Amino-Terminal Sequence of Human Factor B of the Alternative Complement Pathway and Its Cleavage Fragments, Ba and Bb†

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ABSTRACT: Human factor B has been purified in 25% yield by using an improved isolation procedure based on $(NH_4)_2SO_4$ precipitation and CM-Sephadex, hydroxylapatite, and QAE-Sephadex chromatography. Chemical purity was ascertained by analytical polyacrylamide gel electrophoresis. The preparation was also shown to be hemolytically active. Factor B could be cleaved into Ba and Bb with factor $\bar{D}$ in the presence of either cobra venom factor or C3b. The Bb fragment was purified and isolated by DEAE-cellulose chromatography as well as preparative polyacrylamide gel electrophoresis. The Ba fragment was purified and isolated by high-pressure liquid chromatography as well as preparative polyacrylamide gel electrophoresis. The amino acid compositions of factor B, Ba, and Bb were determined. Trypsin cleavage of factor B was also attempted. The number and size of the resultant cleavage peptides were dependent on the ionic strength of the digestion buffer. Digestion in high ionic strength buffer gave rise to predominantly one fragment of 58 000 daltons, while digestion in low ionic strength buffer gave rise to predominantly two fragments of 58 000 and 44 500 daltons. Automated Edman degradation has established an identical extended NH_2-terminal sequence for factor B and Ba. This overall sequence was deduced to be Thr-Pro-Trp-His-Leu-Ala-Arg-Pro-Gln-Gly-X-Cys-X-Leu-Glu-Gly-Val-Glu/Val-Ile-Lys-Gly-Gly-His-Phe-X-Leu-Leu-X-Glu-X-X-Ala-Leu-Glu-Tyr-Val-. Some heterogeneity was detected, but the Ba fragment is clearly derived from the NH_2 terminal of native factor B. In addition, an NH_2-terminal sequence of Lys-Ile-Val-Leu-Asp-Pro-X-Gly-X-Met-Asn-Ile-Tyr-Leu-Val-Leu-Asp-Gly- was determined for Bb. The present sequence data do not reveal any significant structural similarities among the NH_2 termini of human factor B, Ba, or Bb with any of the known serine proteases.

Factor B is a key complement protein. During alternative pathway activation, a single arginyllysyl peptide bond on factor B is cleaved by the catalytic action of factor $\bar{D}$ (Stroud et al., 1979; Lesavre et al., 1979) in the presence of C3b. As a result, two unequal fragments are generated, designated Bb and Ba (Götze & Müller-Eberhard, 1971). The Bb fragment carries the active site of the protein (Götze & Müller-Eberhard, 1971). It forms an integral part of the alternative pathway C3 ($\overline{C3bBb}$) and C5 [$\overline{(C3b)_nBb}$] convertases. These enzymes are responsible for the cleavage of C3 and C5, respectively, to their functionally active fragments, C3a and C3b and C5a and C5b. Factor B, therefore, not only regulates the critical formation of C3b but also is responsible for initiating the activation of the late-acting complement components.

The only complement fragments to be totally sequenced up to now have been C3a (Hugli, 1975) and C5a (Fernandez & Hugli, 1976). In addition, most of the sequence of the A, B, and C chains of C1q (Reid & Thompson, 1978; Reid, 1979) has been determined. Partial NH_2-terminal sequences of other classical complement components including the a and b chains of $\overline{C1s}$ as well as the b chain of $\overline{C1r}$ (Sim et al., 1977), the α, β, and γ chains of C4 (Bolotin et al., 1977; Andrews & Capra, 1978), the α (C3a) and β chains of C3 (Tack et al., 1979b), and the α chain (C5a) of C5 (Tack et al., 1979a) have also been published. The only alternative complement components to be sequenced so far, however, are the NH_2 termini of factor $\bar{D}$ (Davis et al., 1979a,b; Volanakis et al., 1980) and Bb (Niemann et al., 1978; Lesavre et al., 1979).

In an attempt to relate the structure of factor B with its biological activity, we are currently trying to determine as much of its primary amino acid sequence as possible. The biological aspects of factor B which we are particularly interested in relating to its amino acid structure are (1) the factor $\bar{D}$ cleavage region, (2) the substrate binding site of the Bb fragment, and (3) peptides that might have either chemotactic (Hamuro et al., 1978) or macrophage spreading (Götze et al., 1979; Bianco et al., 1979) activity as has been attributed to the Ba and Bb cleavage products, respectively, in other systems. We are also interested in correlating, if possible, the amino acid sequence of factor B with its reported genetic polymorphism as well as substantiating its hypothesized structural similarities with other functionally analogous proteins, such as C2. As a first step toward this goal, therefore, we report here the extended NH_2-terminal amino acid sequence of human factor B as well as its cleavage fragments, Ba and Bb.

Experimental Procedure

Materials

Serum. Outdated plasma was obtained from the Birmingham Red Cross and used immediately or clotted and stored as serum at −20 °C.

Antiserum. Antiserum was raised in rabbits against highly purified human factor B according to the schedule of Livingston (1974). On immunoelectrophoresis this antiserum gave a single β-mobility precipitation arc with pure factor B as well as whole human serum.

Other Materials. CM-Sephadex C-50 and QAE-Sephadex A-50 were obtained from Pharmacia. DEAE-cellulose (DE52) was obtained from Whatman. Electrophoretic grade acrylamide, *N*,*N*′-methylenebis(acrylamide), $NaDodSO_4$,[1] sucrose,

