

FOURTH COMPONENT OF HUMAN COMPLEMENT: DESCRIPTION OF A THREE POLYPEPTIDE CHAIN STRUCTURE*, †

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The present work was performed to initiate a comprehensive study of the relationship between structure and function of the human C4 molecule. Activated C4 fulfills several different functions, including (a) binding through a labile-binding site to molecules and particles such as immunoglobulins, immune complexes, viruses, bacteria, and animal cells (1), (b) interaction through a specific and stable-binding site with an immune adherence receptor present on a variety of cells (2-4), (c) interaction with activated C2 to form the enzyme C3 convertase (5), and (d) interaction with activated C2 and C3 to form C5 convertase (6).

Isolation and description of C4 was first accomplished in 1963 (1). The protein was found to be a β_1 -globulin with a sedimentation coefficient of 10S. Its cytotoxic activity was abolished by treatment with hydrazine or potassium bromide which also changed its electrophoretic mobility. During the cytolytic reaction a small percentage of C4 molecules was shown to be physically bound to the target cell, whereas the majority of C4 molecules accumulated in the fluid phase in inactivated form. This reaction is catalyzed by the C1 \bar{s} subunit of C1 (7). Later it was recognized that a low molecular weight activation peptide, C4a, was released during the binding reaction and that the major fragment, C4b, was the portion of the molecule which was bound to the target cell surface (8-10). The mol wt of native C4 was estimated to be 202,000 daltons and that of C4a to be approximately 10,000 daltons (9, 10).

The purpose of this paper is twofold: (a) To describe a modification of the method of isolation of human C4, and (b) to propose a molecular model of C4 which is based on an analysis of its polypeptide chain structure.

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Materials and Methods

Materials. The following materials were purchased: phenylmethylsulfonylfluoride (PMSF)¹ (Calbiochem, San Diego, Calif.), TEAE-cellulose (Gallard-Schlesinger Chemical Mfg. Corp., Long Island, N. Y.), Biogel A-0.5 M (Bio-Rad Laboratories, Richmond, Calif.), Pevikon C-860 (Mercer Consolidated Corp., Yonkers, N. Y.), QAE-Sephadex (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.), reference proteins for SDS gel electrophoresis (Worthington Biochemical Corp., Freehold, N. J.), SDS (Sigma Chemical Co., St. Louis, Mo.), and dithiothreitol (DTT) (Bio-Rad).

Serum. Serum was obtained from freshly drawn O⁺ human blood, purchased from the San Diego Blood Bank, San Diego, Calif. Usually 2 U of blood were used for one preparation.

Preparation of Pseudoglobulin. To prevent activation of C1, the sera of 2 U of blood were not pooled, but processed separately. To each serum unit PMSF was added (dissolved in 5 ml isopropyl alcohol) to a final concentration of 0.25 mg PMSF/ml serum. Pseudo- and euglobulin fractions were partitioned by dialysis at 4°C against 3 × 10 liters of 0.0125 M Tris-HCl buffer, pH 7.0. The precipitated euglobulins were removed by centrifugation at 16,000 g for 30 min at 4°C. After PMSF was again added to the supernatant pseudoglobulin, this fraction was used as the starting material for the isolation of C4.

TEAE-Cellulose Chromatography. The pseudoglobulin fraction from 1 U of serum (approximately 200 ml) was applied to a 5.0 x 80-cm column of TEAE-cellulose equilibrated with 0.02 M phosphate buffer, pH 7.3 (buffer A). Immediately after the first unit had entered the column, the second unit of pseudoglobulin was applied. The column was washed with 6 liters of buffer A containing enough sodium chloride to adjust the conductivity to 9 mmho/cm (buffer B). C4 was then eluted by a salt concentration gradient. The mixing chamber, a 3,000 ml Florence flask, containing 3,000 ml of buffer B, was connected by a siphon to a 3,000 ml Erlenmeyer flask containing 2,500 ml of 0.45 M sodium chloride in buffer B. 20-ml fractions were collected at a flow rate of 200 ml/h. C4 containing fractions were pooled, filtered through a Millipore filter with a pore size of 0.45 μm and concentrated to 5.0 ml. The concentration of all protein solutions was effected by utilizing an Amicon concentration device (Amicon Corp., Lexington, Mass.) with a UM 10 filter.

