

The Activation of Human Complement Component C5 by a Fluid Phase C5 Convertase*

(Received for publication, March 30, 1983)

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Complement component C5 is converted to C5a and C5b by the cobra venom factor-dependent C3/C5 convertase CVF,Bb (EC 3.4.21.47). The C5 convertase produces selective proteolytic cleavage of an arginyl-leucine peptide bond at positions 74–75 in the α chain of C5. Circular dichroism studies in both the far and near UV regions provide evidence that a conformational change accompanies the C5 activation process. When C5 is activated by CVF,Bb in the presence of complement component C6, the C5b,6 complex is formed. However, when C6 is added after C5 has been converted to C5b, the C5b,6 complex fails to form. Therefore, the activation of C5 results in a transient binding site for C6. Hydrophobic sites are probably exposed upon C5 activation because C5b undergoes aggregation when C5 is converted to C5b in the absence of C6. Transmission electron micrographs of the C5 molecule indicate a multilobal, irregular ultrastructure with estimated dimensions of $104 \times 140 \times 168 \text{ \AA}$. Aggregated C5b has the appearance of globular particles with a diameter range of 350–700 \AA . Although C5 shares a number of features with the third component of complement, including a similar ultrastructure and partial sequence homology, C5 is devoid of the unusual thiol ester linkage found in C3. It is the labile thiol ester that permits covalent attachment between C3 and nucleophilic acceptors. In contrast, interactions between C5 and C6 or C5 and membranes remain non-covalent.

Human complement component C5¹ is a two-chain plasma glycoprotein ($M_r \approx 196,000$) that is activated by limited proteolysis by C5 convertase. On activation, the α chain ($M_r \approx 116,000$) is cleaved, forming a new α' chain ($M_r \approx 105,000$) and the activation peptide, C5a ($M_r = 11,000$), while the β chain ($M_r \approx 80,000$) remains unchanged (3, 4). The proteolytic cleavage of C5 is the only known enzymatic event in assembly of the cytolytic membrane attack complex of complement (for reviews on the membrane attack mechanism of complement,

* This is Publication 2881 from the Department of Immunology, Research Institute of Scripps Clinic, La Jolla, CA. This work was supported by a research fellowship from the Chapter of the California Affiliate of the American Heart Association and by National Institutes of Health Grants AI17354, HL07195, and HL16411. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¹ Terminology for the complement components conforms to the recommendations of the World Health Organization Committee on Complement Nomenclature (1, 2).

see Refs. 5 and 6). The activation peptide, C5a, possesses potent spasmogenic and chemotactic activity (7). The C5a molecule constitutes one of three complement-derived anaphylatoxins, the others being C3a ($M_r = 9,000$) and C4a ($M_r = 9,100$) (for reviews on anaphylatoxins, see Refs. 8 and 9).

Four different C5 convertases have been reported that are capable of converting specifically C5 to C5a and C5b. Two of these are physiological complement enzymes and exclusively surface-associated: the classical pathway C5 convertase, C4b,2a,3b (EC 3.4.21.44) (3, 10) and the alternative pathway C5 convertase, C3b,Bb,C3b, (EC 3.4.21.47)² (4, 11). Both enzymes are unstable and undergo decay dissociation with a half-life at 37 °C of approximately 1.5–3 min (10). The pro-perdin-stabilized alternative pathway C5 convertase has a half-life at 37 °C of 10–34 min (4, 11).

Two fluid phase C5 convertases have been described: the classical pathway enzyme, C4b,2a^{oxy},3b (EC 3.4.21.44) and the cobra venom factor-dependent C5 convertase, CVF,Bb (EC 3.4.21.47). The modified C5 convertase, C4b,2a^{oxy},3b, contains C2a that is derived from C2 oxidized by iodine. The oxidation stabilizes the C4b,2a^{oxy} complex (12, 13). Native C3 was required, in addition to C4b,2a^{oxy} in order for C5 cleavage to occur (13). CVF,Bb is a noncovalent association product of CVF³ and the complement fragment Bb (14, 15). The catalytic subunits of these multimolecular proteases are C2a and Bb. These subunits have been partially sequenced and were found to be atypical serine proteases (16–18).

The fluid phase C5 convertase CVF,Bb was selected for these studies because it is stable (half-life at 37 °C = 7 h) (19) and does not require C3 for cleavage of C5. The objectives of the current investigation have been to characterize the activation of C5 and to obtain more information concerning the structural characteristics of C5 and C5b.

MATERIALS AND METHODS

Naja naja atra venom, lactoperoxidase, imidazole, 2-thiobarbituric acid, Coomassie blue R, DEAE-Sephadex, and 4-vinylpyridine were products of Sigma. Sodium [¹²⁵I]iodide (17.4 mCi/ μg) was purchased from Amersham Corp. Sephadryl S-300 was obtained from Pharmacia Fine Chemicals, Piscataway, NJ. Spectrapor dialysis tubing ($M_r = 2000$ cutoff) was purchased from Scientific Products, Irvine, CA. Guanidine HCl (ultrapure) was obtained from Bethesda Research Laboratories. Uranyl formate was purchased from Eastman.

Human complement components C5, factor B, and factor D were purified as described previously (20). CVF was purified from the crude venom according to Eggertsen *et al.* (21). Human complement component C6 was purified as described in Ref. 22. Protein concentrations were determined using the following extinction coefficients: for factor

² The EC numbers for complement enzymes do not differentiate between the cobra venom factor-dependent and C3b-dependent C3/C5 convertase of the alternative pathway of complement (11).