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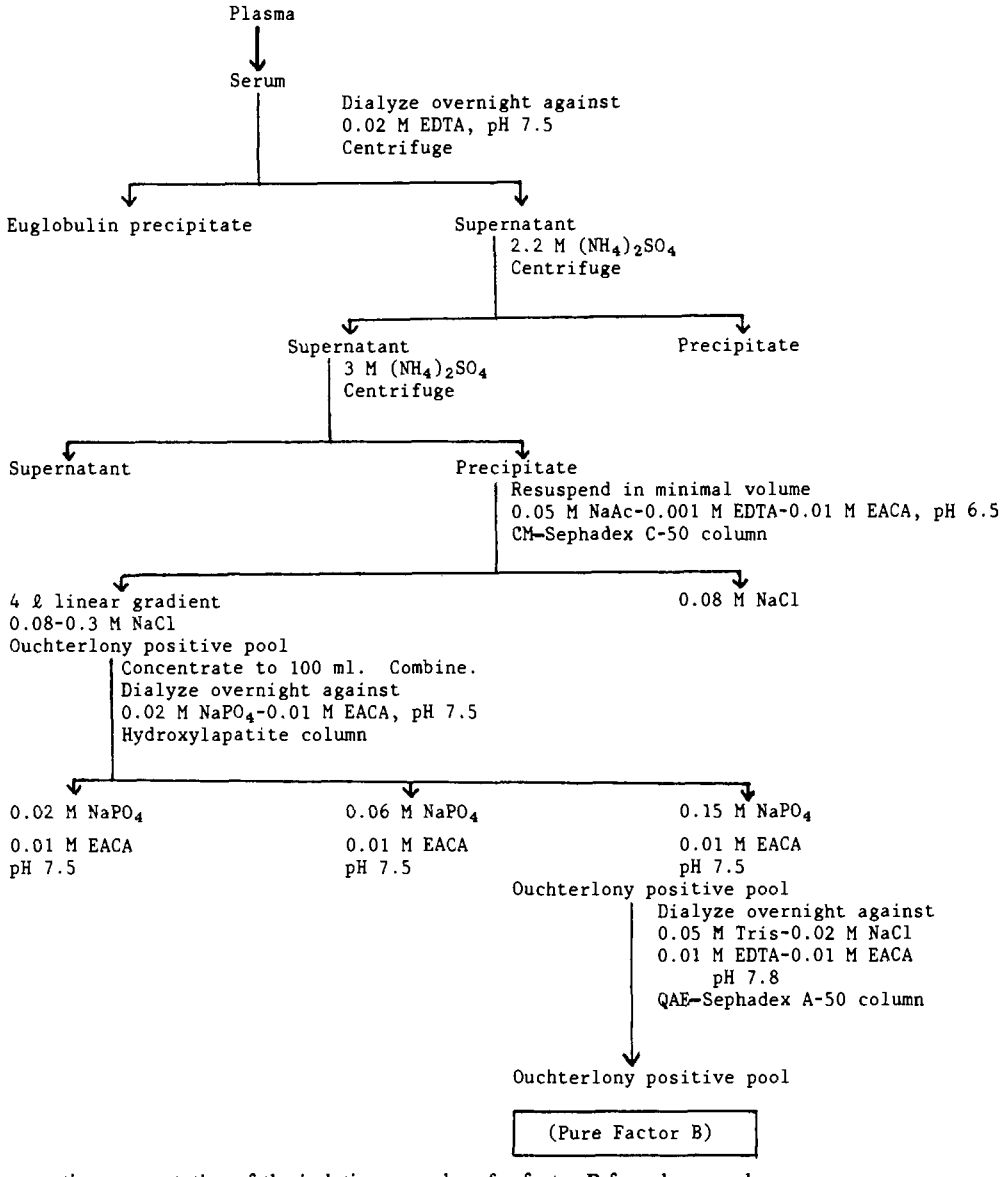

FIGURE 1: Diagrammatic representation of the isolation procedure for factor B from human plasma.

and Bio-Gel HTP (hydroxylapatite) were obtained from Bio-Rad. All column supports were prepared according to the instructions supplied by the manufacturer. Temed (*N,N,N',N'*-tetramethylethylenediamine) was obtained from Eastman. TosPheCH_2Cl–trypsin and soybean trypsin inhibitor were purchased from Worthington. All other chemicals were reagent grade or of the highest quality available.

Methods

Isolation of Factor B. The isolation procedure for a typical preparation of factor B from human plasma is outlined in Figure 1 and Table I. All operations were performed at 4 °C, and all buffers contained ϵ-aminocaproic acid to minimize the degradation of factor B. Initially, ~3.5 L of plasma was clotted and the serum euglobulins were precipitated by low ionic strength dialysis against 0.02 M EDTA, pH 7.5. After centrifugation, solid $(NH_4)_2SO_4$ was added to the supernatant. The fraction which precipitated between 2.2 and 3 M was redissolved in a minimal volume of 0.05 M sodium acetate–0.01 M ϵ-aminocaproic acid–0.001 M EDTA, pH 6.5, and dialyzed extensively against the same buffer. The sample was then centrifuged before application to a 5 × 30 cm CM-

Table I: Purification of Factor B

fraction	total protein[a] (mg)	yield[b] (%)	purifn (x-fold)
A			
serum	66 125	100.0	
supernatant after euglobulin precipitation	61 110	109.6	1.19
3 M $(NH_4)_2SO_4$ precipitation	19 224	65.4	2.25
CM-Sephadex pool	255	62.4	161.86
B			
serum	113 750	100.0	
supernatant after euglobulin precipitation	61 020	90.8	1.69
3 M $(NH_4)_2SO_4$ precipitation	17 100	58.7	3.90
CM-Sephadex pool	392	54.5	158.29
A + B			
hydroxylapatite pool	338	48.2	126.97
QAE-Sephadex pool	123	25.2	182.64

[a] Based on $E_{1\,cm}^{1\%} = 20$ at 280 nm. This value was verified for factor B by amino acid analysis. [b] Measured by a hemolytic tube assay using rabbit erythrocytes.

[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; LC, high-performance liquid chromatography; M_r, relative molecular weight; TosPheCH_2Cl, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; EDTA, ethylenediaminetetraacetic acid; PhNCS, phenylthiohydantoin; TLC, thin-layer chromatography; iPr_2P-F, diisopropyl fluorophosphate.

