Chemical Characterization of Human Factor B of the Alternate Pathway of Complement Activation†

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ABSTRACT: Factor B of the alternate pathway participates in the activation of complement component C3. Factor B and fragments thereof have been isolated from serum and partially characterized. Highly purified factor B is homogeneous by agarose electrophoresis but electrophoretic techniques with higher resolution and isoelectric focusing reveal two types of charge heterogeneity for factor B. The heterogeneity is thus partly dependent upon a genetic polymorphism but in addition a nongenetical microheterogeneity is observed. The sedimentation constant for factor B is 5.9 S and the Stokes' molecular radius is 40 Å. The molecular weight of factor B was assessed by several techniques and a value of about 90 000 was established with all methods. These data demonstrated that factor B consists of a single polypeptide chain. The NH_2-terminal amino acid residue was shown to be proline. Factor B contains about 7.3% carbohydrate. Two fragments of factor B were also encountered in serum. The quantitatively dominating component had a molecular weight of 63 000, was basic in character, and did not exhibit any high degree of charge heterogeneity. Occasionally a basic fragment with the molecular weight 47 000 was also obtained. Immunological analyses revealed that both fragments displayed less antigenic sites than intact factor B. In addition to the 63 000-dalton fragment, urine contained two other fragments of factor B. These fragments, with molecular weights of 27 000 and 21 000, were acidic and displayed a similar type of charge heterogeneity as intact factor B. The two low molecular weight urinary fragments reacted with antisera against factor B but not with antisera against the 63 000-dalton fragment. The NH_2-terminal amino acid of the low molecular weight fragments was proline, suggesting that they were derived from the NH_2-terminal part of factor B. The factor B derived fragments of serum and urine displayed no hemolytic activity. Limited digestion of factor B with trypsin gave rise to two main proteolytic fragments with the apparent molecular weights 60 000 and 30 000. These fragments displayed characteristics similar to those of the serum and urinary fragments, and like these they were not able to induce hemolysis. It is concluded that factor B consists of a single polypeptide chain which is folded into two compact domains. The domains are connected by a stretch of the chain which is highly susceptible to proteolysis. The smaller, NH_2-terminal domain may be released on activation of factor B by proteolysis with factor D.

A key step in the complement reaction sequence is the activation of C3 (see Müller-Eberhard, 1975). This can occur principally in two ways. The classical activation mechanism involves factors C1, C4, and C2 but an alternate pathway is also existent. Factors A, B, and D can accordingly participate in the formation of a C3 convertase (for a review, see Müller-Eberhard, 1971). Factor B (C3 proactivator) was first isolated by Götze and Müller-Eberhard (1971). They estimated the molecular weight of factor B to 80 000 and showed that on activation the protein lost an acidic fragment with an apparent molecular weight of about 20 000 leaving the C3-cleaving activity associated with a basic polypeptide with the molecular weight 60 000.

Haupt and Heide (1965) and Boenish and Alper (1970a) isolated independently a serum glycoprotein with β_2 to γ mobility on electrophoresis. The latter authors noted that the isolated protein probably represented a fragment of a larger glycoprotein, which subsequently was successfully isolated (Boenish and Alper, 1970b). This protein, termed glycine-rich β-glycoprotein, was shown to display two major electrophoretic forms, S and F, which strongly indicated a genetically controlled polymorphism in the glycine-rich β-glycoprotein (Alper et al., 1972). In addition several minor, nongenetically determined electrophoretic forms of the protein were apparent which led to the suggestion that glycine-rich β-glycoprotein consisted of several subunits (Alper et al., 1972). In a subsequent study Alper and associates (Alper et al., 1973) demonstrated that glycine-rich β-glycoprotein indeed is identical with factor B of the alternate pathway.

Despite numerous studies on the functional properties of factor B, data on the physical and chemical characteristics of the protein are largely lacking. This paper describes the isolation of intact factor B and fragments thereof from human serum and urine and some physical and chemical data are given. Studies have also been undertaken to explore some of the features of the charge heterogeneity of factor B.