³ The abbreviations used are: CVF, cobra venom factor; SDS, sodium dodecyl sulfate.

B, the $E_{280 \text{ nm}, 1 \text{ cm}}^{1\%} = 12.7$ (23); for CVF, the $E_{280 \text{ nm}, 1 \text{ cm}}^{1\%} = 10.8$ (21); and for component C6, the $E_{280 \text{ nm}, 1 \text{ cm}}^{1\%} = 9.3$ (22).

The hemolytic activities of C5 and C5b,6 were measured by methods previously published (4, 22). Component C5 was radiolabeled with ^{125}I (24). The specific activity was 100,000 cpm/ μg of C5.

Conversion of C5 to C5b—Component C5 was converted to C5a and C5b by CVF,Bb at either a 1/15 or a 1/20 molar ratio of enzyme to substrate. The two fragments were generated and purified by the following procedure. Into a polypropylene tube was added 0.9 mg of CVF along with 0.6 mg of factor B and 10.0 μg of factor D in 4.0 ml of 10 mM Tris-HCl buffer at pH 8.0 containing 50 mM NaCl and 50 mM MgCl₂. This mixture was incubated for 15 min at 37 °C to allow formation of CVF,Bb, and this then was added to 25 mg of C5 in 90 ml of 10 mM Tris-HCl buffer, pH 8.0, containing 150 mM NaCl. After 12 h at 37 °C, the digest was dialyzed twice against 10 volumes of 10 mM Tris-HCl, pH 8.0, containing 40 mM NaCl. The dialysate was applied to a DEAE-Sephacel column (1.1 × 18 cm), and the column was rinsed with 100 ml of 10 mM Tris-HCl at pH 8.0 containing 40 mM NaCl. A 300-ml linear gradient, from 40 to 540 mM in NaCl and buffered with 10 mM Tris-HCl, pH 8.0, was applied to the column. Subsequently, the column was rinsed with 60 ml of 10 mM Tris-HCl buffer at pH 8.0 and 1.0 M NaCl followed by 60 ml of 10 mM Tris-HCl buffer at pH 8.0 and 6 M guanidine HCl.

The monomeric form of C5b was separated from aggregated C5b by gel filtration on a Sephadryl S-300 column (2.6 × 95 cm) in 10 mM imidazole HCl at pH 7.4 and 350 mM NaCl.

Gel Electrophoresis—SDS-polyacrylamide gel electrophoresis (25) was performed as modified according to Kisiel *et al.* (26). SDS-polyacrylamide slab gel electrophoresis was performed by the method of Laemmli (27).

Formation of the C5b,6 Complex— ^{125}I -labeled C5 (23.6 μg) was activated by a 1/20 molar ratio of CVF,Bb in the presence or absence of C6 (24 μg). The reaction was carried out for 12 h at 37 °C in a final volume of 230 μl in 10 mM Tris-HCl, pH 8.0, 75 mM NaCl.

Sucrose density gradient ultracentrifugation was carried out using linear gradients of 10–40% sucrose in 10 mM imidazole HCl at pH 7.4 and 150 mM NaCl employing a Beckman SW 50.1 rotor. The samples were centrifuged at 35,000 rpm for 12 h at 10 °C.

CD Spectroscopy—The circular dichroism spectra were recorded employing a Jobin Yvon Dichrographe III interfaced with a Nicolet Model 535 signal averager. The instrument was standardized with isoandrosterone. Samples for the far UV region (190–250 nm), which were at a concentration of 0.15–0.19 mg/ml in 20 mM sodium phosphate buffer at pH 8.0 or 5 mM Tris-HCl, pH 8.0, 75 mM NaCl, were scanned in 0.1-cm path length cuvettes. Samples for the near UV region (250–350 nm), which were at a concentration of 1 mg/ml in 10 mM Tris-HCl, pH 8.0, and 150 mM NaCl, were scanned for circular dichroism in 1-cm cuvettes. Prior to spectral analyses, each protein solution was passed through a 0.45- μm Millipore filter. The reference spectra were subtracted (electronically) from the sample spectra by using the signal averager. The results of the CD measurements have been expressed in terms of the mean residue ellipticity (θ) in units of degrees-cm²/dmol. The mean residue weight of C5 and C5b was determined to be 112 from compositional analyses. The percentages of α -helix and β -sheet were estimated by assuming nominal values of $\theta_{222} = -30,000$ degrees-cm²/dmol for 100% α -helix at 222 nm and $\theta_{216} = -9,200$ degrees-cm²/dmol for 100% β -sheet at 216 nm (28).

Determination of the Physical Parameters of C5, C5b, and C5a—The sedimentation coefficients of C5 and C5b were determined employing a Beckman Model E analytical ultracentrifuge equipped with a photoelectric scanner. The sedimentation velocity determinations were performed at 50,750 rpm at 20.75 °C. The sedimentation values were corrected for standard conditions to obtain $s_{20,w}$.

The diffusion coefficients of C5 and C5b were determined by gel filtration on a calibrated Sephadryl S-300 column (29, 30). The partial specific volumes were calculated from the amino acid composition (31) and the carbohydrate content (32).

The frictional ratios were determined as described in Ref. 33 and extinction coefficients as described in Ref. 34. The extinction coefficient for C5a was determined by amino acid analysis.

Isoelectric points were determined in an LKB 2117 Multiphor apparatus on a 5% polyacrylamide gel slab with an Ampholine concentration of 2.4% (w/v) with a pH range of 3.5–9.5.