Sephadex C-50 column equilibrated with the same acetate buffer. The column was washed with 2 L of the above acetate buffer containing 0.08 M NaCl to remove unbound protein and then eluted with a 4-L linear gradient of 0.08–0.3 M NaCl in the same buffer. The factor B enriched pool was concentrated to ~100 mL and stored at −20 °C until another 3.5 L of plasma was processed to this point. The two CM-Sephadex pools were combined, equilibrated with 0.02 M sodium phosphate–0.01 M ε-aminocaproic acid, pH 7.5, centrifuged, and applied to a 5 × 10 cm hydroxylapatite column equilibrated with the same phosphate buffer. After the column was washed with this buffer until the A_{280} was approximately zero, it was eluted successively with 0.06 M sodium phosphate–0.01 M ε-aminocaproic acid, pH 7.5, and 0.15 M sodium phosphate–0.01 M ε-aminocaproic acid, pH 7.5, buffer. Factor B emerged with the latter buffer. These fractions were pooled and dialyzed against 0.05 M Tris–0.01 M ε-aminocaproic acid–0.01 M EDTA–0.02 M NaCl, pH 7.8, buffer. The sample was chromatographed isocratically on a 5 × 15 cm QAE-Sephadex A-50 column equilibrated with the same Tris buffer. Yields of pure factor B were routinely ~25%, which is a substantial improvement over previously published procedures (Boenisch & Alper, 1970b; Hunsicker et al., 1973; Curman et al., 1977).

Other Complement Components. Purified C3b, generated by the enzymatic action of $\overline{C4b2a}$ on C3, was a generous gift of Dr. Robert M. Stroud. Cobra venom factor and partially purified factor $\bar{D}$ were prepared as previously described (Volanakis et al., 1977). Additional cobra venom factor was a generous gift of Dr. Brian J. Johnson.

Immunochemical Methods. At each step of its purification factor B was monitored immunochemically by using the immunodiffusion technique of Ouchterlony (1958) and quantitated by using the single radial immunodiffusion method (Mancini et al., 1965).

Hemolytic Assay. The hemolytic agarose diffusion plate assay with guinea pig erythrocytes (Martin et al., 1976) or a modified tube assay using rabbit erythrocytes (Platts-Mills & Ishizaka, 1974) was employed for quantitative measurement of the functional activity of factor B during the isolation procedure. In the tube assay factor B activity was determined to be that concentration of sample which produced 50% lysis of 10^7 rabbit erythrocytes.

Cleavage of Factor B. Factor B was hydrolyzed by factor $\bar{D}$ in the presence of cobra venom factor or C3b as described by Volanakis et al. (1977). A weight ratio of 1(factor $\bar{D}$):50(cobra venom factor) or 5(C3b):50(factor B) was found to be optimal, that is, the minimal amount of factor $\bar{D}$ and cobra venom factor or C3b required for maximum cleavage of factor B. Cleavages were performed for 2 h at 37 °C. Trypsin cleavage of factor B was also performed according to the method of Curman et al. (1977).

Purification of Factor B Cleavage Fragments. Bb was purified from cleaved factor B preparations by DEAE-cellulose (DE52) chromatography according to the method of Götze & Müller-Eberhard (1971). Ba was obtained by LC (Niemann et al., 1979). Most of the Bb and residual factor B was insoluble in the LC solvent (20% acetic acid) and could be removed by centrifugation.

Polyacrylamide Gel Electrophoresis. At each step in its isolation the purity of factor B was followed by analytical polyacrylamide gel electrophoresis in the presence as well as the absence of $NaDodSO_4$. In addition, the ultimate purity of each preparation was checked by 5–20% gradient slab gel electrophoresis prepared according to the method of Laemmli (1970). Reduction of proteins was carried out with 2% β-mercaptoethanol. Bovine serum albumin (M_r 68 000), ovalbumin (M_r 45 000), and cytochrome *c* (M_r 12 500) were run as molecular weight markers. Protein bands were visualized after Coomassie Blue R-250 staining. Alkaline (pH 8.6) gels were also run in the absence of $NaDodSO_4$ to monitor purification. The identity of factor B on these gels was ascertained on an unstained gel by immunofixation using specific factor B antiserum.

Preparative quantities of the factor B cleavage fragments, Ba and Bb, were resolved and eluted from 3-mm 5–20% $NaDodSO_4$ gradient slab gels. In this procedure, a small aliquot of the mixture to be separated was first iodinated with chloramine-T in phosphate buffer, pH 7.5, containing 1% $NaDodSO_4$ (Hunter & Greenwood, 1962; Bhown et al., 1980). Trace quantities of the iodinated proteins [$(1–2) \times 10^6$ cpm] were mixed with a large excess (~1 mg) of unlabeled sample and applied to each preparative gel. Following electrophoresis at 10 °C, the gel was cut into four 3-cm vertical strips, frozen, and sliced into 1-mm horizontal sections. The slices were counted, and the appropriate fractions were pooled, minced, and subjected to electrodialysis to elute each of the proteins of interest from the gel (Bhown et al., 1980).

Elution of Preparative $NaDodSO_4$–Polyacrylamide Gradient Slab Gels. Elutions were performed in an Isco Model 1750 concentrating apparatus (Bhown et al., 1980). Electrophoresis was performed at 1 W (1 sample cup/apparatus) or 3 W (2 sample cups/apparatus) for 3 h at 4–6 mA. Following electrophoresis, the buffer layers were carefully removed and the samples combined and lyophilized.

Amino Acid Analysis. Samples for amino acid analysis were hydrolyzed in vacuo in 6 N HCl, at 110 °C, for 20 h and analyzed on an updated Durrum D-500 amino acid analyzer (Benson, 1972). Analyses were not corrected for threonine, serine, or tyrosine destruction. Tryptophan was not determined.

Sequencing. Edman degradations were performed in a Beckman Model 890C sequencer employing the slightly modified (Mole et al., 1977) low Quadrol program of Brauer et al. (1975). Between 10 and 20 nmol of each protein was required to sequence between 20 and 40 NH_2-terminal amino acid residues. Repetitive yields were generally between 96 and 99%. All sequences were determined at least twice on different preparations to substantiate each assigned amino acid residue. PhNCS amino acids were identified by at least two independent methods, usually LC and TLC. Because of the high glycine concentration in the $NaDodSO_4$ electrophoresis buffer, samples isolated from gels were run through five to six cycles without the addition of heptafluorobutyric acid before being sequenced.

