3. Gel filtration of C1r on Sephadex G-200. C1r emerged in advance of IgG with a molecular weight, by comparison with the marker proteins, of 185,000 daltons.

TABLE I

Physicochemical parameters of C1r

Sedimentation coefficient (0.001 M EDTA)[a]	7.5S
Diffusion coefficient (D)[b]	3.5×10^{-7} cm²/sec
Molecular weight (S,D)[c]	188,000
Frictional ratio[d]	1.56

[a] Sucrose density ultracentrifugation.

[b] Determined according to the method of Andrews (12).

[c] A partial specific volume of $\bar{v} = 0.73$ was assumed.

[d] Nomographically determined according to Wyman and Ingalls (13).

C1s, and C4; under these conditions C4 was inactivated. In these tests, neutralized trypsin was not able to inactivate C4.

Complexing of C1r with other C1 subcomponents. Various amounts of C1 subcomponents were incubated together in the presence of 0.001 M calcium for 15 min at 30°C before ultracentrifugation through sucrose gradients containing 0.001 M calcium. On ultracentrifugation of an approximately equimolar ratio of 66 μg of trace-radiolabeled C1q and 31 μg of C1r, two peaks of C1r activity were found (Fig. 5). One peak of C1r, representing about 20% of the C1r activity detected in the

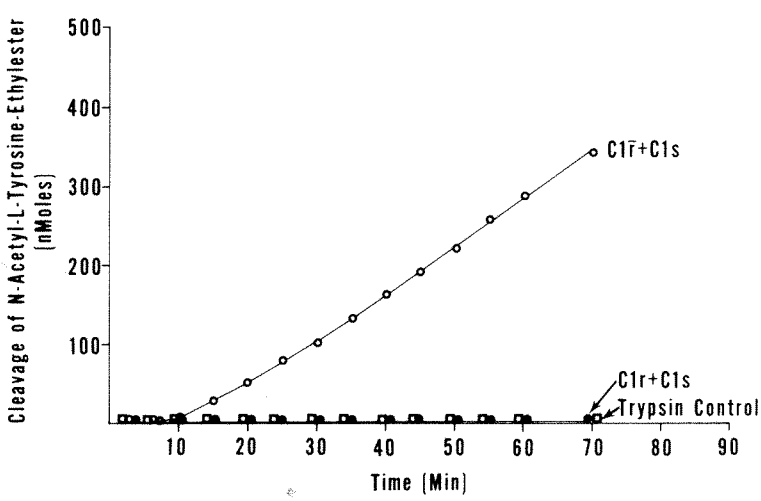

Figure 4. Activation of C1r by trypsin. In this experiment 50 μg of C1r were incubated with 2% trypsin (w/w) for 5 min at 30°C. An excess of SBTI was then added, followed by the addition of 12.5 μg of proenzyme C1s and 0.001 M ALTEE. Ability of C1r to activate C1s was acquired only after pretreatment of C1r with trypsin.

found to be in the proenzyme form when tested for ability to endow proenzyme C1s with the capacity to cleave C4. C1r was activated, however, by incubation with 2% trypsin for 5 min at 30°C before addition of excess SBTI, proenzyme gradient, coincided with the 11S location of the radiolabeled C1q. The bulk of C1r sedimented with the characteristic 7.5S rate observed on ultracentrifugation of C1r in the absence of C1q.