Isolation of the Chains of C5 and C5b—The chains of C5 and C5b were isolated by preparative SDS-polyacrylamide gel electrophoresis. The chains were S-pyridylethylated (35) after separation.

Amino Acid and Carbohydrate Composition—Amino acid analysis was carried out by the method of Moore and Stein (36) and Spackman

et al. (37). Hexosamine was determined after the proteins were hydrolyzed with 2 M HCl for 16 h at 110 °C. Half-cystine was determined as cysteic acid by the method of Hirs (38). The amino acid and hexosamine samples were analyzed on a Durum D500 amino acid analyzer. Tryptophan content was quantitated by the method of Edelhoch (39). Sialic acid was determined by the method of Warren (40) and neutral sugar was analyzed by the method of Dubois *et al.* (41).

Automated NH₂-terminal Sequence Analyses—The NH₂-terminal sequence analyses of 38 nmol of S-pyridylethylated α' chain of C5b and 39 nmol of S-pyridylethylated C5b were performed using a Beckman Model 890 D Sequencer. The technique of Edman and Begg (42) was employed using a 0.1 M Quadrol program. The first cycle was double-coupled, and the phenylthiohydantoin derivatives were identified by high performance liquid chromatography using a Waters μBondapak C-18 column. A secondary identification method was sometimes used, namely hydrolysis of the phenylthiohydantoin derivatives to free amino acids by 47% hydriodic acid *in vacuo* for 4 h at 150 °C. NH₂-terminal residues were quantitated based on the original protein content as determined by amino acid analyses.

Electron Microscopy of C5 and C5b—Samples of C5 and C5b in 0.1 M ammonium acetate, 0.05 M ammonium carbonate, 0.1 mM EDTA, pH 7.4, were adsorbed to thin carbon films by the original Valentine procedure (43) and negatively stained with 1% (w/v) uranyl formate using the pleated sheet technique (44, 45). Pleated regions of the grid were photographed in a Hitachi 12A transmission electron microscope at a primary magnification of 64,000 diameters using an accelerating voltage of 75 kV, a 200 μm C2 aperture, and a 50- μm objective aperture. Magnification calibration of the electron microscope was performed by standard methods (46).

RESULTS

Activation of C5 by CVF,Bb in the Absence or Presence of C6—Conversion of C5 to C5b by CVF,Bb was followed as a function of time at 37 °C over a 5-h period. The conversion of C5 was accompanied by a progressive loss of C5 hemolytic activity. The SDS-polyacrylamide gel electrophoresis patterns of the products are presented in Fig. 1. Conversion of C5 ($M_r \approx 196,000$), at a concentration of 0.7 mg/ml, to C5b ($M_r \approx 185,000$) results in cleavage of the α chain ($M_r \approx 116,000$) into the α' chain ($M_r \approx 105,000$) and C5a ($M_r = 11,000$).

When C6 was present during the activation of C5, the C5b,6 bimolecular complex was formed as evidenced by the appearance of an 11.2 S peak observed on sucrose density gradient ultracentrifugation. Furthermore, 70,000 CH₅₀ units⁴ of C5b,6 hemolytic activity/mg of C5b,6 protein were measured, indicating a functional complex. However, if C6 was added after C5 cleaved to C5b, no C5b,6 was detected by sucrose gradient ultracentrifugation and no C5b,6 hemolytic activity was detected (Fig. 2). The C5b,6 complex was resistant to 1 M NaCl but was dissociated by 0.5 M KSCN.

Isolation of C5b by Chromatography on DEAE-Sephacel—C5b was separated from the reaction mixture that contained C5a, C5b and CVF,Bb by DEAE-Sephacel column chromatography. The elution profile is shown in Fig. 3. The Bb fragment of factor B and C5a passed through the column, whereas C5b was retained and then eluted with a sodium chloride gradient (fractions 105–116). The material in fractions 82–90 has not been identified, although it may contain some uncleaved factor B, and fractions 91–100 contained uncleaved C5. Some C5b that bound very tightly to the DEAE-Sephacel column was eluted by 1 M NaCl (fractions 161–163) and 6 M guanidine (fractions 183–184).

CD Spectroscopy of C5, C5b + C5a, and Purified C5b—From the results presented in Fig. 2, it was deduced that the cleavage of C5 is accompanied by a conformational change. Direct evidence for such a conformational change was obtained by

⁴ Complement hemolytic activity is expressed in CH₅₀ units where one CH₅₀ unit of activity produced 50% hemolysis of 5×10^7 sheep erythrocytes under standard conditions.

CD spectroscopy. Fig. 4 shows the far UV CD spectra for C5 and for the reaction mixture C5b + C5a. C5 was cleaved by CVF,Bb within the cuvette in order to minimize a possible artifact that could have arisen from a differential loss of either C5a or C5b. The spectra indicate that there is a small but measurable loss of apparent secondary structure on C5 conversion. Also shown is the CD spectrum of purified C5b. Fragment C5b has substantially less apparent secondary structure than native C5. The difference between the CD spectrum of C5 and C5b can be attributed, in part, to the CD spectrum of C5a. It is known that the activation peptide C5a contains approximately 50% α -helix (47). The near UV CD spectra of C5 and C5b + C5a present further physical evidence that a conformational change is accompanying the activation of C5 (Fig. 5). Differences between the spectra of C5 and C5b + C5a indicate that the protein conformational change involves several aromatic side chains.