Results

Purity of the Isolated Factor B. Figure 2 demonstrates that factor B after QAE-Sephadex chromatography was of high purity since it gave rise to a single protein-staining band on electrophoresis both in the presence and in the absence of $NaDodSO_4$.

Cleavage of Factor B. As shown in Figure 3, factor B was converted to Bb and Ba by the catalytic action of factor $\bar{D}$ in the presence of cobra venom factor as well as C3b. Cleavage occurred only in the presence of both factor $\bar{D}$ and cobra venom factor or C3b. No significant cleavage of factor B was detected when it was either incubated alone or in the presence of only factor $\bar{D}$, cobra venom factor, or C3b.

Factor B was also subjected to limited proteolytic digestion by trypsin to examine if fragments similar to those produced

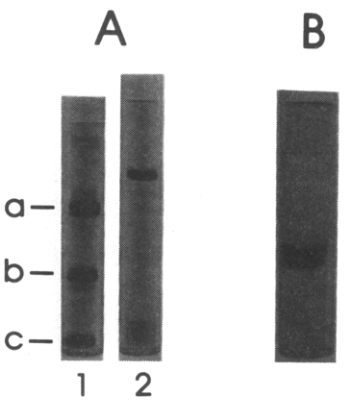

FIGURE 2: Electrophoresis of factor B after QAE-Sephadex chromatography. (A) $NaDodSO_4$ gels of (1) molecular weight standards [(a) bovine serum albumin, 68 000; (b) ovalbumin, 48 000; (c) cytochrome *c*, 12 500] and (2) a typical factor B preparation. (B) Alkaline (pH 8.6) gel of same factor B preparation.

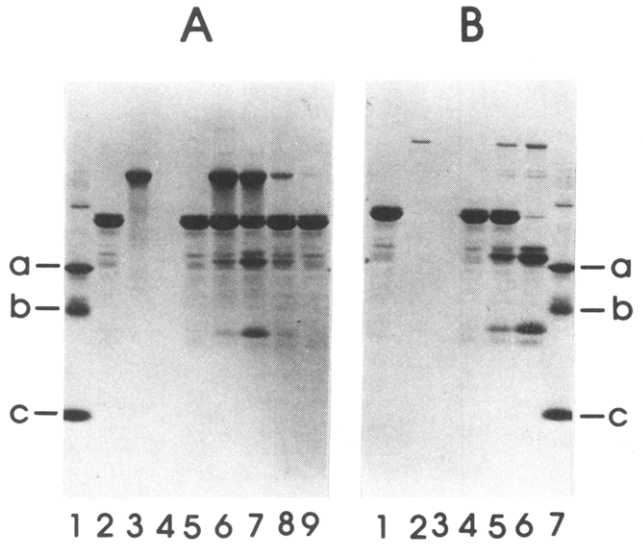

FIGURE 3: Cleavage of factor B by factor $\bar{D}$. (A) In the presence of cobra venom factor. $NaDodSO_4$ electrophoresis of (1) molecular weight standards, (2) factor B, (3) cobra venom factor, (4) factor $\bar{D}$, (5) factor B + factor $\bar{D}$, (6) factor B + cobra venom factor, (7) factor B + factor $\bar{D}$ + cobra venom factor, (8) factor B + factor $\bar{D}$ + cobra venom factor (0.625 μg), and (9) factor B + factor $\bar{D}$ + cobra venom factor (0.0625 μg). (B) In the presence of C3b. $NaDodSO_4$ electrophoresis of (1) factor B, (2) C3b, (3) factor $\bar{D}$, (4) factor B + factor $\bar{D}$, (5) factor B + C3b, (6) factor B + factor $\bar{D}$ + C3b, and (7) molecular weight standards. Electrophoresis was performed under nonreducing conditions after a 2-h incubation at 37 °C. The anode was at the bottom. Unless otherwise indicated, the amount of protein on the gel was the following: factor B = 6.25 μg; cobra venom factor = 6.25 μg; C3b = 0.625 μg; factor $\bar{D}$ = 0.125 μg. a = bovine serum albumin; b = ovalbumin; c = cytochrome *c*.

by factor $\bar{D}$ cleavage in the presence of cobra venom factor or C3b could be generated. Figure 4 shows that factor B is very sensitive to trypsin in both low (5 mM $NaPO_4$) and high (0.2 M NH_4HCO_3) ionic strength alkaline (pH 8.0) buffers. After only a brief period of exposure to this enzyme, two main fragments were generated with apparent molecular weights corresponding to Bb and Ba as determined by $NaDodSO_4$ electrophoresis. The 63 000-dalton protein-staining band just above "e" in Figure 4 coelectrophoresed with highly purified Bb. It appears almost immediately (at 1 min) in both buffers but is apparently further degraded by 15 min to a smaller (M_r 58 000) molecular weight species which seems to be relatively resistant to further digestion (protein-staining band "e" in Figure 4). The 33 000-dalton protein-staining band "g" in Figure 4 similarly coelectrophoresed with highly purified Ba.