Gel Filtration. The concentrated TEAE-cellulose pool was passed over a 5.0 x 100-cm column of Biogel A-0.5 M equilibrated with 0.1 M phosphate buffer, pH 7.3, at 4°C. 5-ml fractions were collected at a flow rate of 50 ml/h. C4 containing fractions were pooled, passed through a Millipore filter, concentrated to 4 ml, and dialyzed against 2 × 1 liters barbital buffer, pH 8.6, ionic strength 0.05.

Preparative Electrophoresis. The concentrated C4 pool from the Biogel column was applied to a 1 x 20 x 50-cm block of Pevikon C-860 equilibrated with pH 8.6 barbital buffer. Electrophoresis was performed at 4°C for 24 h at 3.5 V/cm. C4 containing eluates of block segments were pooled, filtered through Millipore filters, and concentrated to 10 ml. The concentrate was dialyzed against 3 × 1 liters of QAE-Sephadex chromatography starting buffer.

QAE-Sephadex Chromatography. The Pevikon block C4 pool was applied to a 2.3 x 65-cm column of QAE-Sephadex A-50 equilibrated with 0.004 M phosphate buffer, pH 7.3, containing sodium chloride to give a conductivity of 22 mmho/cm. After adsorption of the applied material, the column was washed with 400 ml of starting buffer and C4 was eluted with a salt concentration gradient. The mixing chamber, a 1,000 ml Florence flask, containing 1,000 ml of QAE starting buffer, was connected by a siphon to a 1,000 ml Erlenmeyer flask containing 850 ml of starting buffer, the conductivity of which had been adjusted to 40 mmho/cm. 6-ml fractions were collected at a flow rate of 25 ml/h.

Detection of C4 Hemolytic Activity. This was done by adding 5- to 50-μl aliquots of C4 containing fractions to a system consisting of 0.5 ml gelatin containing veronal buffer, 20 μg isolated C3, 0.1 ml hydrazine-treated human serum diluted 1/5, and 0.4 ml erythrocytes (E) sensitized with antibody (A) at a concentration of 5 × 10⁹/ml. After incubation for an appropriate time which did not allow 100% lysis to occur in any reaction mixture, the reaction was stopped by addition of 2.0 ml ice-

¹Abbreviations used in this paper: A, antibody; DTT, dithiothreitol; F, Forssman antigen; PMSF, phenylmethylsulfonylfluoride; Sbl, labile-binding site; Sbs, stable-binding site.

cold saline and centrifugation. The degree of lysis was determined spectrophotometrically at 541 nm.

Treatment of C4 with C1 Esterase. Purified C4 was digested with purified C1 esterase according to the method described (7).

Analytical Electrophoresis. Immunoelectrophoresis was performed according to Scheidegger (11). Disc electrophoresis was performed using the method of Davis (12) employing 6% running gels and Tris-HCl-glycine buffer, pH 8.7.