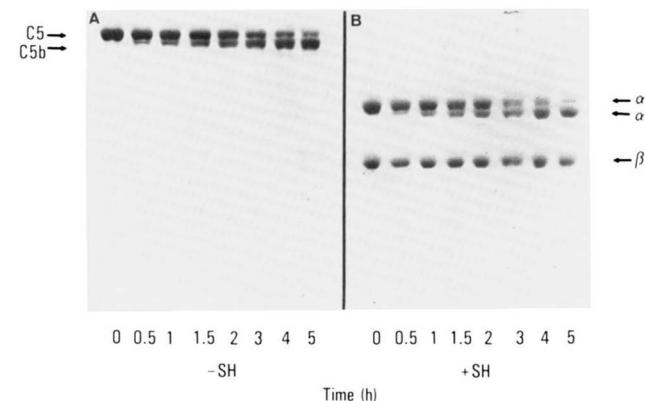


FIG. 1. A time course of C5 conversion to C5b by CVF,Bb as monitored by SDS-polyacrylamide (9%) gel electrophoresis. Component C5 (0.7 mg/ml) was activated by CVF,Bb at a molar ratio of CVF,Bb to C5 of 1:20 in 10 mM Tris-HCl, pH 8.0, and 75 mM NaCl at 37 °C. Aliquots were removed from the reaction mixture, assayed for C5 hemolytic activity, and analyzed by SDS-polyacrylamide gel electrophoresis. Each track contains 14 μ g of protein, and the polypeptides were stained with Coomassie blue R. Time of incubation is indicated for each sample, and the patterns with and without the addition of 1% (v/v) 2-mercaptoethanol are shown.

trum of C5b was observed to be identical with that of the C5b + C5a, indicating that C5a makes a negligible contribution to the circular dichroism of C5 at wavelengths between 250 and 320 nm.

The Physical Parameters of C5, C5a, and C5b—The physical properties of C5, C5a, and C5b are presented in Table I. The molecular weights as determined by physical methods (C5, $M_r = 186,900$ and C5b, $M_r = 171,000$) are somewhat lower than those obtained from SDS-polyacrylamide gel electrophoresis (C5, $M_r = 196,000$ and C5b, $M_r = 185,000$). The molecular weights determined by physical methods are considered more accurate because C5 and C5b are glycoproteins. SDS-polyacrylamide gel electrophoresis tends to provide overestimates of molecular weights of glycoproteins, since glycoproteins bind less detergent than apoproteins (48).

Assuming normal hydration, the frictional ratios of both C5 ($f/f_0 = 1.45$) and C5b ($f/f_0 = 1.46$) indicate that both proteins are asymmetric. C5a ($f/f_0 = 1.21$) is likely to be globular. The isoelectric point of C5b is somewhat more acidic than C5, which is consistent with the fact that the activation peptide C5a is cationic ($pI = 8.6$).

The CD spectral analyses were used to obtain an estimate

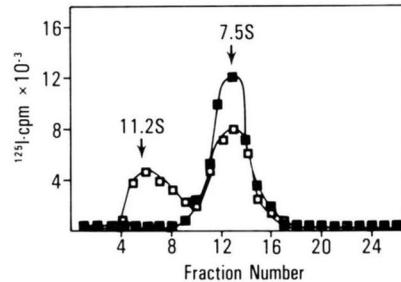


FIG. 2. The association of C5b with component C6. 125 I-labeled C5 (23.6 μ g) was activated by CVF,Bb as described in the legend of Fig. 1 either in the absence (■) or in the presence (□) of C6 (24 μ g). After 12 h at 37 °C, 24 μ g of component C6 were added to the reaction mixture that did not contain C6. The final volume of the reaction mixtures was 230 μ l. Aliquots (200 μ l) were layered over 5-ml linear sucrose density gradients (10–40%) in 10 mM Tris-HCl, pH 8.0, containing 150 mM NaCl, and the samples were centrifuged at 35,000 rpm for 12 h in a Beckman SW 50.1 rotor.