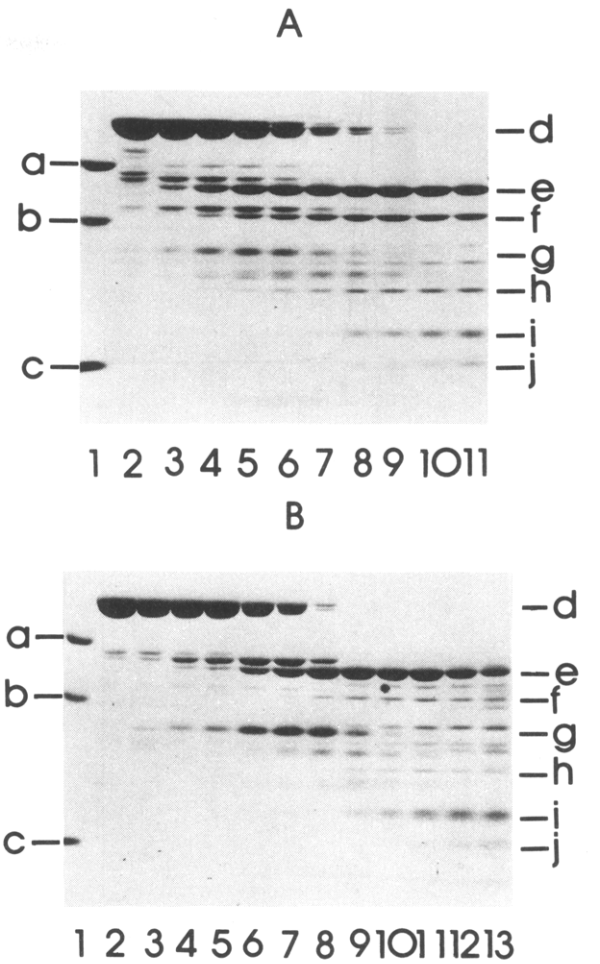

FIGURE 4: Cleavage of factor B by trypsin. (A) $NaDodSO_4$ electrophoresis of factor B incubated for the following times with trypsin in 5 mM $NaPO_4$, pH 8.0: (1) molecular weight standards; (2) 0 time; (3) 1 min; (4) 5 min; (5) 10 min; (6) 15 min; (7) 30 min; (8) 45 min; (9) 60 min; (10) 90 min; (11) 120 min. (B) $NaDodSO_4$ electrophoresis of factor B incubated for the following times with trypsin in 0.2 M NH_4HCO_3, pH 8.0: (1) molecular weight standards; (2) unincubated factor B; (3) factor B incubated for 120 min without trypsin; (4) 0 time; (5) 1 min; (6) 5 min; (7) 10 min; (8) 15 min; (9) 30 min; (10) 45 min; (11) 60 min; (12) 90 min; (13) 120 min. The anode was at the bottom. Molecular weights: a = 68 000 (bovine serum albumin); b = 45 000 (ovalbumin); c = 12 500 (cytochrome *c*); d = 93 000 (factor B); e = 58 000 (Bb′, smaller molecular weight peptide apparently resulting from the further digestion of the above Bb protein-staining band); f = 44 500; g = 33 000 (Ba); h = 24 500; i = 17 000; j = 13 000.

It also appears almost immediately (at 1 min) in both buffers, reaches a peak at 5–15 min, and then is similarly further degraded to lower molecular weight species (probably the M_r 17 000 protein-staining band "i" in Figure 4). In addition, while prolonged digestion in high ionic strength buffer gave rise to predominantly one fragment of 58 000 daltons, digestion in low ionic strength buffer gave rise to predominantly two fragments of 58 000 and 44 500 daltons. It was concluded, therefore, that trypsin cleavage would not be a satisfactory method for producing either the quantity or the quality of Bb and Ba cleavage peptides necessary for initial NH_2-terminal sequencing.

Purification of Factor B Cleavage Fragments. Bb was isolated after fractionation of a cleaved factor B preparation by DEAE-cellulose chromatography (Götze & Müller-Eberhard, 1971). Peak 1 in Figure 5A demonstrates the purity of the recovered Bb. This purified Bb retained the ability to precipitate with factor B antiserum and gave a single protein-staining band of appropriate apparent molecular weight