SDS-Polyacrylamide Gel Electrophoresis. Molecular weights of C4 and its subunits were determined in 7% polyacrylamide gels containing 0.1% SDS in phosphate buffer, pH 7.25, according to the method of Weber and Osborn (13). 1 vol of protein solution was mixed with 1 vol of phosphate buffer containing 8.5 M urea, 2% SDS, and 0.014 M DTT. The mixture was held at 37°C for 45 min, when an equal volume of 50% glycerol in water was added which was saturated with bromphenol blue. Electrophoresis was performed using 25- μ l aliquots in 6 x 100-mm gel columns for 3 h at 8 milliamperes/gel in a Hoefer gel electrophoresis apparatus (Hoefer Scientific Instruments, San Francisco, Calif.). The electrode vessels were filled with 0.1 M phosphate buffer, pH 7.25, containing 0.1% SDS. The gels were stained twice, first with 0.2% amido Schwartz dissolved in methanol-water-acetic acid (4.5:4.5:1), for 30 min, then destained by diffusion in the same solvent and stained the second time for 18 h with 0.025% Coomassie Blue in 10% TCA. Destaining was carried out in 10% TCA and gels were stored for evaluation in 7% acetic acid. Proteins used as mol wt markers were reduced α_2 -macroglobulin (185,000), reduced β -galactosidase (135,000), reduced C1s inhibitor (105,000) (supplied by Dr. Peter Harpel, New York Hospital—Cornell Medical Center, New York), reduced phosphorylase A (92,500), reduced fibrinogen (70,000, 60,000, and 50,000), reduced glucose-6-phosphate dehydrogenase (36,000), reduced C1s light chain (31,600), and reduced chymotrypsinogen (25,500). When radiolabeled C4 was used, gels were sliced into 2-mm wide discs and analyzed for radioactivity in a Packard auto-gamma scintillation spectrometer (Packard Instrument Co., Downers Grove, Ill.).

Radiolabeling of Purified C4. Purified C4 was radiolabeled with ^{125}I by the method of McConehey and Dixon (14). 250 μg C4 protein in 1 ml saline was treated with 5 μg chloramine T and 200 μCi ^{125}I for 10 min at 4°C after which time 5 μg of sodium metabisulfite was added. Uptake of iodine was approximately 20% and specific radioactivity 0.16 $\mu\text{Ci}/\mu\text{g}$. Excess iodine was removed by dialysis against 3 x 10 liters of 0.85% NaCl followed by dialysis against 1 x 1 liters of 0.1 M phosphate buffer, pH 7.3.

Preparation of Forssman (F) Antigen Containing Erythrocyte Fragments, Antibody (A), and C Complexes. Sheep erythrocyte (E) membrane fragments containing Forssman antigen were prepared as described by Rapp and Borsos (15). FAC1,4 complexes were prepared by the method used for preparation of EAC1,4 as outlined previously using [^{125}I]C4 (8).

Dissociation of C4 from FAC1,4. [^{125}I]C4 was dissociated from FAC1,4 by incubation with SDS-urea or SDS-urea-DTT (see above) for 45 min at 37°C. Membrane fragments were separated from the released [^{125}I]C4 by centrifugation for 10 min at 7,000 g. The C4 containing supernate was subjected to SDS-polyacrylamide gel electrophoresis.

Results

Isolation of C4. Starting material for the purification of C4 consisted of the pseudoglobulin fraction formed by dialysis of serum against low ionic strength Tris-HCl buffer at pH 7.0. This procedure yields 30% more C4 than preparation of the pseudoglobulin fraction at pH 5.4. To prevent possible C1 activation, all operations were performed strictly at 4°C and pooling of individual sera was omitted. Further, to inactivate possibly present active C1s, PMSF was added to the initial sera and to the pseudoglobulin preparations.

Upon application of the pseudoglobulin fraction to TEAE-cellulose, the majority of the proteins eluted from the column with buffer A. C4 could then be separated from the bulk of the other proteins still adsorbed to the cellulose by gradually increasing the salt concentration. C4 activity was found in fractions having a conductivity between 12 and 17 mmho/cm (Fig. 1).