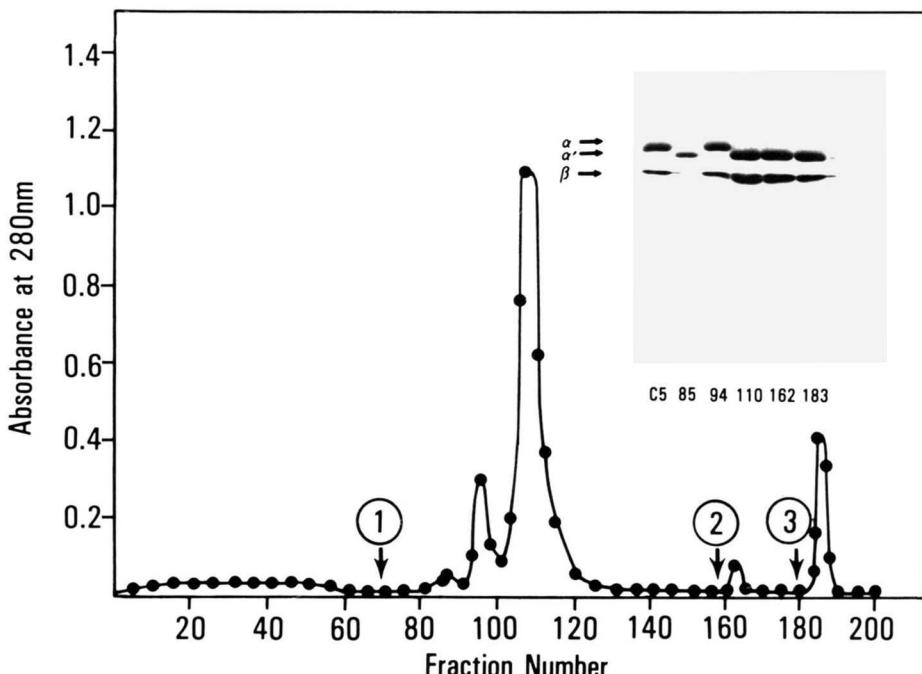


FIG. 3. The isolation of C5b on DEAE-Sephadex. A digestion mixture of C5b and C5a (25 mg) in 10 mM Tris-HCl, pH 8.0, 40 mM NaCl was applied to a DEAE-Sephadex column (1.1 \times 18 cm). The column was rinsed with 100 ml of 10 mM Tris-HCl at pH 8.0 and 40 mM NaCl. A 300-ml linear gradient (arrow 1) was applied from 40 to 540 mM in NaCl in a 10 mM Tris-HCl buffer at pH 8.0. After the gradient, the column was washed successively with 60 ml of 10 mM Tris-HCl, pH 8.0, 1.0 M NaCl (arrow 2), and 60 ml of 10 mM Tris-HCl at pH 8.0 containing 6 M guanidine HCl (arrow 3). Fractions of approximately 2.3 ml were collected. The inset shows SDS-polyacrylamide (9%) gel patterns for reduced samples (5–10 μ g) of the identified fractions eluted from this column.

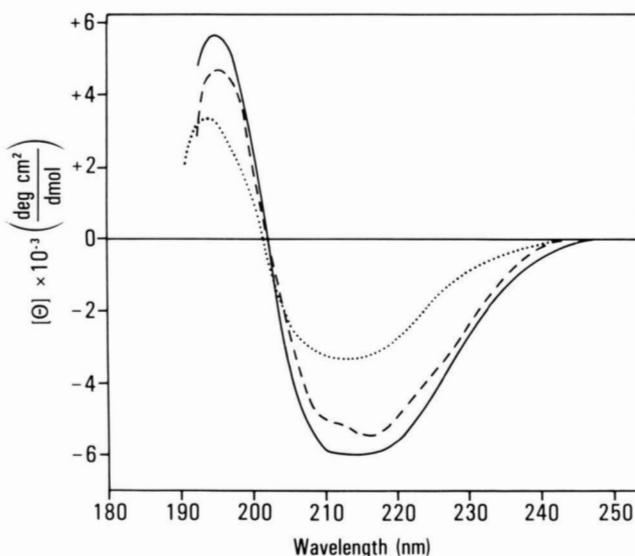


FIG. 4. The CD spectra in the far UV region of component C5, C5b + C5a reaction mixture, and C5b. The CD spectrum of 0.15 mg/ml of component C5 (—) was recorded in 5 mM Tris-HCl at pH 8.0 and 75 mM NaCl. The CD spectrum of the reaction mixture containing C5b + C5a (---) was recorded after incubating C5 (0.15 mg/ml in 5 mM Tris-HCl at pH 8.0 and 75 mM NaCl) with a 1:20 molar ratio of CVF,Bb to C5 in the cuvette for 12 h at 37 °C. A separate C5 sample was incubated with a 1:20 molar ratio of CVF,Bb to C5 for 12 h at 4 °C, a temperature at which no C5 cleavage was detected. The reference spectra of the buffer with CVF,Bb were subtracted from the spectra of the sample mixture. Also shown is the spectrum of purified C5b (· · ·).

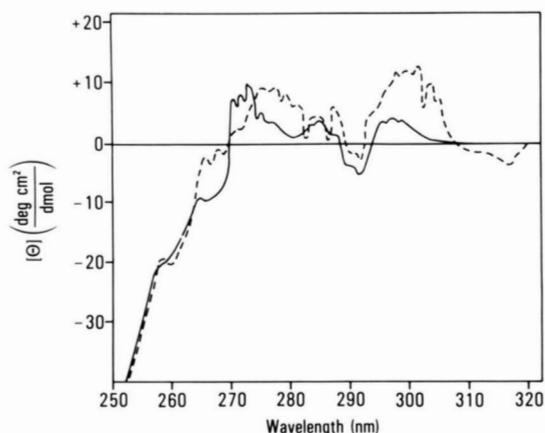


FIG. 5. The CD spectra in the near UV region for component C5 and the reaction mixture containing C5b + C5a. The spectrum of C5 (1.0 mg/ml) (—) was determined in 10 mM Tris-HCl at pH 8.0 and 150 mM NaCl. The spectrum of the reaction mixture of C5b + C5a (---) was recorded after incubating C5 (1.0 mg/ml in 10 mM Tris-HCl, pH 8.0, containing 150 mM NaCl) with a 1:20 molar ratio of CVF,Bb to C5 in the cuvette for 12 h at 37 °C.

of the apparent secondary structure of these molecules. The combined α -helical and β -sheet content was estimated to be 37% for C5, 27% for C5b, and 50% for C5a. By such criteria, C5, C5b, and C5a apparently contain conventional secondary structure.

Isolation of the Chains of C5 and C5b—In order to characterize further the structure of the C5 molecule, the chains of C5 and C5b were isolated by preparative SDS-polyacrylamide gel electrophoresis. Fig. 6 shows analytical SDS-polyacrylamide gel electrophoresis patterns of the α , α' , and β chains of C5 and C5b.

