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Third Component of Human Complement: Structural Analysis of the Polypeptide Chains of C3 and C3b†

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ABSTRACT: The α- and β-polypeptide chains of human C3 have been isolated after total reduction and alkylation by gel filtration on agarose in the presence of sodium dodecyl sulfate. The amino acid and neutral sugar contents of the C3 protein and each chain are reported. Automated Edman degradation has established amino-terminal sequences for the α and β chains of Ser-Val-Gln-Leu-Thr-Glu-Lys-Arg-Met-Asx-Lys-Val-Gly and Ser-Pro-Met-Tyr-Ser-Ile-Gly-Thr-Pro-Asx, respectively. Carboxypeptidase digestions have identified carboxyl-terminal sequences for the α and β chains of (Ala,Val)-Gly-Ser and Pro-Ala-Ala. Proteolytic cleavage of the α chain, characteristic of C3 activation, was studied with trypsin. The α' chain of the resultant C3b fragment was also isolated after total reduction and alkylation by gel filtration. An amino-terminal sequence of Ser-Asn-Leu-Asp-Glu-Asp-Ile-Ile-Ala-Glu-Glu-Asp-Ile-Val was determined for this chain. The previous proposal concerning the assignment of the C3a anaphylactic peptide to the amino terminus of the C3 α chain [Budzko, D. B., et al. (1971) *Biochemistry 10*, 1166–1172] is substantiated by (1) the identity in the amino-terminal sequence of the C3 α chain and the C3a fragment [Hugli, T. E., et al. (1975) *J. Biol. Chem. 250*, 1472–1478], (2) the appearance of a unique amino terminal associated with the C3b α' chain, and (3) unchanged carboxyl-terminal sequences for the C3b α' and β chains.

The third component of human complement (C3) participates in both the classical and alternative pathways of complement activation. Our present knowledge concerning the physicochemical properties of the native protein, its mode of activation, and specificity of interaction with particle surfaces can be summarized as follows: (1) the molecular weight of C3 has been estimated by Budzko et al. (1971) to be 185 000 using the gel electrophoresis method of Hedrick & Smith (1968) in agreement with the value of 187 650 ± 5650 determined by "low-speed" sedimentation equilibrium (Tack & Prahl, 1976); (2) polyacrylamide gel electrophoresis of C3 under denaturing and reducing conditions has shown that the protein is comprised of two polypeptide chains, α and β, with respective molecular weights of $1.1–1.4 \times 10^5$ and $7.5–8.0 \times 10^4$ (Nilsson et al., 1975; Molenaar et al., 1974; Bokisch et al., 1975; Tack & Prahl, 1976); (3) activation of C3 by the classical pathway C3 convertase, $\overline{C4b,2a}$, is effected by cleavage of a single peptide bond in the α chain releasing an activation peptide

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(C3a) with a molecular weight of 9100 (Bokisch et al., 1969; Nilsson & Mapes, 1973; Hugli et al., 1975) which contracts smooth muscle (Dias Da Silva & Lepow, 1967; Cochrane & Müller-Eberhard, 1968) and releases histamine from mast cells (Dias Da Silva et al., 1967; Johnson et al., 1975); (4) the larger C3b fragment, comprised of an $\alpha'\beta$ chain structure, has been identified as a component of the classical pathway C5 convertase, $\overline{C4b,2a,3b}$, and the alternative pathway C3 and C5 cleaving activities (Müller-Eberhard et al., 1967; Sandberg et al., 1970; Götze & Müller-Eberhard, 1971; Fearon et al., 1973; Medicus et al., 1976); (5) "nascent" C3b is capable of binding to cell surfaces (Müller-Eberhard et al., 1966) and immune aggregates (Miller & Nussenzweig, 1975) by an apparent irreversible process; (6) recent studies by Law & Levine (1977) of C3b binding to sheep erythrocytes and zymosan particles have suggested that C3b is bound to these surfaces through both hydrophobic interactions as well as perhaps an ester-like bond; and (7) C3b attached to a cell or particle surface may interact through stable binding sites with specific cell surface receptors, CR1 (immune adherence receptor) and CR2 (C3d receptor), present on primate erythrocytes, neutrophils, monocytes, macrophages, and eosinophils (Nelson, 1953; Huber et al., 1968; Lay & Nussenzweig, 1968; Bianco et al., 1970; Ross et al., 1973; Ross & Polley, 1975; Gupta et al., 1976; Dierich & Bokisch, 1977).