Experimental Procedure

Materials

Serum. Fresh serum and outdated plasma were obtained from the Blood Center, University Hospital, Uppsala, and used immediately or stored at −23 °C until further processing.

Urine. Twenty-four-hour urine specimens were obtained from several patients known to have tubular proteinuria. Most of the urine was collected from patients who had been studied previously (Peterson et al., 1969). Urine collection and sample processing were carried out as described elsewhere (Berggård and Bearn, 1968).

Antisera. Antisera were raised in rabbits against highly purified factor B and the serum-derived 63 000-dalton fragment. The immunization procedure has been described (Pe-

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TABLE I: Purification of Factor B.

Fraction	Total protein (mg)	Factor B[a] (mg)	Yield (%)	Purity (%)
Serum (540 mL)	39 300[b]	153	100	0.39
$(NH_4)_2SO_4$ (2.5-3.5 M)	17 600[b]	107	70	0.61
Sulfoethyl-Sephadex, pH 6.0	272[b]	60	39	22
DEAE-Sephadex, pH 7.5	120[b]	37	24	31
Sephadex G-200	42[b]	27	18	64
Zone electrophoresis, pH 8.9	22.2[c]	22.1	14	100

[a] Measured by a single radial immunodiffusion technique. [b] Determined by the Folin procedure. [c] Estimated from the optical density at 280 nm.

terson et al., 1969). The antisera were shown to be specific without absorptions by tests on double immunodiffusion and immunoelectrophoresis. A rabbit antiserum against factor B was also obtained from Behringwerke AG (Marburg/Lahn, West Germany).

Other Materials. Sepharose 6B, Sephadex G-100 and G-200, DEAE-Sephadex A-50, and sulfoethyl-Sephadex were obtained from Pharmacia Fine Chemicals AB, Uppsala, and prepared according to the instructions supplied by the manufacturer. Trypsin (code TRTPCK) was purchased from Worthington Biochemical Corp. (Freehold, N.J.). Dansyl chloride and dansyl amino acids were obtained from British Drug Houses, Poole, England, which also supplied polyamide sheets for thin-layer chromatography. All other chemicals were reagent grade or of the highest quality available.

Methods

Concentration of Proteins. Concentration of proteins during the isolation procedure was accomplished by ultrafiltration (Berggård, 1961). The recoveries of factor B and the 63 000-dalton fragment after ultrafiltration always exceeded 90%.

Electrophoresis. Polyacrylamide gel electrophoresis at pH 8.9 and 4.5, agarose gel electrophoresis, and sodium dodecyl sulfate–polyacrylamide gel electrophoresis were carried out as described elsewhere (Nilsson and Peterson, 1975). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis in slab form and two-dimensional electrophoresis in polyacrylamide gels combining separation by isoelectric focusing or, alternatively, separation at pH 8.9 with separation in sodium dodecyl sulfate in the second dimension was carried out essentially as described by O'Farrell (1975). Preparative zone electrophoresis was performed in blocks of Pevikon C-870 (Kema Nord AB, Stockholm, Sweden; Müller-Eberhard, 1960).

Isoelectric Focusing. Isoelectric focusing was performed in slabs of polyacrylamide gel. The slabs contained 5% (w/v) acrylamide and 0.4% (w/v) *N,N'*-methylenebisacrylamide (Eastman-Kodak) in 2.4% (v/v) carrier ampholytes (Ampholine, LKB Produkter AB, Stockholm, Sweden) of the desired pH interval. Preparative isoelectric focusing was performed on a water-cooled apparatus with 110-mL internal volume (LKB Produkter AB, Stockholm, Sweden). The experiments were conducted according to the instructions supplied by the manufacturer.