In contrast to the reversible association ob-

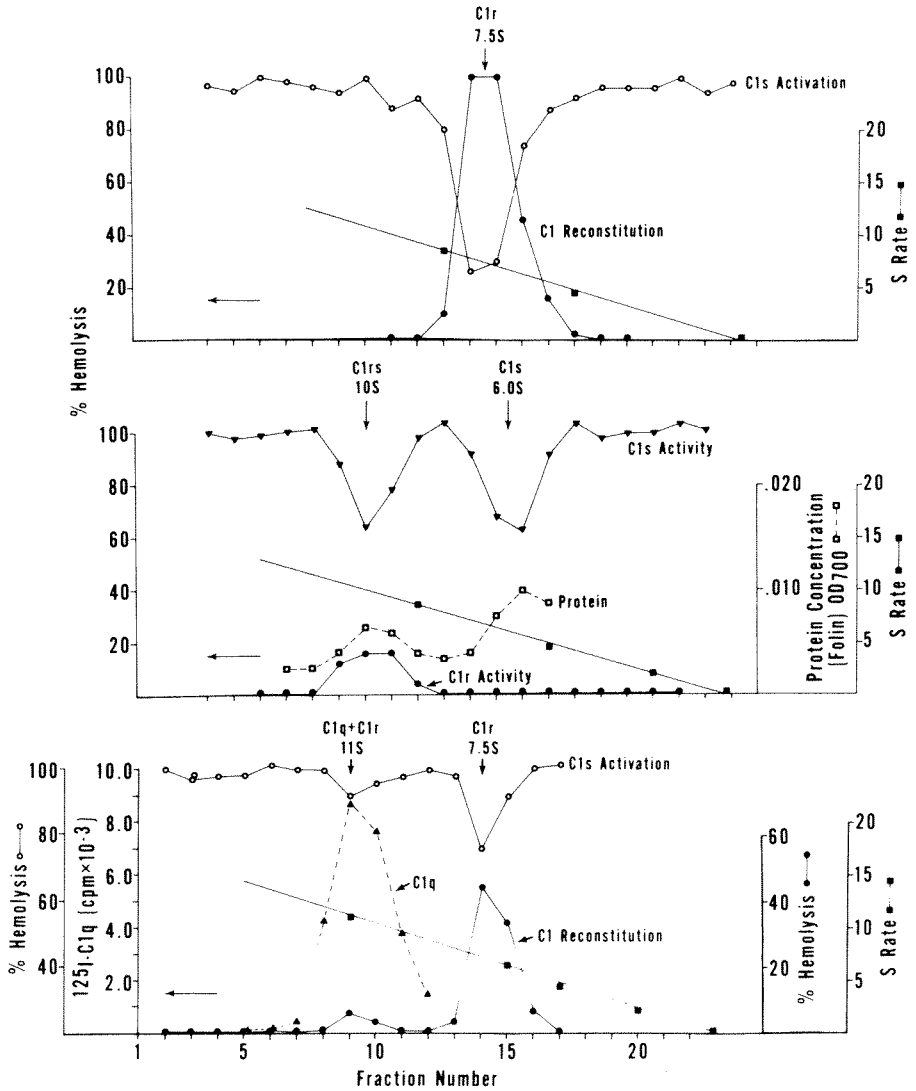

Figure 5. Sucrose gradient ultracentrifuged analysis of C1r and mixtures of C1r with C1s and C1q. C1r alone (*upper panel*), a mixture of 19 μg of C1r and 23 μg of C1s (*middle panel*), or a mixture of 66 μg of ^{125}I-C1q and 31 μg of C1r (*lower panel*) was incubated for 15 min at 30°C before ultracentrifugation. 0.001 M calcium was present in all buffers employed. C1r formed a firm complex with C1s and interacted reversibly with C1q.

served between C1q and C1r, C1r was found to form a firm complex on incubation with C1s. In the study shown in Figure 5, 19 μg of C1r and 23 μg of C1s (corresponding to an approximate molar ratio of C1r to C1s of 1:2) were incubated together before ultracentrifugation. Under these conditions, all of the C1r and approximately half of the C1s were incorporated into a heavier complex which sedimented with a 10S rate. The excess C1s sedimented with a rate of 6S, which corresponds to the rate observed for the C1s dimer on ultracentrifugation of C1s (in calcium containing buffers) in the absence of C1q (6).

DISCUSSION

The results presented here and in the companion paper (6) demonstrate that highly purified C1r functions as an activator of C1s and that activation takes place through proteolytic cleavage of C1s. The results confirm earlier studies by Naff and Ratnoff with partially purified C1r, indicating that C1r is an enzyme able to activate C1s (5). The marked affinity of C1r for C1s (Fig. 5) suggests that C1r and C1s are complexed to each other in the C1 macromolecule, implying that the susceptible bond in C1s is situated in close proximity to the active center of C1r. Consistent with this hypothesis is the fact that equimolar concentrations of C1r and C1s are required to achieve full activation and the lack of turnover of C1s by C1r during activation (6).

In our initial studies on the isolation of C1r we were unable to detect C1r activity, as assessed by ability to activate proenzyme C1s, in fractions of DEAE columns to which euglobulin-

containing C1r was applied. C1r, however, could readily be detected if the column fractions were incubated with C1q, C1s, and calcium and ability to form C1 then measured. This observation suggested to us that C1r might exist as a proenzyme in the native state, and since other complement components which exist in proenzyme forms may be activated by trypsin, including C1s (6, 10), C2 (11), and C3 proactivator (11), we examined the ability of trypsin to activate C1r. C1r present in column fractions was found to be activated by brief treatment with trypsin, a finding which enabled us to detect the native form of C1r during purification.

Isolated C1r was also found to be unable to activate proenzyme C1s (Fig. 4). This ability, however, was readily acquired after activation of C1r by incubation with trypsin. For this reaction 2% trypsin (w/w) was optimal, since smaller amounts failed to achieve full activation of C1r and larger amounts led to loss of ability of C1r to activate proenzyme C1s. Trypsin activation of C1r led to a change in the physicochemical properties of C1r; the sedimentation rate decreased from 7.5S to 6S and electrophoretic mobility in polyacrylamide gels increased. These changes probably reflect a proteolytic attack on the molecule.

The finding that C1r existed in the proenzyme form was unexpected. This observation indicates that binding of C1 via C1q to aggregated immunoglobulin or an immune complex leads to conversion of C1r from a proenzyme form into an active enzyme. At present it is not known whether activation is accomplished via enzymatic attack, such as is observed on treatment of C1r with trypsin, or whether it results from conformational changes in C1r secondary to binding of C1. Although we have not been able to activate proenzyme C1r in free solution in the presence of C1q, calcium, and aggregated IgG, and no evidence for enzymatic activity associated with C1q has been obtained, enzymatic activation remains a possibility. In fact, the somewhat lower molecular weight for C1r, which in all likelihood was activated, reported by de Bracco and Stroud (4) is consistent with proteolytic activation. However, we consider it more likely that C1r is usually activated as a result of conformational changes induced in the molecule by the binding of C1r, via C1q, to immunoglobulin. In this concept, the necessity for attachment of C1 to immunoglobulin and for integrity of the C1 macromolecule in order to achieve activation, as well as the observation that C1r firmly complexes with C1s, can be viewed as facilitating the conformational changes required for activation by providing spatial constraints on the C1r molecule. Additional studies will help to clarify the mechanism of activation of C1r. Examining $\overline{C1r}$ for the hallmarks of an enzymatic attack and determining if it differs in conformation from proenzyme C1r will be of particular interest, as will assessing possible reversibility of conformational changes after release of C1r from its complex with C1q and C1s and determining the relationship of such changes to the reported instability of dissociated activated C1r (4).

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