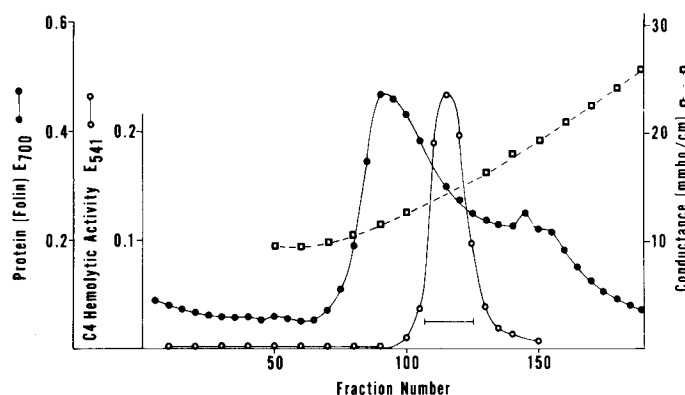


FIG. 1. Second step of C4 isolation: Chromatography of pseudoglobulin fraction on TEAE-cellulose. The pseudoglobulin fractions from 2 U of serum were applied to a 5.0×80 -cm column of TEAE-cellulose equilibrated with 0.015 M phosphate buffer, pH 7.3. A major portion of the pseudoglobulin was eluted by washing the column with buffer B at a flow rate of 150 ml/h at 4°C before a salt gradient was applied at fraction 1. C4 containing fractions were pooled as indicated by the bar.

An additional threefold purification of C4 was achieved by molecular sieve chromatography using Biogel A-0.5 M (Fig. 2). The elution volume of C4 was approximately 1.3 times the void volume.

The C4 pool was next subjected to preparative electrophoresis at pH 8.6. Fig. 3 shows a portion of the elution profile of the Pevikon block. C4 was well separated from ceruloplasmin and albumin but partially overlapped in distribution with that of C3. An approximately fourfold purification was achieved with this isolation step.

Final separation of C4 from C3 and other contaminants was achieved by ion exchange chromatography using QAE-Sephadex. Under the conditions used, C4 binds tightly to the anion exchanger and is eluted only after the conductivity has reached approximately 30 mmho/cm as seen in Fig. 4. This figure also demonstrates correspondence between the distribution of C4 hemolytic activity and that of the protein. A twofold purification was achieved in this last step. The entire isolation procedure is summarized in Table I. The degree of purification, recovery, and yield are listed in Table II.

Purity of C4. The purity of the C4 isolated by the above method was established using a number of different techniques. Ouchterlony analysis gave a single precipitin line with various antisera to whole human serum. A reaction of identity was observed when isolated C4 was analyzed with antiwhole human serum and anti-C4. No line was seen with antisera to albumin, IgG, IgA, IgM, transferrin, ceruloplasmin, C3, C5, and β -lipoprotein. Upon immunoelectrophoresis a single precipitin arc developed with an antiserum to whole human serum and this appeared to be identical to the arc produced by antiserum to C4 (Fig. 5). Polyacrylamide gel electrophoresis showed (Fig. 6) a major component, but also a minor faster and minor slower band. That these bands represent conversion products of C4 was verified by subjecting Cl₂-treated C4 to polyacrylamide gel electrophoresis. After this treatment the major species of molecules disappeared and gave rise to the fast- and slow-migrating protein zones.

FOURTH COMPONENT OF HUMAN COMPLEMENT

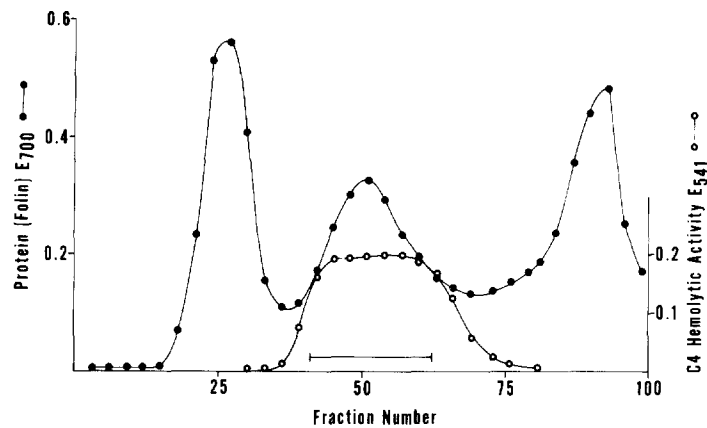


FIG. 2. Third step of C4 isolation: Molecular sieve chromatography through Biogel A-0.5 M of C4 containing pool from second step. The concentrated protein pool from the previous step was injected into a 5.0×100 -cm column of Biogel A-0.5 M equilibrated in 0.1 M phosphate buffer, pH 7.3. The column was eluted at a flow rate of 50 ml/h at 4°C. C4 containing fractions were pooled as indicated by the bar.