The participation of the C3 protein in immune surveillance mechanisms (Gigli & Nelson, 1968; Mantovani et al., 1972; Stossel et al., 1975; Wellek et al., 1975; Reynolds et al., 1975; Ehlenberger & Nussenzweig, 1977) and immune response pathways (Pepys, 1972, 1974; Bitter-Suermann et al., 1973; Hartmann & Bokisch, 1975; Sandberg et al., 1975; Koopman et al., 1976; Lewis et al., 1977) has been well documented; however, little information is available concerning the structural correlates of its biological activities. An improved method for the preparation of human C3 has permitted the isolation of gram quantities of protein for structural analyses (Tack & Prahl, 1976). Automated Edman degradation has revealed the presence of two amino-terminal sequences in C3 which is in agreement with the two-chain structure observed on polyacrylamide gels with sodium dodecyl sulfate and 2-mercaptoethanol present. In this report we describe the preparative isolation of the component α and β chains and their structural characterization. In addition, trypsin has been used to mimic the action of the C3 convertase, $\overline{C4b,2a}$, and the molecular parameters of the conversion to C3b investigated. The α' chain of C3b produced by trypsin has been isolated and its amino-terminal structure delineated.

Experimental Procedures

Materials. Platelet-poor plasma from human blood was obtained from the Washington Regional Blood Center of the American National Red Cross and C3 prepared as described previously (Tack & Prahl, 1976). Sepharose 4B and CL-4B were purchased from Pharmacia Chemicals. EDTA,[1] Tris, mannose, fucose, sialic acid, glucosamine, L-rhamnose, and galactose were products of Sigma Chemicals. Guanidine hydrochloride, DTT, 3 N mercaptoethanesulfonic acid, and sequenator reagents were from Pierce Chemical Co. Bovine pancreatic trypsin, soybean trypsin inhibitor, and carboxypeptidases A and B were purchased from Worthington Biochemicals. Sodium dodecyl sulfate was a product of British Drug House Ltd. Bio-Rad Laboratories was the source of AG2-X10 resin (200–400 mesh), polyacrylamide gel electrophoresis reagents, and hydroxylapatite (Bio-Gel HT).

Chain Separation. The reduction and alkylation of C3 was performed in 6 M guanidinium chloride as previously reported (Tack & Prahl, 1976). The sample was then dialyzed against 0.1 M acetic acid to remove salts and finally equilibrated in 0.1 M ammonium bicarbonate (pH 7.9), 1% in sodium dodecyl sulfate. Separation of the α and β chains was effected by downward flow elution on a 2.5 × 160 cm column of Sepharose CL-4B equilibrated with 0.1 M ammonium bicarbonate (pH 7.9), 0.2% in sodium dodecyl sulfate. The flow rate was maintained at 3–4 mL/h with a Pharmacia peristaltic pump and 3.0-mL fractions were collected. Fractions were screened by polyacrylamide gel electrophoresis according to the method of Weber & Osborn (1969).

Sedimentation Equilibrium Analysis of the α and β Chains. Ultracentrifugation studies were carried out in a Beckman Model E analytical ultracentrifuge equipped with photoelectric scanner, multiplexer, and electronic speed control. The "low speed" sedimentation equilibrium method was used to assess the molecular weights of the C3 α and C3 β subunits (Teller, 1973). Each chain was dialyzed against 0.01 M sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl and 0.1% sodium dodecyl sulfate and diluted with dialysate to give solutions of 0.4 and 0.2 absorbance unit/mL at 280 nm (cell path, 1 cm). Equilibrium runs were performed in the six-hole An-G Ti rotor at a speed of 9000 rpm using 12-mm double sector cells equipped with absorption window holders and sapphire windows. The protein concentration (C) was expressed as units of absorbance at 280 nm and the buoyant molecular weight of the protein–sodium dodecyl sulfate complex $M_c(1 - \bar{\nu}_p\rho)$ calculated from the equation

$$M_c(1 - \bar{\nu}_p\rho) = \frac{2RT}{\omega^2}\frac{\mathrm{d}(\ln C)}{\mathrm{d}r^2}$$

where $\mathrm{d}(\ln C)/\mathrm{d}r^2$ was derived from the slope of a ln C vs. r^2 plot. The molecular weight of the protein (M_p), corrected for bound sodium dodecyl sulfate, was obtained by dividing the buoyant molecular weight by the expression $(1 - \bar{\nu}_p\rho)^+ \chi(1 - \bar{\nu}_d\rho)$, where χ is the weight of sodium dodecyl sulfate bound/unit weight of protein and $\bar{\nu}_d$ is the partial specific volume of the detergent (Hersh & Schachman, 1958). A value of 0.863 mL g^{-1} was used for $\bar{\nu}_d$ (Anacher et al., 1964). The partial specific volumes of the α and β chains, $\bar{\nu}_p$, were calculated to be 0.733 and 0.734 mL g^{-1}, respectively, from the corresponding amino acid compositions according to the method of Cohn & Edsall (1943). The value of χ used in these calculations was 0.36 g/g of protein as determined by Poillon et al. (1969) for tryptophan oxygenase using the interferometric procedure of Olins & Warner (1967).