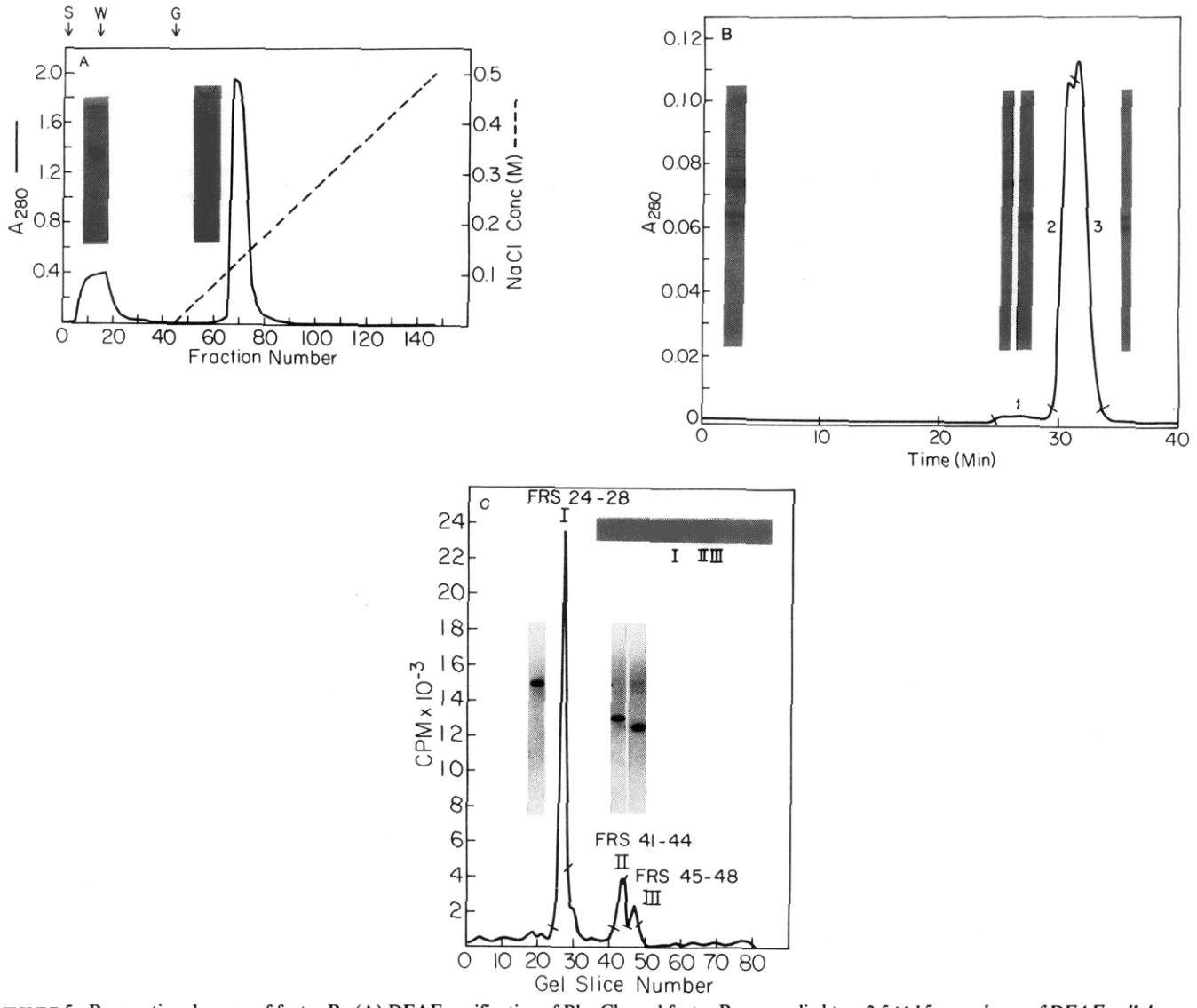

FIGURE 5: Preparative cleavage of factor B. (A) DEAE purification of Bb. Cleaved factor B was applied to a 2.5 × 15 cm column of DEAE-cellulose. The first A_{280} peak as determined by $NaDodSO_4$ electrophoresis represents the highly purified Bb cleavage fragment. The second A_{280} peak was determined by $NaDodSO_4$ electrophoresis to represent highly purified residual uncleavage factor B. S = sample application; W = wash; G = gradient. (B) LC purification of Ba. Ba was resolved from the cleavage mixture by LC. $NaDodSO_4$–polyacrylamide gradient gels adjacent to the A_{280} profile illustrate the protein composition of each pool. The first gel represents the unfractionated factor B cleavage mixture. Pool 1, eluting between 25 and 29 min (gel 2), contains C3b, factor B, and Bb. Pool 2, eluting between 29 and 31 min (gel 3), contains purified Ba. Pool 3, eluting between 31 and 34 min (gel 4), contains a further breakdown product of Ba (Ba′) as well as some Ba trail-over from the preceding adjacent pool. The anode was at the bottom. (C) $NaDodSO_4$ electrophoresis purification of Bb and Ba. Both Bb and Ba were separated and recovered from preparative $NaDodSO_4$–polyacrylamide gradient gels as described under Experimental Procedure. Protein-staining bands I–III above the graph correspond to radiolabeled pools I–III, respectively. The anode was at the right. Reelectrophoresis of pools I–III on $NaDodSO_4$–polyacrylamide gradient gels resulted in the protein-staining bands indicated on the graph. The anode was at the bottom. I = Bb; II = Ba; III = Ba′ (smaller molecular weight peptide apparently resulting from the further breakdown of Ba).

on $NaDodSO_4$ electrophoresis.

Ba was isolated after fractionation of the factor B cleavage mixture by LC. The resolution of the Ba fragment by this technique is illustrated in Figure 5B. $NaDodSO_4$ electrophoresis of pool 2 demonstrated the high degree of purity of the isolated Ba fragment since it gave rise to a major protein-staining band with only a single trace (less than 10%) of lower molecular weight contaminant.

The Bb and Ba fragments from an extended cleavage with factor $\bar{D}$ were also resolved on and eluted from preparative gradient slab gels. The radiolabeled and staining pattern is illustrated in Figure 5C. Peak I has been determined by amino acid analysis and sequencing to be Bb. Peak II similarly has been determined to be Ba. Peak III, on the basis of amino acid composition and limited sequencing data, appears to be a breakdown product of Ba. This additional lower molecular weight fragment (Ba′) most probably arises as a result of very minor impurities in the factor $\bar{D}$ preparation since the weight ratios of factor B and C3b used in the cleavage mixture were the same as those employed in the analytical gel system. Only the factor $\bar{D}$ ratio was increased fivefold in these preparative gels to enhance the cleavage and minimize the amount of residual uncleaved factor B.

Amino Acid Composition. The amino acid composition of factor B and its hydrolysis products, Ba and Bb, is presented in Table II. The amino acid composition of factor B as well as its Ba and Bb cleavage fragments agrees well with previously published data (Boenisch & Alper, 1970a,b; Curman et al., 1977; Kerr & Porter, 1978; Lesavre et al., 1979).

NH_2-Terminal Amino Acid Sequence of Factor B and Its Cleavage Fragments, Bb and Ba. Native factor B and its activation fragments, Bb and Ba, were subjected to NH_2-

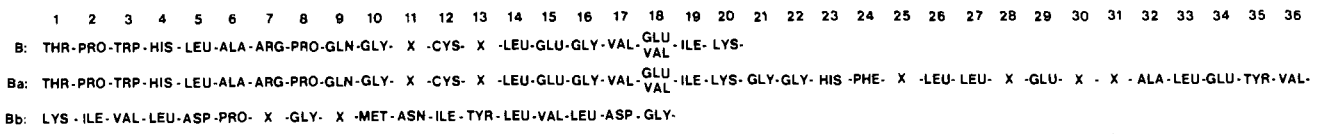

FIGURE 6: NH_2-terminal amino acid sequence of factor B and its cleavage fragments, Ba and Bb. X = unidentified amino acid.

Table II: Amino Acid Composition

	factor B		Bb		Ba	
	residues/ 100 amino acids	residues/ molecule[a]	residues/ 100 amino acids	residues/ molecule[b]	residues/ 100 amino acids	residues/ molecule[c]
Lys	7.73	59	8.80	45	3.09	9
His	2.89	22	2.61	13	2.56	7
Arg	5.22	40	3.84	20	8.08	23
Asp	10.36	79	11.34	58	7.66	22
Thr	5.63	43	4.41	23	7.44	21
Ser	6.90	53	6.07	31	9.90	28
Glu	12.14	93	11.69	61	11.94	42
Pro	6.66	51	6.28	32	4.77	14
Gly	9.11	69	7.56	38	12.30	35
Ala	4.74	36	5.11	26	5.00	14
1/2-Cys	ND[d]	ND	ND	ND	4.03	11
Val	7.52	57	8.04	41	4.86	14
Met	1.60	12	1.59	8	0.50	1
Ile	4.71	36	4.60	24	3.24	9
Leu	7.47	57	7.20	37	5.84	17
Tyr	4.31	33	4.05	21	4.24	12
Trp	ND	ND	ND	ND	ND	ND
Phe	3.00	23	3.12	16	2.48	7