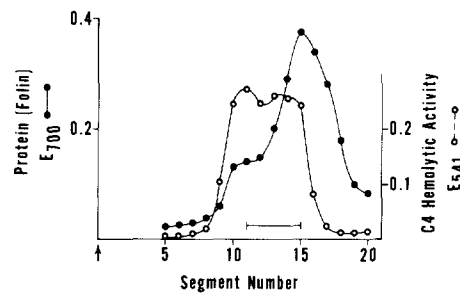


FIG. 3. Fourth step of C4 isolation: Preparative electrophoresis on Pevikon block of C4 containing pool from third step. The concentrated C4 pool from the Biogel column was subjected to electrophoresis at pH 8.6 on a $1 \times 20 \times 50$ -cm block of Pevikon C-860 for 24 h at 60 milliamperes and 3.5 V/cm at 4°C. Origin is indicated by the arrow and the block was sectioned into 1.25-cm segments. Shown is a partial elution profile of a typical block. C4 containing fractions were pooled as indicated by the bar.

Subunit Structure of C4. Purified C4 was subjected to electrophoresis in 7% polyacrylamide gels containing SDS. Fig. 7 shows the results of one such experiment. When treated with SDS and urea (gel a), C4 migrates as a major band with a mol wt $209,000 \pm 5,000$ daltons. Prior treatment of C4 with C1 esterase leads to partial aggregation, but no detectable change in the molecular weight of the monomer (gel b). Reduction of C4 in the presence of SDS and urea gives rise to three distinct polypeptide chains (gel c). The calculated mol wt of these chains are: α , $93,000 \pm 2,000$; β , $78,000 \pm 2,000$; and γ , $33,000 \pm 300$ daltons. If C4 is treated with C1 esterase before reduction, only the α -chain is affected (gel d). The mol wt of C1s cleaved α -chain (α' -chain) is 87,000 daltons. Therefore, the peptide removed from the α -chain was assigned a mol wt of 6,000 daltons. C4a was not detected in these experiments. Alkylation of reduced C4

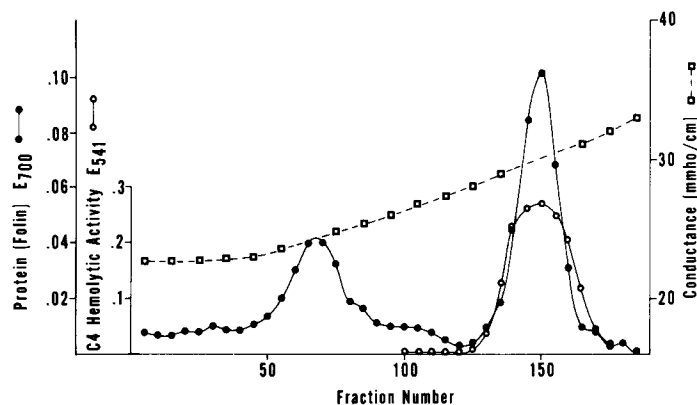


FIG. 4. Fifth step of C4 isolation: Chromatography of C4 containing pool from fourth step on QAE-Sephadex. The concentrated C4 containing pool from the previous step, which had been dialyzed against QAE starting buffer, was applied to a 2.3×65 -cm column of QAE-Sephadex A-50 equilibrated in starting buffer. The column was washed with the same buffer at a flow rate of 20 ml/h at 4°C to remove those proteins which were not bound by the ion exchanger under the given conditions before a salt gradient was started at fraction 1. Note the correspondence of C4 activity and protein distribution in the major peak.