Trypsin Digestion of C3. The C3 protein at 8–10 mg/mL was dialyzed against 0.2 M Tris-HCl buffer (pH 7.4), containing 5 mM EDTA at 4 °C. A stock solution of bovine trypsin at 10 mg/mL in 1 mM HCl was diluted to 1 mg/mL with buffer and added to give a final substrate to enzyme molar ratio of 130:1. After 1 min at 37 °C, soybean trypsin inhibitor was added in a four- to fivefold molar excess with respect to trypsin. The pH of the digest was brought to 4.0 by the dropwise addition of glacial acetic acid; the solution was dialyzed against 0.1 M acetic acid at 4 °C and lyophilized. The lyophylate was dissolved in 0.2 M Tris-HCl buffer (pH 8.2), containing 5 mM EDTA, 20 mM DTT, and 6 M guanidinium chloride. After a 2-h incubation at 37 °C under nitrogen, the sample was alkylated by the addition of iodoacetamide to a final concentration of 42 mM and the incu-

[1] Abbreviations used: EDTA, ethylenediaminetetraacetic acid; DEAE, diethylaminoethyl; Pth, phenylthiohydantoin; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; DTT, dithiothreitol.

bation continued for an additional 30 min at 37 °C. The transfer of the reduced and alkylated protein to the appropriate buffer for separation of the α' and β chains was the same as described earlier for C3 chain isolation.

Carbohydrate Analysis. Samples of C3 and the component α and β chains for neutral sugar analysis were transferred to Pyrex hydrolysis tubes in 0.1 M acetic acid and dried in vacuo over sodium hydroxide. The dried samples were taken up in 2 N HCl and hydrolyzed for 2, 4, and 6 h in sealed and evacuated ($<5 \times 10^{-3}$ torr) tubes at 110 °C. Following hydrolysis, L-rhamnose was added as an internal standard, the hydrolysates were dried in vacuo over sodium hydroxide, and the individual monosaccharides were separated by ion-exchange chromatography as their borate complexes (Khym & Zill, 1952) on a JEOL Model JLC-5AH amino acid analyzer converted to a neutral sugar analyzer (Lee et al., 1969). An orcinol–sulfuric acid reagent was used for the detection and quantitation of neutral sugars. Hexosamine determinations for C3 were performed according to the method of Elson & Morgan (1933). Samples were hydrolyzed in 4 N HCl for 2 h at 100 °C in Pyrex screw-cap culture tubes with Teflon liners. Glucosamine was used as the standard for these determinations. The method of Aminoff (1961) was used for sialic acid determinations following hydrolysis in 0.1 N sulfuric acid for 30 min at 85 °C.

Removal of Sodium Dodecyl Sulfate. It was considered necessary to remove bound sodium dodecyl sulfate from the chains for amino acid analyses, carbohydrate analyses, and carboxypeptidase digestions. The method of Lenard (1971) was used for this purpose as described in the preceding paper (Tack et al., 1979). Control experiments with sodium dodecyl [^{35}S]sulfate confirmed the removal of greater than 98% of the detergent, with recoveries of protein in excess of 95%.

Miscellaneous Methodology. Amino acid analyses were performed on a JEOL 6-AH amino acid analyzer following hydrolysis in 6 N HCl or 3 N mercaptoethanesulfonic acid. Automated Edman degradation of the isolated chains was carried out on a JEOL Model 47K sequence analyzer using a modified double-cleavage program with 0.25 M Quadrol. These methods, as well as those of carboxypeptidase A and Y digestions, have been described in detail in an earlier publication (Tack & Prahl, 1976) or in the preceding paper of this issue (Tack et al., 1979).

Results and Discussion

In a previous communication (Tack & Prahl, 1976), the two-chain structure of human C3 was confirmed both by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and 2-mercaptoethanol and the finding of a double sequence on automated Edman degradation of the reduced and alkylated protein. In order that unambiguous amino- and carboxyl-terminal sequences may be assigned to each chain, and the source of the C3a anaphylactic fragment confirmed, isolation and chemical characterization of the chains were undertaken.