Immunochemical Methods. Immunodiffusion in gel was performed as described (Peterson and Berggård, 1971). Immunoelectrophoresis was carried out according to the Scheidegger micromethod (Scheidegger, 1955). The single radial immunodiffusion method (Mancini et al., 1965) was used to quantitate factor B in impure fractions. This technique, or immunodiffusion in gel, was also used to assay the protein during the isolation procedure. Highly purified factor B was used as reference in the determinations.

Immune precipitation of ^{125}I-labeled factor B and related fragments was carried out by mixing the ^{125}I-labeled protein with an appropriate amount of antiserum. The reaction was allowed to proceed for 4 h at ambient temperature. Immune complexes were precipitated by the addition of formalin-treated *Staphylococcus aureus* of the Cowan I strain (Östberg et al., 1976). The precipitates were dissolved by boiling for 1 min in 0.1 mL of 8 M urea containing 2% sodium dodecyl sulfate and 5% β-mercaptoethanol prior to sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

Factor B was visualized by radioimmunofixation following separation by isoelectric focusing in polyacrylamide gel slabs (Alper and Johnson, 1969).

Hemolytic Assay of Factor B and Its Fragments. The hemolytic diffusion plate assay (Martin et al., 1976) was employed for quantitative measurement of the hemolytic activity of factor B and fragments thereof. The serum concentration in the agarose plates was 4.5% and the Mg^{2+} concentration, 9 mM. The hemolytic areas varied linearly with the logarithm of the amount of factor B from 0.1 μg to 20 μg.

Analytical Ultracentrifugation, Calculations of Diffusion Coefficients, Molecular Weights, and Frictional Ratios. Details and references have been presented earlier (Nilsson and Peterson, 1975).

Analytical Gel Chromatography. Analytical gel chromatography was performed as described (Nilsson and Peterson, 1975; Karlsson et al., 1972).

Amino Acid and Carbohydrate Analyses. These analyses were performed as described earlier (Nilsson and Peterson, 1975).

NH_2-Terminal Analyses. NH_2-terminal analyses of reduced and alkylated factor B and related fragments were accomplished by Edman degradation plus dansylation (Gray, 1967). The liberated dansyl amino acids were identified by two-dimensional chromatography on polyamide thin-layer sheets (Woods and Wang, 1967).

Other Methods. Protein was labeled with ^{125}I according to the Chloramine-T method (Hunter and Greenwood, 1962). Factor B was maleylated with [^{14}C]maleic anhydride (New England Nuclear, Dreieichenhein, West Germany).

Determinations of γ-carboxyglutamic acid was kindly performed by Dr. S. Magnusson. Protein concentrations in unpurified fractions were estimated by the modified Folin procedure of Lowry et al. (1951) with human serum albumin as the reference. More highly purified fractions were quantitatively assayed for protein by measuring the absorbance at 280 nm.

Isolation of Factor B. Factor B was isolated from fresh serum or outdated plasma. The isolation procedure for a typical preparation is summarized in Table I. After the initial