TABLE I
Modified Procedure for Isolation of C4

1. Preparation of pseudoglobulin:	Dialysis of fresh serum against 0.0125 M Tris-HCl buffer, pH 7.0, supernate
2. Ion exchange chromatography:	TEAE-cellulose, salt gradient
3. Gel filtration:	Biogel A-0.5 M, 0.1 M PO_4 buffer, pH 7.3
4. Preparative electrophoresis:	Pevikon block; barbital buffer, pH 8.6, T/2 = 0.05
5. Ion exchange chromatography:	QAE-Sephadex, salt gradient

TABLE II
Purification and Yield for Human C4 Isolation

Material	Protein	Effective molecules	Effective molecules/protein
	mg		mg
Serum	36,400	2.4×10^{15}	6.6×10^{10}
Pseudoglobulin	33,800	1.0×10^{15}	3.0×10^{10}
TEAE-cellulose pool	238.7	ND	ND
Biogel pool	55.8	8.5×10^{13}	1.52 ± 10^{12}
Pevikon block pool	23.8	8.0×10^{13}	3.4×10^{12}
QAE-Sephadex pool	8.16	7.5×10^{13}	9.2×10^{12}

C4 activity yield: 3.2%; purification: $139 \times$.

before SDS electrophoresis had no effect on the resultant patterns. Staining with periodic acid-Schiff (PAS) indicated the presence of carbohydrate in each of the three chains.

To verify the three chain structure of C4, two experiments were performed.

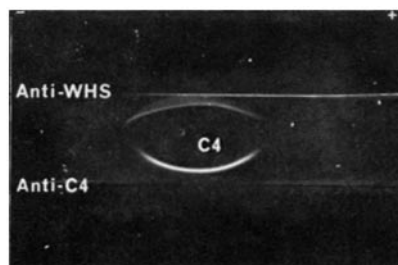


FIG. 5. Immunoelectrophoretic representation of isolated C4. Immunoelectrophoresis of isolated C4 was performed in 1% ion agar at pH 8.6 at 4°C for 2 h. Anode is at the right.

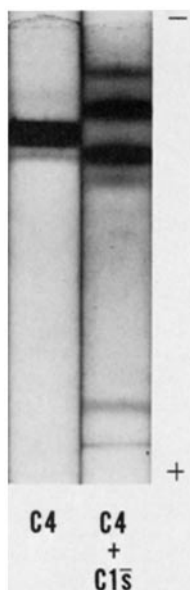


FIG. 6. Disc gel electrophoresis of C4 and C1s-treated C4. 25 μ g of isolated C4 or isolated C4 treated with 1% (wt/wt) C1s were subjected to electrophoresis in 6% polyacrylamide gels. Anode is at the bottom.

First, gel c was scanned at 550 nm and the adsorbance of the stained bands was calculated and compared with the molecular weight of the corresponding polypeptide chain. As shown in Table III, the ratios of the amount of stain correspond well to the molecular weight ratios for the respective polypeptide chains.

Second, the chain structure of C4b was examined after it had been specifically bound to immune complexes through the action of C1 and then dissociated from them. Membrane fragments of sheep erythrocytes containing F were treated with A to F, C1, and [125 I]C4. The FAC_{1,4} complexes were washed and dissociated in SDS and urea either with or without DTT. The supernate was then subjected to SDS gel electrophoresis and the gels were analyzed for the distribution of radioactivity. The pattern of radioactivity of the reduced eluted material was very similar to that obtained with reduced C4b. Specifically, in addition to α' - and β -chains, the lower molecular weight γ -chain was clearly present (Fig. 8).