Isolation of C3 Chains. In these studies no attempts were made to achieve a preferential reduction of interchain disulfide bonds, but rather the protein was fully reduced and alkylated in 6 M guanidinium chloride prior to chain separation. Although C3 prepared in this manner was soluble in 1 M acetic acid, resolution of the chains was not obtained by gel filtration on a 2.5 × 160 cm column of G-200 in the presence of this solvent or in 1 M propionic acid, suggesting that the chains remained associated or aggregated. It was not possible to evaluate the larger pore agarose gels as the protein adsorbed to the matrix at this low ionic strength. Efforts to fractionate the chains on hydroxylapatite in 0.1% sodium dodecyl sulfate (Moss & Rosenblum, 1972) or DEAE-cellulose in the presence of 8 M deionized urea proved difficult to reproduce. Gel filtration on Sepharose 4B (or CL-4B) in 6 M guanidinium chloride or 0.1 M ammonium bicarbonate (pH 7.9), containing 0.2% sodium dodecyl sulfate, was most effective. A typical pattern in the presence of the latter denaturant system is shown in Figure 1. As revealed by polyacrylamide gel electrophoresis in sodium dodecyl sulfate, considerable α-chain contamination of the β-chain pool was evident in this particular experiment. These cross-contaminants were effectively removed by repetitive gel filtration, where encountered. The pools were conveniently concentrated by lyophilization and the detergent was removed on an AG2-X10 resin column.

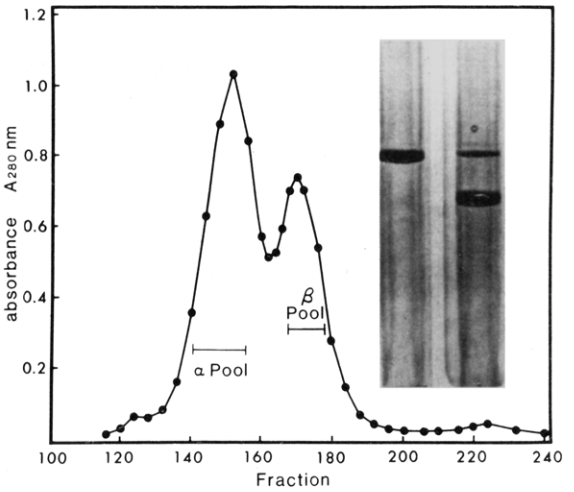

FIGURE 1: Gel filtration of reduced and alkylated human C3 on a 2.5 × 160 cm column of Sepharose CL-4B equilibrated and developed with 0.1 M ammonium bicarbonate (pH 7.9), containing 0.2% sodium dodecyl sulfate. The absorbance at 280 nm is shown. The insert, from left to right, shows the gel electrophoretic patterns in sodium dodecyl sulfate for the α and β chain pools as indicated.

Table I: Buoyant and Calculated Molecular Weights of the C3 α and C3 β Chains by Analytical Ultracentrifugation[a]

run	slope ($d(\ln C)/dr^2$)	buoyant mol wt ($M_c(1-\bar{\nu}_p\rho)$)	calcd mol wt (M_p)	
α chain				
1	0.758	41 826	133 204	133 300 ± 5300
2	0.759	41 882	133 382	
β chain				
1	0.437	24 114	76 796	75 400 ± 3020
2	0.421	23 231	73 984	

[a] The partial specific volumes ($\bar{\nu}_p$) of the α and β chains were calculated to be 0.733 and 0.734 mL g^{-1}, respectively, from amino acid compositional data according to Cohn & Edsall (1943). Runs 1 and 2 for the α and β chains correspond to concentrations of 0.4 and 0.2 absorbance unit/mL at 280 nm.

Molecular Weight Determination by Sedimentation Equilibrium. Weight average molecular weights for the C3 α and C3 β chains were determined by sedimentation equilibrium ultracentrifugation in 0.1% sodium dodecyl sulfate at two protein concentrations using absorption optics and a split-beam photoelectric scanner. Plots of ln C vs. r^2 from equilibrium scans at 280 nm for each chain are shown in Figure 2. A linear least-squares fit of the data was obtained using the NIH M Lab/PDP 10 interactive system described by Knott & Schrafer (1972). The linearity of each plot across the liquid column indicates the monodisperse nature of the complex for each chain produced in the presence of this detergent. Slope values, buoyant molecular weights ($M_c(1-$