TABLE I
Physical parameters of the human complement components C5, C5b, and C5a^a

Parameter	C5	C5b	C5a ^a
$s_{20,w}$ (Svedberg units)	7.9	7.5 ^c	
$D_{20,w} (10^{-7} \text{ cm}^2/\text{s})$	3.9	4.0 ^c	12.1
\bar{v} (ml/g)	0.736	0.734	0.713
Molecular weight	186,900 ^b	171,700 ^{b,c}	11,200
f/f_0	1.45	1.46 ^c	1.21
$E_{280 \text{ nm}, 1 \text{ cm}}^{1\%}$	10.8	10.9	3.5
pI	4.7–5.5	4.5–5.3	8.6
Percentage ordered structure	37	27	50
Percentage α -helix	17	10	50
Percentage β -sheet	20	17	0

^a Data from Hugli and Muller-Eberhard (8).

^b Computed from $s_{20,w}$, $D_{20,w}$ (diffusion coefficient), and \bar{v} (partial specific volume).

^c Determined for C5b monomer.

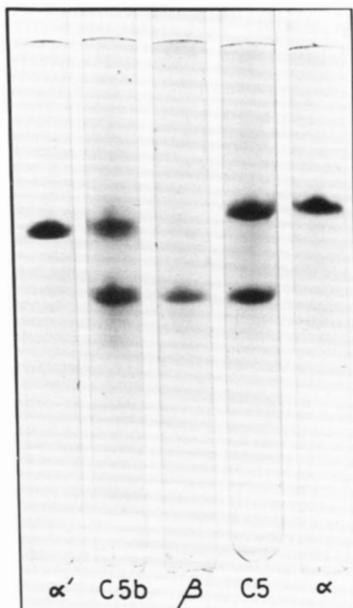


FIG. 6. SDS-polyacrylamide (7%) gel electrophoresis patterns for C5 and C5b and the isolated chains of C5 and C5b. Material applied to the gels from left to right is as follows: the α' chain of C5b, reduced C5b, the β chain of C5 (or C5b), reduced C5, and the α chain of C5. Each gel contains about 10–15 μ g of protein, and the gels were stained with Coomassie blue R.

Amino Acid and Carbohydrate Compositions of C5, C5a, and C5b and α , α' , and β Chains—Amino acid and carbohydrate compositions were determined for the intact protein and for the α , α' , and β chains of C5 and C5b. The results are presented in Table II. C5 is calculated to contain 2.7% oligosaccharide and C5b has 1.6% oligosaccharide. One noteworthy feature in the composition of C5 is the relatively low half-cystine content (1.7% of total amino acid residues) in comparison with other plasma glycoproteins. Since C5 has been reported to contain only between 0.3 to 2 free sulfhydryl groups (4, 49, 50), most of the half-cystine residues in the intact molecule exist in disulfide linkages.

NH₂-terminal Sequence Analysis of C5b and the α Chains of C5b—Prior publications (47, 51) have shown that C5a is derived from the NH₂ terminus of the α chain of C5, based on identical NH₂-terminal sequences for intact C5, the C5 α chain, and C5a. We have determined the NH₂-terminal sequences of S-pyridylethylated C5b and the S-pyridylethylated α' chain of C5b by automated Edman degradations (Table

III). The sequences obtained for intact C5b and the α' chain are identical, thus confirming an earlier report (51) that the β chain of C5 contains a blocked NH_2 terminus. The yields of leucine recovered from the NH_2 terminus of C5b and the α' chain of C5b were 0.77 and 0.81 eq, respectively. The repetitive yields were 91–92% for 13 steps of sequence. There was no sequence homology with either the α' chain of C3b (52) or that of component C4b (53).

Aggregation of Complement Fragment C5b—When C5, at concentrations greater than 0.25 mg/ml, was activated by CVF,Bb, aggregates of C5b were formed. Formation of the aggregated C5b was dependent on the initial C5 concentration as well as the salt concentration of the activation reaction. If C5 (1.5 mg/ml) in 10 mM Tris-HCl and 50 mM NaCl at pH 8.0 was activated by CVF,Bb, about 70% of the C5b was found in an aggregated state as evidenced by the gel filtration experiment shown in Fig. 7. In contrast, no aggregates of C5b were observed when C5 was activated at a concentration of 75 $\mu\text{g}/\text{ml}$ in 150 mM NaCl. The aggregates of C5b could not be dissociated by 1 M NaCl, but they were completely dissociated by 1% sodium dodecyl sulfate. Isolated monomeric C5b

was not observed to form such aggregates on standing. These results suggest that aggregated C5b forms as a consequence of the transient exposure of hydrophobic sites upon activation of C5. It is possible that the metastable hydrophobic sites of C5b enable this protein to form a complex with C6.

Electron Microscopy of Component C5 and Aggregated C5b—Results of electron microscopy of negatively stained C5 and aggregated C5b are shown in Fig. 8. In Fig. 8A is shown a typical field of well dispersed C5 molecules which exhibit an irregular multilobal structure. Orthogonal projections were selected from a large field view of C5, and these are shown in Fig. 8, B and C. In the series shown in Fig. 8B, the C5 molecule appears heart-shaped with dimensions of 140 \times 151 Å, and in the other series (Fig. 8C), the molecule has the appearance of a distorted prolate ellipsoid with dimensions of 104 \times 168 Å. It is hypothesized that a 90° rotation can convert one projection image into the other. The field images of aggregated C5b are shown in Fig. 8D. The aggregates are of similar size varying in diameter from 350 to 700 Å and exhibiting no regular or ordered structure. Monomer C5b appeared virtually identical with C5 (results not shown).