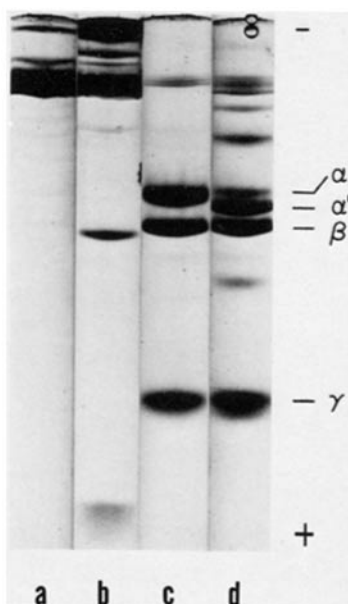


FIG. 7. Demonstration of three C4 polypeptide chains and of the effect of C15 on C4 structure. Reduced and unreduced samples of C4 were subjected to electrophoresis through 7% polyacrylamide gels containing SDS. Gel (a) contains unreduced C4 which migrates mostly as a single component of mol wt 209,000 daltons. Heavier material probably represents polymerized C4i. Gel (b) shows the effect of C15 treatment on unreduced C4. The position of the major component does not show any major changes, although more heavy material has been produced. The faster moving zone is C15 and the diffuse band at the bottom of the gel is a contaminant in the C15 preparation. Gel (c) shows that reduction in the presence of SDS dissociates C4 into three distinct polypeptide chains of mol wt 93,000, 78,000 and 33,000 daltons. Gel (d) represents C15-treated C4 which was then reduced. Only the α -chain shows a reduction of molecular weight. The band intermediate between β and γ is C15 heavy chain. C15 light chain is partially covered by the γ -chain.

TABLE III
Comparison of Molecular Weight and Staining Ratios of Component Chains of C4 after SDS-Polyacrylamide Gel Electrophoresis

Subunits	Molecular weight ratio	Stain ratio
α/β	$93,000/78,000 = 1.19$	1.15
α/γ	$93,000/33,000 = 2.8$	2.4
β/γ	$78,000/33,000 = 2.4$	2.1

Discussion

The data presented in this paper indicate that C4 is composed of three polypeptide chains which differ in molecular weight and are linked by disulfide bonds and noncovalent forces. The mol wt of the α -, β -, and γ -chains are 93,000, 78,000 and 33,000 daltons, respectively. The sum of the mol wt of the chains,

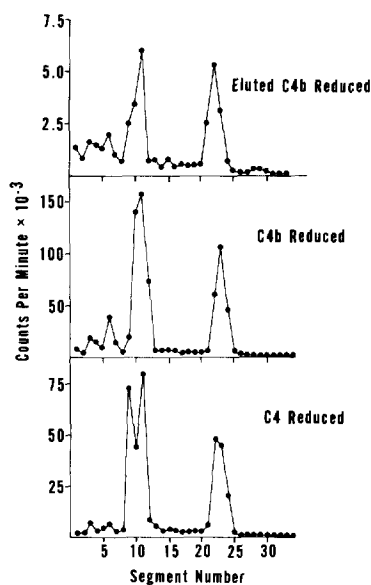


FIG. 8. Verification of multiple polypeptide chains in C4b specifically bound to immune complexes. ^{125}I C4 which had been specifically bound to immune complexes was eluted by treatment with SDS-urea-DTT and subjected to SDS polyacrylamide gel electrophoresis. The gels were sliced and analyzed for radioactivity. Counts per minute are plotted against segment number. The origin is at the left, the anode is to the right. The eluted, reduced C4b (top panel) gives a pattern very similar to C1 $\bar{5}$ -treated C4 which was then reduced (middle panel). Reduced, native C4 is shown in the bottom panel.

204,000 daltons is in close agreement with the mol wt of the undissociated C4 molecule, which is 209,000 daltons. These values also indicate that each chain occurs only once in the intact molecule. This concept is further supported by the estimated molar ratios of the subunits. Since a composition of three subunits is rare for protein molecules, the possibility of contamination had to be excluded. The following facts lend support to the three chain model: (a) The method of isolation employed in this study produced C4 which was pure by physical and immunochemical criteria. (b) The three subunit SDS gel pattern of reduced C4 has been established for eight different C4 preparations. (c) The three subunit pattern was observed for fully active as well as C1 $\bar{5}$ -treated C4. (d) C4b which was specifically bound to immune complexes and was then dissociated showed the same type of pattern as control C4b.