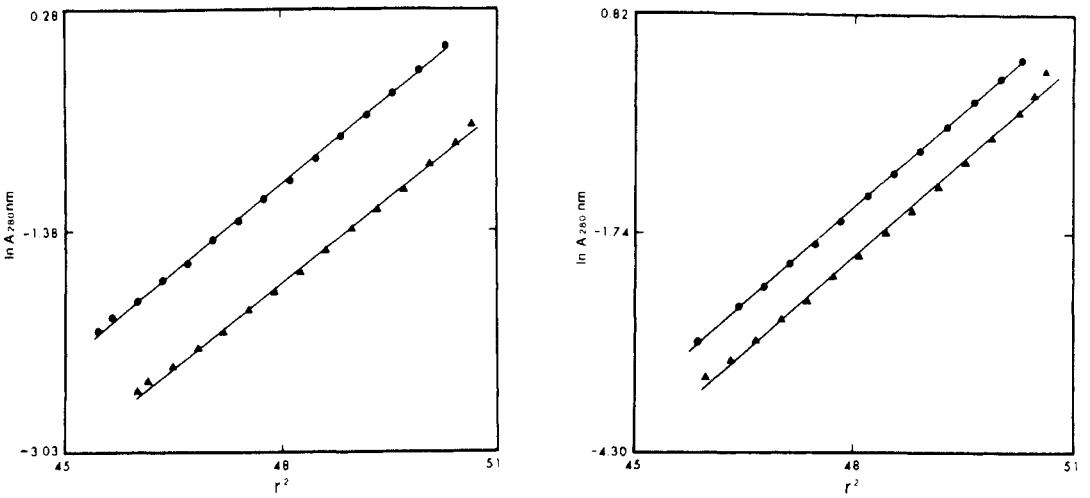

FIGURE 2: The ln A_{280nm} vs. r^2 plots at sedimentation equilibrium for the C3 β (left side) and C3 α (right side) in 0.1% sodium dodecyl sulfate, containing 0.01 M sodium phosphate buffer (pH 7.0) and 0.1 M NaCl. Each chain was sedimented to equilibrium at two concentrations: 0.4 absorbance unit/mL (●) and 0.2 absorbance unit/mL (▲).

$\bar{\nu}_p\rho$)), and calculated molecular weights (M_p) are presented in Table I. The β-chain molecular weight of 75 400 ± 3020 is in excellent agreement with that determined by sodium dodecyl sulfate gel electrophoresis [(7.5–8.0) × 10^4]. The α-chain molecular weight of 133 000 ± 5300 appears a little high but still within the accepted range of (1.1–1.4) × 10^5.

A value of χ of 0.36 g/g of protein was used for these calculations for the following reasons: (1) this value was experimentally determined by Poillon et al. (1969) for tryptophan oxygenase under similar conditions of detergent concentration, ionic strength, and temperature; (2) Piatigorsky et al. (1974) used this value for calculation of the subunit molecular weight of δ-crystallin from embryonic chick lens fibers and this was in good agreement with the molecular weight determined from gel electrophoresis studies in sodium dodecyl sulfate; and (3) the concentration of sodium dodecyl sulfate monomer in a 0.1% (3.5 mM) solution containing 0.01 M sodium phosphate buffer (pH 7.0) and 0.1 M NaCl would be ~1 mM and, therefore, a saturation complex of 0.4 g/g of protein could be expected (Reynolds & Tanford, 1970). It has been recently appreciated that the weight ratio of bound sodium dodecyl sulfate to proteins is not only dependent upon the concentration of detergent and ionic strength (Allen, 1974; Robinson & Tanford, 1975) but also on the nature of the protein itself (Robinson & Tanford, 1975). The latter workers, in a study of porcine liver cytochrome b_5, reported that, whereas the intact protein bound 1.3 g of sodium dodecyl sulfate/g of protein at saturation, the polar and hydrophobic fragments of the protein at saturation bound 0.7 and ~3 g/g, respectively. In that the value of χ in these studies is not an empirical value, the above observations must be taken into consideration.

Amino Acid and Carbohydrate Analyses of C3 Chains. The amino acid compositions of an individual preparation of human C3 and the isolated α and β chains of that preparation are given in Table II. Although most residues fall within 3% of those reported by us earlier (Tack & Prahl, 1976), several exceptions must be noted which include an increase in aspartic acid and a decrease in methionine and tryptophan values of 5, 30, and 17%, respectively. The earlier values of methionine were high due to an integrating problem and now are in better accord with the values reported by Budzko et al. (1971) and Molenaar et al. (1974). The reduced tryptophan value in these studies presumably represents an inherent difficulty with its hydrolytic stability. Comparative perusal of the compositions

Table II: Amino Acid Compositions of Human C3 and the Component α and β Chains[a]

	C3[b]	C3α[c]	C3β[c]	(C3α + C3β)/C3
Lys	116.7	74.0	40.9	0.98
His	27.8	17.7	10.0	1.00
Arg	81.1	51.5	28.8	0.99
S-CMCys	21.8	16.9	4.1	0.96
Asp	152.6	104.2	54.8	1.04
Thr	100.0	57.5	49.7	1.07
Ser	106.7	58.4	50.7	1.02
Glu	219.2	142.5	81.0	1.02
Pro	79.4	43.1	38.1	1.02
Gly	98.9	50.6	47.5	0.99
Ala	98.6	63.7	33.6	0.99
Val	146.2	69.9	72.0	0.97
Met	30.9	21.0	11.6	1.06
Ile	78.9	46.9	32.3	1.00
Leu	152.9	99.8	62.3	1.06
Tyr	55.6	33.8	23.7	1.03
Phe	60.8	36.7	25.1	1.02
Trp	12.0	10.2	2.4	1.05
total residues	1640	998	669	1.02