[a] Based on a molecular weight of 93 000 (dependent on the percent of each amino acid), a carbohydrate content of 8.95% [an average of published (Boenisch & Alper, 1970b; Curman et al., 1977) values], and the analysis of over 40 different factor B preparations. [b] Based on a molecular weight of 63 000 (dependent on the percent of each amino acid), a carbohydrate content of 9.9% [an average of published (Boenisch & Alper, 1970a; Haupt & Heide, 1965) values], and the average of two independent determinations. [c] Based on a molecular weight of 33 000 (dependent on the percent of each amino acid), a carbohydrate content of 6.3% ($B_{8.95\%} \times {}_{93\,000} - Bb_{9.9\%} \times {}_{63000}$), and the average of two independent determinations. [d] ND = residues not determined.

terminal amino acid sequence analysis. The results are summarized in Figure 6. Purified factor B (20–50 nmol) was subjected to automated Edman degradation for 40 cycles. The NH_2-terminal amino acid was clearly threonine. After residue 20, however, the sequence became uninterpretable. Purified Ba (8 nmol) was similarly subjected to automated Edman degradation for 43 cycles. After residue 36 this sequence also became uninterpretable. The sequence identity between the NH_2 terminal of Ba and factor B, however, clearly indicates that the Ba fragment is indeed derived from the NH_2 terminal of native factor B. Evidence was also obtained for two different amino acid residues at cycle 18. This supports the hypothesis that the electrophoretic heterogeneity of factor B (Alper et al., 1972; Curman et al., 1977; Boenisch & Alper, 1970b; Haupt & Heide, 1965) may be associated with amino acid residues located in the Ba region of the intact protein (Curman et al., 1977). Purified Bb (20 nmol) was also subjected to automated Edman degradation for 30 cycles. Again after residue 18 this sequence similarly became uninterpretable. This sequence agrees with and extends that recently determined by Lesavre et al. (1979). No amino acid heterogeneity was detected.

Discussion

A high-yield procedure for the purification of factor B of the alternative complement pathway has been described. Factor B was subsequently converted to its Bb and Ba cleavage products by the catalytic action of factor $\bar{D}$ in the presence of cobra venom factor or C3b. Our data substantiate the observation that this cleavage does indeed occur only in the presence of both factor $\bar{D}$ and cobra venom factor or C3b. Limited digestion of factor B with trypsin was used to generate similar molecular weight fragments. However, numerous additional intermediate size peptides also appear to be generated (Curman et al., 1977). Trypsin digestion, therefore, was not found to be satisfactory to produce either the quantity or the quality of Bb and Ba cleavage peptides necessary for this initial sequence study. In general, the major problems with using such a protease to produce the Bb and Ba cleavage fragments from factor B are (1) lack of specificity, (2) difficulty in controlling the digestion, resulting in nonproductive intermediate molecular weight peptides with the confusing fraying of peptide NH_2 termini (Curman et al., 1977), and (3) nonreproducibility, resulting in a variable extent of cleavage which produces unpredictable cleavage patterns even under standard incubation conditions. Clearly the more specific cleavage of factor B with factor $\bar{D}$ and cobra venom factor or C3b is preferable for further structural analyses and absolutely essential for biological activity studies involving the cleavage products, Bb and Ba.

Trypsin cleavage of native factor B, however, does promise to provide much useful structural, and possibly biological, information on the more trypsin-resistant peptides obviously derived from the further proteolytic digestion of the Bb and Ba cleavage fragments. Our preliminary results (see Figure 4) suggest that the digestion of native factor B by trypsin is enhanced in lower ionic strength buffers, possibly due to the protein assuming a less compact conformation and exposing susceptible peptide bonds to proteolytic attack. Digestion in high ionic strength buffer, for example, gave rise to predominantly one fragment of 58 000 daltons, while digestion in low ionic strength buffer gave rise to predominantly two fragments of 58 000 and 44 500 daltons. Varying the ionic strength of the trypsin digestion buffer, therefore, could prove to be useful in controlling the size of the predominant resultant peptides produced for further analysis.

It has previously been reported that although the 60 000-dalton fragment appears to be highly resistant to further proteolysis by trypsin, the 30 000-dalton fragment is not and appears to be rapidly degraded into smaller molecular weight components (Curman et al., 1977). In agreement with these previous observations, we also noticed the appearance of a 17 000 apparent molecular weight peptide corresponding to the disappearance (at 30 min) of our Ba-like 33 000-dalton fragment. If human Ba is found to have chemotactic activity, as has been reported for guinea pig Ba (Hamuro et al., 1978), one might expect it to have a structure and/or amino acid composition that is susceptible to proteolytic digestion so that the chemotactic message could be easily turned off once it was no longer required. When analyzed by gradient gel electrophoresis, however, our 63 000-dalton fragment appears to be equally as labile as our 33 000-dalton fragment. A closely migrating peptide (M_r 58 000) which is probably derived from Bb and might easily be identified as the 60 000-dalton fragment by less sensitive electrophoretic techniques does remain resistant.

The amino acid composition of both factor B and its cleavage fragments, Bb and Ba, agrees well with previously published values (Boenisch & Alper, 1970a,b; Curman et al., 1977; Kerr & Porter, 1978; Lesavre et al., 1979). The compositions of these proteins are only remarkable, as previously noted (Boenisch & Alper, 1970a,b), for their relatively high glycine content. The amino acid analysis of the Ba fragment is also unusual, however, for its high cystine and low methionine content. The actual number of cystine residues found in the Ba fragment agrees well with the theoretically predicted value (previously published factor $B_{Cys} - Bb_{Cys}$ values), even though this determination was not performed on carboxymethylated material and is probably low. The theoretically predicted number of methionine residues for the Ba fragment (factor $B_{Met} - Bb_{Met}$), on the other hand, is ~4. Only one methionine residue, however, was detected in this peptide, even though other infrequently occurring amino acid residues, such as phenylalanine, were detected at theoretically predicted levels. This suggests that the actual number of methionine residues in this fragment may be even lower than expected, which might be attributable to amino acid heterogeneity in this region of the factor B molecule. Ba is also low in overall neutral amino acids such as valine, leucine, and isoleucine when compared to factor B and Bb. In addition, although the overall basic amino acid composition of these three proteins is similar, Ba is unique in that it contains a higher percentage of arginine rather than lysine. Knowledge of these amino acid differences between factor B and its cleavage products, Bb and Ba, will prove useful in future chemical and enzymatic digestion studies.