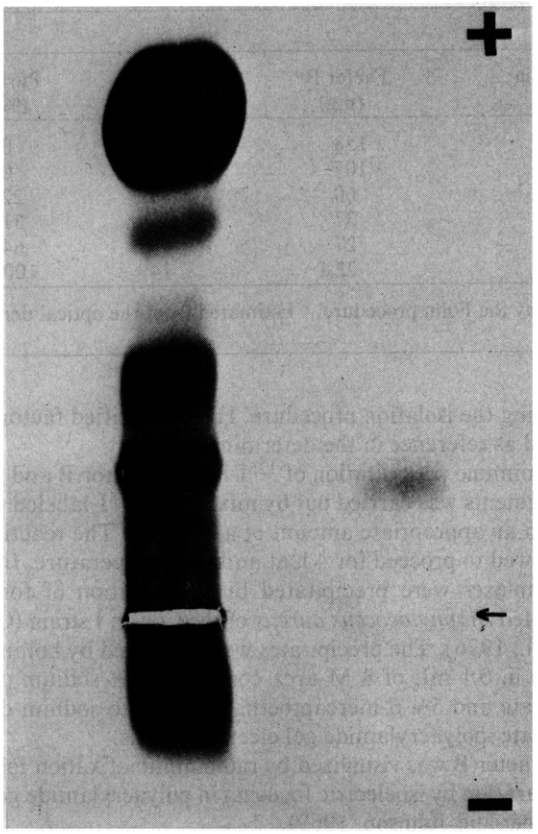

FIGURE 1: Agarose gel electrophoresis in sodium barbital buffer, pH 8.6, of normal human serum (left) and highly purified factor B (right). The arrow denotes the origin.

$(NH_2)_2SO_4$-precipitation step, the material was subjected to ion-exchange chromatography on a column of sulfoethyl-Sephadex equilibrated with 0.01 M sodium acetate buffer, pH 6.0, containing 5 mM EDTA. The column was eluted with a linear NaCl gradient from 0 to 0.5 M. The factor B containing material appeared at about 0.35 M NaCl. After completion of the gradient, the column was eluted with the acetate buffer containing 1.5 M NaCl. In some preparations, minor amounts of factor B reacting material were eluted at the high ionic strength. This material was further purified on a column of Sephadex G-200 equilibrated with 0.02 M Tris-HCl buffer, pH 8.0, containing 0.15 M NaCl. The fractionation step yielded a 63 000-dalton fragment of factor B in highly purified form (see below).

Further purification of the major factor B containing fraction from the sulfoethyl-Sephadex column was achieved by DEAE-Sephadex chromatography in 0.02 M Tris-HCl buffer, pH 7.5, containing 2 mM EDTA. Elution was performed with a linear NaCl gradient from 0 to 0.2 M in the pH 7.5 buffer. Factor B emerged at a NaCl concentration of about 0.1.

The next purification step, gel chromatography on a column of Sephadex G-200 equilibrated with 0.02 M Tris-HCl buffer, pH 8.0, containing 0.15 M NaCl and 2 mM EDTA, gave a factor B containing fraction which was contaminated by only one unrelated protein identified as hemopexin.

The final purification step consisted of electrophoresis in borate buffer, pH 8.9. The factor B containing fraction from the gel chromatography step gave rise to two well-resolved peaks. The protein with the slowest anodal mobility was the only one reacting with antiserum against factor B. Fractions comprising this material were pooled and concentrated and evidence is given below that this material constituted highly purified factor B.

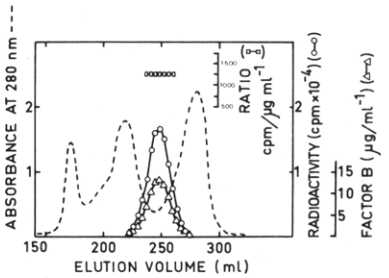

FIGURE 2: Chromatography on Sephadex G-200 of normal human serum mixed with trace amounts of ^{125}I-labeled highly purified factor B. The column (145 × 2 cm) was equilibrated with 0.02 M Tris-HCl buffer, pH 8.0, containing 0.15 M NaCl. Fractions of 4.0 mL were collected at 30-min intervals. The distribution in the effluent of factor B, present in the serum, was determined by a single radial immunodiffusion technique.

Results

Purity of the Isolated Factor B. Figure 1 demonstrates that factor B was of high purity since it gave rise to a single protein zone on agarose electrophoresis. The protein migrated somewhat more slowly than serum transferrin. Analysis of the isolated factor B on polyacrylamide gel electrophoresis at pH 8.9 revealed several protein zones. This type of electrophoretic heterogeneity was also evident on prolonged agarose electrophoresis and represents microheterogeneity of the protein (see below).