[a] Reported as moles of amino acid per mole of protein. [b] Calculated on the basis of a carbohydrate-free molecular weight of 185 000 using 1.5 wt % as the estimated carbohydrate content. [c] C3 α and C3 β molecular weights of 115 000 and 75 000, respectively, were uncorrected for carbohydrate content.

of the chains reveals that, on a mol % basis, the β chain is richer in threonine, serine, glycine, and valine, but has reduced levels of aspartic acid, glutamic acid, alanine, half-cystine, and tryptophan. On comparison of the α- and β-chain compositions reported here with those of Taylor et al. (1977), significant variations are apparent in half-cystine and tryptophan values. These differences may be attributable in part to the methods used for quantitation; i.e., half-cystine was determined by us as *S*-carboxymethylcysteine or aminoethylcysteine and tryptophan as the free amino acid following hydrolysis in 3 N mercaptoethanesulfonic acid, whereas Taylor et al. (1977) determined half-cystine as cysteic acid and tryptophan by standard methods following alkaline hydrolysis according to Hugli & Moore (1972). In view of the calcium binding ability of C3, Dr. Gary Nelsesteun (Department of Biochemistry, University of Minnesota) examined a hydrolysate of C3 following reduction with [^{3}H]diborane (Zytokovicz & Nelsesteun, 1975) for the presence of 5,5′-[^{3}H]dihydroxyleucine, the reduction product of γ-carboxyglutamic acid. These

Table III: Amino-Terminal Sequences of C3 α, C3 β, and C3b α′ Chains[a]

C3 α:	Ser-Val-Gln-Leu-Thr-Glu-Lys-Arg-Met-Asx-Lys-Val-Gly-X-Tyr
C3 β:	Ser-Pro-Met-Tyr-Ser-Ile-Gly-Thr-Pro-Asx
C3b α′:	Ser-Asn-Leu-Asp-Glu-Asp-Ile-Ile-Ala-Glu-Glu-Asp-Ile-Val-X-Arg-Ser-Glx-Phe-Ile

[a] Derived from automated Edman degradation. Methods of identification and quantitation are available as supplementary material.

studies have indicated an absence of this residue in the C3 protein, in agreement with the observations of Hauschka (1977).

Neutral sugar analysis of human C3 revealed the presence of mannose, fucose, and trace amounts of galactose. Maximal values of mannose and fucose observed on time course hydrolysis with 2 N HCl were 0.8 and 0.3 wt %, respectively. The α and β chains were found to contain both neutral sugars; the mannose and fucose content of the α chain was 1.2 and 0.6 wt %, and that of the β chain 0.4 and 0.2 wt %, respectively. The content of neutral sugars, determined independently for the component chains, compared favorably with that observed for the C3 protein. The method of Elson & Morgan (1933) was used to determine the level of amino sugars in C3; five individual assays established a range for hexosamine content from 0.24 to 0.35 wt %. Assay by the method of Aminoff (1961) for sialic acid failed to reveal the presence of this sugar residue. On consideration of the sensitivity of the assay, ≥5 μg of sialic acid, the maximal amount of sialic acid which could be present in C3 would be <0.01 wt %. In view of the hypothesis that terminal sialic acid residues are important in slowing the hepatic clearance of glycopeptides by a galactose-specific receptor (Ashwell & Morell, 1974), this finding was initially unexpected, although the subsequent identification of only trace amounts of galactose in C3 obviates the issue.

Amino and Carboxyl Termini of the C3 Chains. The α and β chains of human C3 were subjected to automated Edman degradation commencing with 50–100-nmol samples, and the derived sequences are shown in Table III. Methods of identification and quantitation for the Pth derivatives are available as supplementary material (see paragraph at the end of this paper concerning supplementary material). Due to the large size of these proteins and problems with maintaining a uniform film in the spinning cup, we were unable to go beyond residue 15 in the α chain, with an unidentified residue at position 14, and 10 residues in the β chain. The double sequence that had been observed with C3 (Tack & Prahl, 1976) could be clearly identified as the sum of the two chains. The amino-terminal sequence of the C3 α chain was in agreement with that reported by Hugli et al. (1975) for the C3a anaphylatoxin, supporting the earlier suggestion that the amino-terminal end of this chain was the source of this activation fragment, following the action of trypsin on C3 (Budzko et al., 1971).