TABLE II
Amino acid and carbohydrate content of components C5, C5b, and C5a and the α , α' , and β chains

	C5	C5b	C5a ^a	α	α'	β
Amino acids						
Lysine	121.8	111.0	8	67.9	63.9	44.9
Histidine	33.2	28.5	2	19.0	17.7	12.0
Arginine	56.4	54.1	5	33.6	32.5	18.3
Aspartic acid	160.7	160.3	6	88.4	80.5	73.5
Threonine	106.9	100.0	3	60.3	60.4	40.3
Serine	126.2	119.6	4	70.0	71.7	53.0
Glutamic acid	165.8	160.6	9	112.2	99.3	74.4
Proline	71.1	70.3	1	35.6	32.3	36.0
Glycine	96.5	103.4	3	61.3	56.1	42.4
Alanine	100.0	92.3	8	62.0	52.2	41.7
Half-cystine	28.0	23.2	7	20.1	15.4	5.8
Valine	135.2	123.5	5	81.8	66.2	61.2
Methionine	23.1	21.4	1	11.7	9.9	7.7
Isoleucine	109.6	99.5	5	59.2	58.0	44.8
Leucine	159.2	154.2	4	100.1	93.8	59.8
Tyrosine	73.5	73.1	2	42.7	42.9	36.7
Phenylalanine	74.3	72.2	1	39.4	38.2	26.1
Tryptophan	14.7	15.0	0	7.9	8.1	6.1
Total amino acid residues	1656	1581	74	973	899	685
Molecular weight polypeptide	186,000	177,000	8,285	108,000	101,000	76,000
Monosaccharides						
Hexose	10.9	5.7	6	ND ^b	ND	ND
Glucosamine	9.5	7.1	4	7.8	5.0	0.5
Sialic acid	5.1	2.4	3	4.5	2.1	0
Total monosaccharide units	25–26	15	13			
Molecular weight carbohydrate	5,200	3,000	2,700			
Molecular weight component	191,200	180,000	10,985			

^a Compiled from Fernandez and Hugli (47).

^b ND, not determined.

TABLE III
The NH_2 -terminal sequences of C5b and the α' chain of C5b

Residue number	1	2	3	4	5	6	7	8	9	10	11	12	13
C5b	NH ₂ -Leu-	[His] ^a -	Met	-Lys-	Thr	-Leu-	Leu	-Pro-Met/Val-	[Ser]	-Lys-Pro-Glu-			
C5b α' chain	NH ₂ -Leu-	[His]-	Met	-Lys-	[Thr]	-Leu-	Leu	-Pro-	Val	-	ND ^b	-Lys-ND	-Glu-
C3b α' chain ^c	NH ₂ -Ser-	Asn	-Leu	-Asp-	Glu	-Asp-	Ile	-Ile-	Ala	-	Glu	-Glu-Asp	-Ile-
C4b α' chain ^d	NH ₂ ----	Ala	-Leu	---	Glu	---	Ile	-Leu-	Gln	-	Glu	-Glu-Asp	-Leu-

^a Brackets indicate tentative assignment.

^b ND, not determined.

^c Data from Tack *et al.* (52).

^d Data from Press and Gagnon (53) and gaps were introduced to maximize homology between C3b and C4b α' chains.

DISCUSSION

This work was directed toward obtaining a better understanding of the activation of C5. Since C4 and C3 are structurally similar to C5, it is instructive to compare and contrast the activation mechanism of these proteins with that of C5.

It is proposed that activated C5b has a metastable binding site for C6 (Fig. 2). Activated C5b must associate with C6 within a short period of time after activation or C5b decays and can no longer function in the assembly of the membrane attack complex, C5b-9. Metastable binding sites have been shown to exist in C3b, C4b, C5b, and C5b-7 (54). Recent work has suggested that C3 and C4, as well as the protease inhibitor α_2 -macroglobulin, contain an intrachain thiol ester. After activation, the putative thiol ester becomes readily accessible for reaction with nucleophiles or water. After either transacylation or hydrolysis of the thiol ester, a free sulphydryl group becomes exposed (49, 55-64). Therefore, the metastable states of C3b and C4b are considered to be transition conformers in

which the thiol ester is exposed. Although similar to C3 and C4 in other structural respects, C5 does not contain an intra-chain thiol ester (4, 49, 62, 65).

Accordingly, both C3b and C4b can associate covalently with a wide range of nucleophilic acceptors (57, 59, 63-68), whereas the binding of C5b to zymosan or cell membranes is noncovalent (4, 65). Therefore, the activation state of C5b differs from that of either C3b or C4b.

It is not known what specific chemical or mechanistic features constitute the labile binding site on activated C5b. However, results of circular dichroism analyses from both the far UV region, which monitors polypeptide backbone conformation, and the near UV region, which monitors aromatic amino acids and cystine chromophores, demonstrated that conformational alteration accompanies C5 cleavage. These CD results are qualitatively similar to those observed for C3 and C4 activation (66-68).

C5 has relatively few disulfide bonds. There are three disulfide bonds in C5a, the α chain has 15 half-cystines, and the β chain only 6 half-cystines (Table II). This comparatively low level of stabilizing disulfide bridges may provide a partial explanation for the irreversible conformational change imparted on C5 after cleavage to C5a and C5b. In addition, the relatively low number of disulfide bonds could account for instability of C5 when exposed to chaotropes such as potassium thiocyanate (4, 69).

A conspicuous similarity in activation of C3, C4, and C5 is that all three proteins are activated by limited proteolysis involving a single cleavage site in the respective α chains. Activation gives rise to a new α' chain accompanied by the release of a small activation peptide (e.g. C4a, $M_r = 9,000$; C3a, $M_r = 9,100$; and C5a, $M_r = 11,000$). C5a has about 35% sequence homology with C3a, and C5a is 20% homologous with C4a (70). However, contrary to expectations, the NH₂-terminal portion of the α' chain of C5b exhibits no apparent homology with corresponding sequences published for the α' chains of C4b (52, 53) (Table III).