To examine if the charge heterogeneity of the isolated factor B was accompanied by a size heterogeneity as well, the isolated protein was labeled with ^{125}I and a trace amount of the radioactive protein was mixed with a sample of freshly drawn serum. The mixture was subjected to gel chromatography on a column of Sephadex G-200. Figure 2 shows that the radioactivity was eluted as a symmetrical peak. Genuine factor B of serum occurred in the same elution position as shown by quantitative single radial immunodiffusion analysis. These data suggest that the isolated factor B is of high purity and most likely represents the intact protein. The activity of the isolated protein was tested in the hemolytic diffusion plate assay (Martin et al., 1976) and the activity of the isolated material corresponded to the activity of an identical amount of factor B present in freshly drawn serum.

Physical and Chemical Characteristics of Factor B. In Table II some physical and chemical characteristics of factor B are summarized.

Sedimentation velocity determinations were carried out at protein concentrations ranging from 0.1 to 1.2%. Factor B behaved as a single homogeneous component in the ultracentrifuge. The sedimentation constant of factor B decreased somewhat with increasing concentrations.

The value obtained for Stokes' molecular radius for factor B (40 Å) was significantly larger than the value for albumin (35.5 Å) but considerably less than that for IgG (52 Å).

The frictional ratio, 1.28, is in accordance with a globular structure for factor B.

Molecular weights were estimated from sedimentation equilibrium ultracentrifugation data recorded at various concentrations. The values for factor B seemed to be independent of concentration in the range from 0.1 to 2.0 mg per mL. Linear relationships between log C and r^2 suggested that factor B was homogeneous.

To investigate the possibility of a subunit structure for the isolated factor B, it was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and to gel chromatography on a column of Sepharose 6 B equilibrated with 6 M guanidine hydrochloride. The reduced and alkylated factor B always

TABLE II: Physical–Chemical Properties of Factor B.

Sedimentation constant, $s^0_{20,w}$	5.9 S
Partial specific volume,[a]	0.721 mL/g
Stokes' molecular radius[b]	40 Å
Diffusion constant, $D_{20,w}$[b]	5.4
Frictional ratio (f/f_0)	1.28
Molecular weight	
Sedimentation equilibrium[c]	
0.2 mg/mL	87 700
0.4 mg/mL	91 400
0.8 mg/mL	89 600
Sedimentation–Stokes' radius	93 000
Polyacrylamide gel electrophoresis[d]	86 000
Gel chromatography[e]	89 000
$E_{280}^{1\%}$	12.7
NH_2-terminal residue	Proline
Carbohydrate content	7.25%

[a] Calculated from the amino acid and carbohydrate compositions. [b] Estimated by analytical gel chromatography. The diffusion constant is given as 10^{-7} cm^2 s^{-1}. [c] The speeds used for the ultracentrifugations were 20 000, 18 000, and 16 000 rpm, respectively, for the three initial concentrations of factor B. [d] Determined in 0.1% sodium dodecyl sulfate on reduced and alkylated factor B. [e] Determined in 6 M guanidine hydrochloride on reduced and alkylated protein.

TABLE III: Amino Acid Composition of Factor B.[a]

Amino acid	Residue/100 residues	Residues/molecule[b] (to nearest integer)
Lys	7.19	53
His	2.31	17
Arg	5.16	38
Asp	10.18	75
Thre[c]	5.43	40
Ser[c]	7.46	55
Glu	11.67	86
Pro	5.16	38
Gly	8.68	64
Ala	4.88	36
½-cystine[d]	3.12	23
Val[e]	7.33	54
Met	1.36	10
Ile[e]	4.48	33
Leu[e]	6.65	49
Tyr	4.34	32
Phe	2.71	20
Trp[f]	1.90	14

[a] Except where noted all figures are average values of one 24-h and one 72-h hydrolysis from two determinations on separate preparations. [b] Calculations were based on a molecular weight of 83 000 for the polypeptide portion of factor B. [c] Values were obtained by extrapolation to zero time of hydrolysis. [d] Determined after performic acid oxidation. [e] 72-h hydrolysis value. [f] Determined spectrophotometrically.

appeared as a protein with an apparent molecular weight in the range of 86 000 to 89 000. This value is in good agreement with the ultracentrifugation data and strongly indicates that factor B consists of a single polypeptide chain.