The ability of carboxypeptidase A (CpA) to release 1.0 mol of alanine/mol of C3, and a mixture of CpA and carboxypeptidase B (CpB) to release 1.32 mol of alanine/mol of C3 in 0.1% sodium dodecyl sulfate was previously reported (Tack & Prahl, 1976). When the α chain of C3 was subjected to CpB or mixed CpA/CpB digestion in the presence of detergent, only 0.1 mol of alanine/mol of chain was released; neither CpA nor carboxypeptidase Y (CpY) alone released

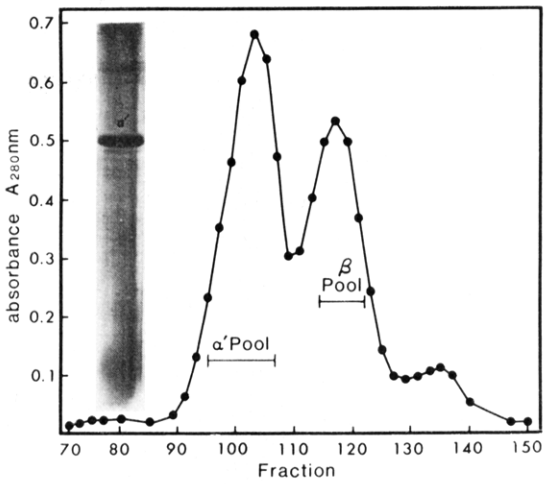

FIGURE 3: Gel filtration of reduced and alkylated C3b on a 2.5 × 160 cm column of Sepharose CL-4B equilibrated and developed with 0.1 M ammonium bicarbonate (pH 7.9), containing 0.2% sodium dodecyl sulfate. The insert shows the gel electrophoretic pattern of the α′ chain pool as indicated.

detectable amounts of amino acids in the presence of detergent. In contrast, digestion of the β chain with CpA, CpB, or mixed CpA/CpB released 0.96–1.01 mol of alanine/mol of chain in the presence of 0.1% sodium dodecyl sulfate. Digestion with CpY increased the amount of alanine to 1.10 mol/mol of β chain. If sodium dodecyl sulfate was first removed from the protein (Lenard, 1971) and omitted from the digest buffer, the results of CpA digestions were quite different. CpA was now able to release both serine and glycine from the α chain in ratios of 0.48 and 0.28 mol/mol of chain, respectively. With CpY, in addition to serine and glycine, both valine and alanine were identified in ratios of 0.80, 0.48, 0.34, and 0.32 mol/mol of chain. A tentative carboxyl-terminal sequence for the C3 α chain of (Ala,Val)-Gly-Ser is proposed. The alanine observed on digestion with CpB is believed due to low levels of contamination with β chain and/or endopeptidase activity in the commercial CpB preparation. Although digestion of the β chain with CpA in the absence of detergent still resulted in the release of 1.0 mol of alanine/mol of chain, when CpY was used, the amount of alanine released increased to 1.42 mol/mol with a trace of proline at low CpY concentrations (240:1 molar ratio) and to 2.20 mol of alanine and 1.14 mol of proline/mol of β chain at higher CpY concentrations (50:1 molar ratio). A tentative sequence of Pro-Ala-Ala is proposed for the C3 β chain. The presence of a proline residue in the third position is assumed responsible for the inability of CpA to go beyond the carboxyl-terminal alanine in either the presence or absence of detergent.

Tryptic Digestion of C3 and Characterization of the Products. The activation of C3 by either the classical or alternative pathway C3 convertase is coincident with the proteolytic cleavage of the α chain giving rise to the C3a and C3b fragments with respective molecular weights of 9100 and 180 000 (Bokisch et al., 1969). Activation of C3 may be studied with trypsin, with the elaboration of anaphylactic activity and a similar pattern of fragments on polyacrylamide gel electrophoresis in sodium dodecyl sulfate (Bokisch et al., 1969; Nilsson et al., 1975). Although Bokisch et al. (1969) reported that a 13:1 molar ratio of C3 to trypsin would effect a complete conversion of C3 to C3a and C3b within several minutes, we have found that a 130:1 molar ratio of C3 to trypsin would effect a stoichiometric conversion within 1–2 min. The C3a fragment produced at the higher trypsin concentrations was a mixture of residues 1–77 and 1–69