Our preliminary amino acid sequencing data indicated that the NH_2 terminal of native factor B might be blocked or that denaturation may be required in order for this protein to be sequenced. Subsequent sequencing attempts proved the latter to be the case. Thus, after reduction and alkylation we were able to obtain sequence data on the first 20 NH_2-terminal amino acid residues which are shown in Figure 6. The NH_2-terminal amino acid of this protein has previously been reported to be both threonine (Kerr & Porter, 1978) and proline (Curman et al., 1977). Our sequence data confirmed that threonine is the NH_2-terminal amino acid, whereas proline was identified as the penultimate amino acid residue. An interesting feature of this molecule is how quickly its sequence became uninterpretable. This was probably due to fragmentation of this rather large protein (~850 residues) during sequencing but may also indicate inherent amino acid heterogeneity. Factor B, for example, has long been known to exhibit electrophoretic heterogeneity (Boenisch & Alper, 1970b; Alper et al., 1972; Curman et al., 1977). At least part of this heterogeneity seems to be attributable to genetic polymorphism which most probably resides in the amino acid sequence since neuraminidase treatment fails to obligate the observed heterogeneity (Curman et al., 1977). The latter interpretation would confirm the suggestion of others (Alper et al., 1972; Boenisch & Alper, 1970b; Haupt & Heide, 1965) that the majority of the observed heterogeneity of factor B may be due to amino acid sequence differences in the NH_2-terminal portion of factor B.

The NH_2-terminal region of the Ba fragment also proved difficult to sequence. It is now clear, however, that this fragment is indeed derived from the NH_2 terminal of native factor B. The most outstanding feature of this sequence is its obvious identity with the NH_2-terminal region of native factor B. The amino acid heterogeneity at residue 18 supports earlier observations which suggested that electrophoretic heterogeneity of factor B resides in the Ba fragment and is probably not solely carbohydrate dependent (Boenisch & Alper, 1970b; Alper et al., 1972; Curman et al., 1977).

In contrast to native factor B and the Ba fragment, the NH_2-terminal region of the Bb fragment was easily sequenced. The most characteristic feature of this sequence is its hydrophobicity. It remains unclear whether factor B can be inhibited by iPr_2P-F under appropriate incubation conditions and therefore should be classified as a serine protease (Fearon et al., 1974; Götze, 1975; Medicus et al., 1976; Vogt et al., 1977). Bb does contain the active site of the C3 and C5 convertases, both of which have trypsin-like specificities. The partial amino acid sequence of the Bb fragment, however, reveals little structural similarity between its NH_2 terminal and that of the light chain of human $\overline{C1r}$, $\overline{C1s}$, plasmin, factor D, or any of the other known serine proteases. Final resolution of this question of course ultimately depends on amino acid sequence analysis of the active site of the enzyme.

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Covalent Structure of Collagen: Amino Acid Sequence of α1(III)-CB5 from Type III Collagen of Human Liver†

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ABSTRACT: Type III collagen was prepared from human liver by limited pepsin digestion, differential salt precipitation, and carboxymethylcellulose chromatography. Ten distinct peptides were obtained by cyanogen bromide digestion. The peptide α1(III)-CB5 was further purified by carboxymethylcellulose chromatography, and its amino acid sequence was determined. Automatic Edman degradation of intact α1(III)-CB5, tryptic and thermolytic peptides, and hydroxylamine-derived fragments was used to establish the total sequence. The mammalian collagenase site contained in the α1(III)-CB5 sequence was ascertained by digestion of native type III collagen with purified rheumatoid synovial collagenase. Collagenase cleavage occurred at a single Gly–Ile bond, one triplet before the corresponding specific cleavage site of type I collagen. The present work brings the known sequence of human liver type III collagen to include α1(III)-CB3-7-6-1-8-10-2-4-5. These correspond to the homologous region of α1(I)-CB0-1-2-4-5-8-3-7 residues 11–804.

Collagen is the most abundant extracellular connective tissue protein of most vertebrates. It exists as a triple-stranded helix of three α chains, each containing over 1000 amino acid residues (Piez, 1976; Gallop et al., 1972; Traub & Piez, 1971). At least three genetically distinct, interstitial collagens occur in mammals and are referred to as types I, II, and III.

Both type I and type III collagens occur simultaneously in most connective tissues with the exception of bone which contains only type I. Type II collagen is found solely in cartilage and vitreous humor (Miller & Lunde, 1973; Chung & Miller, 1974; Epstein, 1974; Swann et al., 1972; Stuart et al., 1979). Type III collagen contains three identical α chains of 95 000 mol wt held together by disulfide linkages. Ten CNBr peptides of type III collagen of human skin and liver have been isolated and characterized (Chung et al., 1974; Seyer & Kang, 1977), and complete amino acid sequences of eight peptides, α1(III)-CB3-7-6-1-8-10-2-4, representing the first 558 residues from the NH_2 terminus have been reported (Seyer & Kang, 1977, 1978). The present report describes the amino acid sequence of α1(III)-CB5 of human liver, a 237-residue CNBr peptide which contains the collagenase cleavage site. The sequence of only one peptide from human type III collagen, α1(III)-CB9, remains to be determined.

During the preparation of this manuscript, the entire sequence of bovine skin type III collagen was reported (Fietzek et al., 1979; Dewes et al., 1979a,b; Bentz et al., 1979; Lang et al., 1979; Allman et al., 1979). The last report represents the first complete amino acid sequence analysis of an individual collagen chain.

Materials and Methods

Preparation of α1(III)-CB5. Human cirrhotic livers were obtained after autopsy, and type III collagen was prepared as previously described (Seyer et al., 1976, 1977). The CNBr peptides were obtained by digestion of purified type III collagen in 70% formic acid and separated by ion-exchange

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