Treatment of C4 with C1 $\bar{5}$ was shown previously to result in cleavage of C4 and dissociation of a low molecular weight fragment, C4a (9, 10). After the action of C1 $\bar{5}$ only the α -chain showed a reduced molecular weight. This observation indicates that C4a is derived from the α -chain of C4, and suggests that the α -chain contributes to the generation of the labile-binding site (Sbl) through which activated C4 participates in C-dependent cytotoxicity. The mol wt difference between the α - and α' -chain is 5,000–7,000 daltons. It is not clear, therefore, why isolated C4a was found in earlier studies in this laboratory to have a mol wt of 11,000–15,000 daltons (9). The possibility is considered that C4a was isolated as a

dimer. However, Patrick and Lepow reported a mol wt of 8,600 daltons (10). Since C4-dependent immune adherence has previously been reported (2-4), the cell bound C4b fragment is envisioned to be endowed with a stable-binding site (Sbs) which reacts with specific cellular receptors. A schematic drawing of the proposed model of C4 is shown in Fig. 9.

It is of interest to compare the proposed structure of C4 with the proposed structure of C3 (16). C3 contains two polypeptide chains (α and β) joined by disulfide bonds and noncovalent forces, the mol wt of which are 120,000 and 75,000 daltons, respectively (17). Activation of C3 is affected by the action of C3 convertase (C4,2) (5), a trypsin-like enzyme, which cleaves a small peptide (C3a) (18, 19) from the N-terminus of the α -chain (20). Once activated, the C3b portion of the molecule is capable of binding to cells through the newly exposed Sbl (21). Bound C3b also contains a second Sbs which reacts with the immune adherence receptor of a variety of cells (22).

In several respects C4 is similar to C3. Both molecules contain β -chains of approximately the same size and α -chains that are heavier than the β -chains. In both molecules the Sbl is exposed by action of the respective activating enzyme

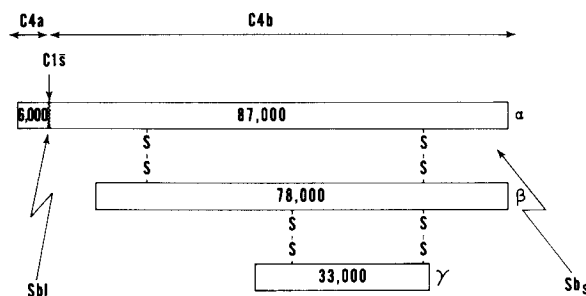


FIG. 9. Schematic presentation of proposed tertiary structure of C4.

on the α -chain with concomitant formation of a small peptide. Both b fragments, in their bound form, are endowed with immune adherence reactivity. In addition, Sobel and Bokisch (3) and Ross and Polley (4) have shown that in some instances the reaction of bound C3b with specific cellular receptors can be inhibited with soluble C4b and vice versa, suggesting a structural similarity of the Sbs of both molecules. These observations are compatible with the view that both molecules evolved from a common ancestral protein. However, the structural similarities between C3 and C4 diverge with the finding of the γ -chain of C4. It is conceivable that this subunit is the structural equivalent to a specific C4 function, namely to serve as acceptor of activated C2 in the formation of C3 convertase. More work is needed to examine this possibility.

Summary

The fourth component of human complement (C4) was shown to be composed of three distinct polypeptide chains linked by disulfide bonds and noncovalent forces. The sum of the molecular weights of the chains equalled that of the intact

molecule. The mol wt of the α -, β -, and γ -chains were respectively, 93,000, 78,000, and 33,000 daltons. Action of C1s on C4 affected only the α -chain, reducing its mol wt to 87,000 daltons. The size of the activation peptide, C4a, is therefore estimated to be 6,000 and that of the major fragment C4b, 198,000 daltons. Periodic acid-Schiff-stained SDS polyacrylamide gels of reduced C4 revealed carbohydrate to be associated with all three chains. A modification of the original method of isolation of C4 is presented.

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