Electron micrographs of negatively stained C5 indicate that the protein is irregular in shape and contains several lobes

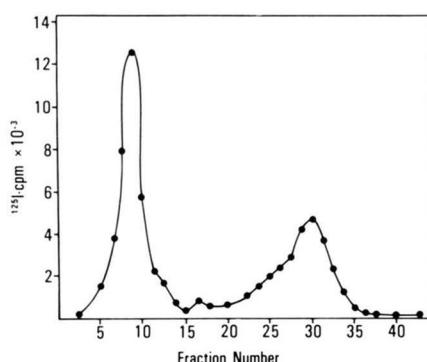


FIG. 7. Gel filtration of C5b and aggregated C5b. ^{125}I -labeled component C5 (1.5 mg/ml in 10 mM Tris-HCl at pH 8.0 and 50 mM NaCl) was activated completely to C5b by a 1:15 molar ratio of CVF_{Bb} to C5 after 12 h at 37 °C. The reaction mixture was applied to a Sephadex S-300 column (2.6 × 95 cm) in 10 mM imidazole/HCl at pH 7.4 containing 350 mM NaCl. The first peak contains about 60% of the C5b in an aggregated form.

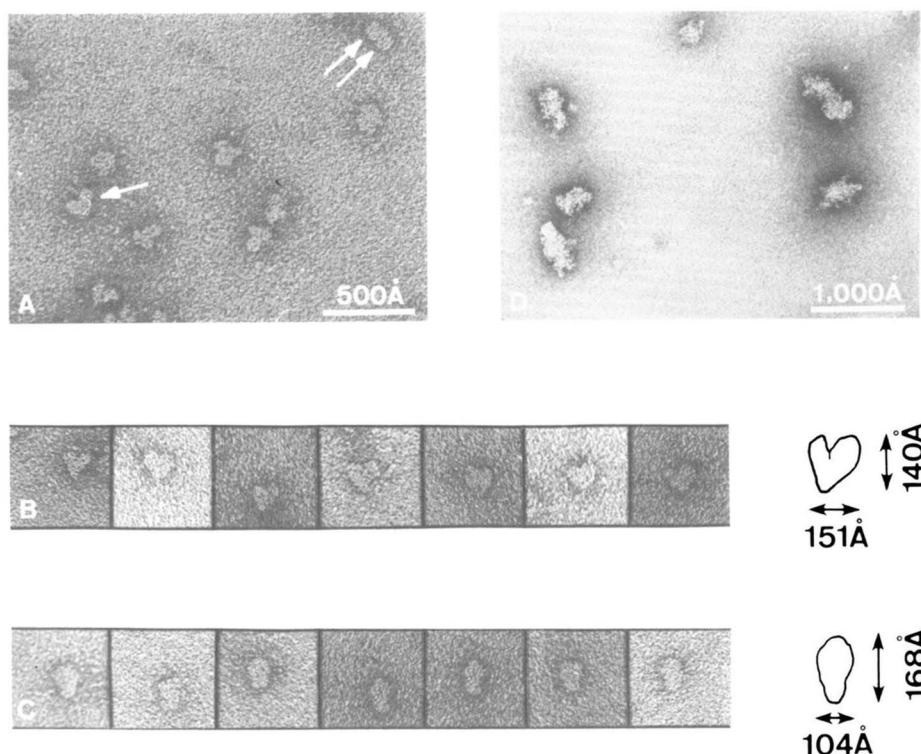


FIG. 8. Electron micrographs of component C5 and aggregated C5b negatively stained with uranyl formate. *A*, a field view of C5 (magnification $\times 260,000$). The arrows indicate two typical projection images which we take to be orthogonal. *B*, a series of one of the selected projection images of C5 (magnification $\times 260,000$). An example of this image in the field view of C5 is marked by the single arrow in *A*. *C*, another series of projection images of C5 (magnification $\times 260,000$). An example of this image in the field view of C5 is shown by the double arrows in *A*. *D*, a field view of negatively stained aggregated C5b (magnification $\times 130,000$).

(Fig. 8). The ultrastructure is consistent with a frictional ratio of 1.45 for this molecule. The conspicuous lobes seen on the images of the C5 molecule suggest the existence of several binding sites or functional domains on the surface of this protein. The ultrastructure of C5 is notably similar to that of C3 (71).

In summary, the structure and activation mechanism of complement component C5 show both similarities and differences when compared with component C3. C5 shares with C3 similarities in chain structure, certain physical properties, amino acid composition, and ultrastructure. Both C3 and C5 are activated by the same complement enzymes, namely C2a and Bb, by limited proteolyses at the α chain positions 77–78 and 74–75, respectively. The activation peptides C5a and C3a are homologous. In contrast, partial NH₂-terminal sequences of the α' chains of C5b and C3b show no similarity. Although both C5b and C3b undergo metastable activation states, the labile conformer of C3b has an exposed thiol ester, whereas C5b does not contain a thiol ester, but rather C5b expresses a transitory binding site that is specific for C6. It is this C5b,6 complex that serves as a foundation for the sequential assembly of the membrane attack complex C5b-9.

Acknowledgments—We gratefully acknowledge the excellent technical assistance of Kevin Ferreri in obtaining amino acid compositions and automated NH₂-terminal sequence analyses. We thank Ellye Lukaschewsky for assistance in preparing the manuscript.

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