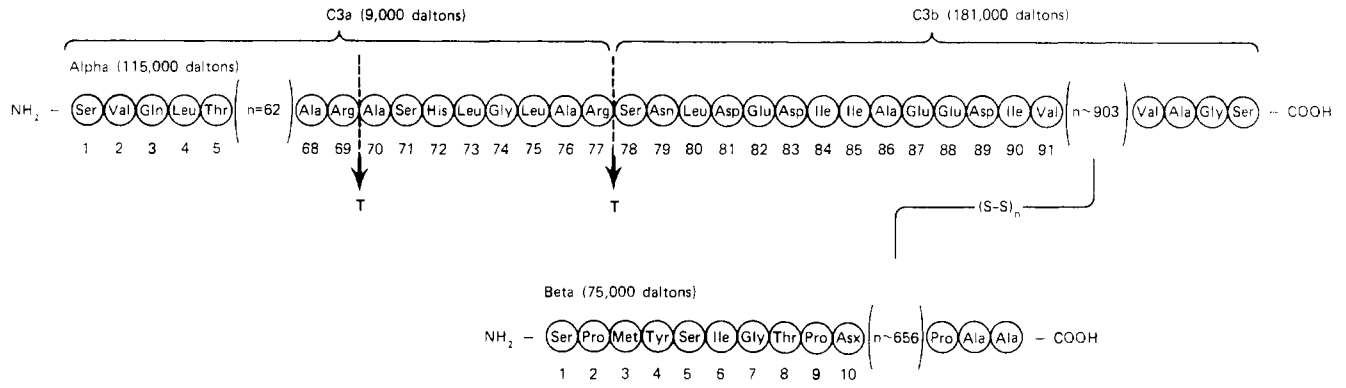

FIGURE 4: Human C3 polypeptide chain structure; initial bovine trypsin (T) cleavage sites in the α chain.

(numbering of Hugli, 1975). While the C3a formed at the lower trypsin concentrations still contained the 1–69 species, the lower level was preferred here in order to reduce the possibility of secondary cleavages in C3b.

A trypsin digest of C3 (130:1 molar ratio) was desalted against 0.1 M acetic acid, lyophilized, and taken up in 6 M guanidinium chloride (pH 8.2) for reduction and alkylation as described in detail earlier for native C3. Separation of the C3b α' and β chains on a Sepharose CL-4B column in 0.2% sodium dodecyl sulfate is shown in Figure 3 together with a polyacrylamide gel of the resultant α' pool. When subjected to automated Edman degradation, a sequence unrelated to that of the α chain was determined as shown in Table III. The carboxyl terminus of the α' chain was identical with that of the α chain as revealed by carboxypeptidase digestion, thereby establishing that the cleavage site of trypsin (and presumably the classical and alternative pathway C3 convertases) is located exclusively near the amino terminus of this chain. Unchanged amino and carboxyl termini of the β chains of native C3 or trypsin–C3b is in accord with the restriction of proteolytic effects to the α chain (Bokisch et al., 1975).

An effort was made to examine a trypsin digest of C3 (65:1 molar ratio) for small peptide material which might emanate from the inter-C3a/C3b region. The digestion of 100 mg of C3 was stopped after 1 min at 37 °C by the addition of a threefold weight excess of soybean trypsin inhibitor and the pH rapidly dropped to 4.0 with glacial acetic acid. The digest was applied to a 2.5 × 160 cm column of Sephadex G-75 equilibrated with 0.15 M sodium acetate buffer (pH 3.7), containing 0.15 M NaCl according to the procedure of Bokisch et al. (1969). The included volume of the column following elution of the C3a fragment, up to 120% of the total column volume, was pooled and lyophilized. An aliquot was removed, hydrolyzed in 6 N HCl, and examined on the amino acid analyzer. There were no amino acids seen to approach molar amounts based on the amount of C3 digested, although the C3a derived peptide comprising residues 70–77 was identified in less than 25% of theoretical yield, suggesting that the tryptic activation was not accompanied by a stoichiometric yield of a small inter-C3a/C3b peptide.

A model for the structure of human C3 based on the sequence information derived here and by Hugli (1975) is shown in Figure 4. The amino termini of the α chain and C3a are identical. The carboxyl terminus of C3a and the amino terminus of the α' chain of C3b are proposed to be contiguous in view of our inability to identify the release of inter-C3a/C3b peptide material in molar yield. Clearly in the absence of overlap data, this alignment must be considered tentative.

As represented then, the site of initial trypsin attack is seen as involving the cleavage of the Arg-Ser bond in position 77–78, with secondary cleavage at the Arg-Ala bond at position 69–70. It is interesting that, whereas the C3a fragment is markedly basic, containing 17 lysine and arginine residues in 77 residues, the amino terminus of the α' chain reveals 6–7 aspartic/glutamic acid residues in the 20-residue stretch sequenced. The tendency of C3a to remain associated with the C3b fragment at pH 7.4 and physiological ionic strengths was first reported by Bokisch et al. (1969). In our hands dissociation can be effected at neutral pH by increasing the sodium chloride concentration from 0.15 to 1.0 M, suggesting the contribution of ionic bonds to the association of these two fragments. It is tempting to speculate that the tightly coiled C3a portion of the α chain might be folded back onto itself, interacting with the acidic portions of the α' amino terminus, thereby looping out the region containing residues 77–78 which appear to be exquisitely susceptible to proteolytic attack. Until tested, however, such considerations remain speculative.

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Supplementary Material Available

Tables indicating the methods of identification and quantitation of Pth derivatives (3 pages). Ordering information is given on any current masthead page.

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