The amino acid composition and carbohydrate content of factor B are presented in Tables III and IV.

NH_2-terminal amino acid analyses were carried out by the dansyl technique on factor B. Dansyl-*O*-tyrosine and dansyl-ϵ-lysine were observed in all analyses. The only other dansylated amino acid present in hydrolysates of factor B was proline. It was therefore concluded that the NH_2-terminal amino acid of factor B is proline.

Charge Heterogeneity of Factor B. It has been well documented that factor B occurs in two major genetic forms denoted FF and SS in the homozygous states (Alper et al., 1972). These electrophoretic variants are well resolved by isoelectric focusing in polyacrylamide gel. Figure 3 depicts such a separation. Although the analyzed sera were obtained from individuals homozygous at the factor B locus three to four protein zones were apparent in each case. The overall pattern of the protein zones was, however, compatible with a genetically defined charge difference. In separate analyses each protein zone was isolated and sodium dodecyl sulfate–polyacrylamide gel electrophoresis revealed that the protein in all zones had the same size and this was indistinguishable from that of genuine factor B. Attempts were therefore made to explore if the nongenetic charge heterogeneity might result from differences in the content of sialic acid. Figure 3 shows, however, that multiple protein zones were apparent even after exhaustive digestion of the isolated factor B with neuraminidase, although the overall isoelectric focusing pattern after the enzymatic treatment was displaced toward the basic region.

TABLE IV: Carbohydrate Composition of Factor B.[a]

Carbohydrate	Found g/100 g	Found Residues/molecule[b]	To nearest integer (residues/molecule)[b]
Galactose	1.03	5.11	5
Mannose	1.42	7.07	7
Glucosamine	2.34	9.47	9
Galactosamine	<0.02	<0.10	0
N-Acetylneuraminic acid	2.33	6.74	7
Fucose	0.13	0.73	1

[a] All figures are average values from three separate determinations. [b] Calculations were based on the assumed molecular weight 89 400 for factor B.

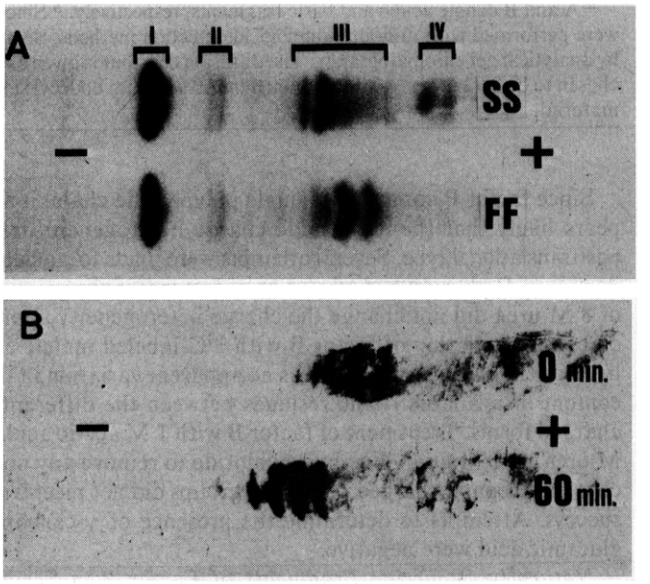

FIGURE 3: Isoelectric focusing of factor B on polyacrylamide slab gels. (A) Samples (8 μL plasma) were focused for 4 h and fixed with radioactively labeled antibodies to factor B. The autoradiogram shows the patterns of the genotypes SS and FF. (I) The 63 000-dalton component. (II) Unidentified degradation products. (III) Intact factor B. (IV) The 30 000-dalton component. (B) Purified factor B (1 mg/mL) was digested with neuraminidase (40 μg/mL) in 0.02 M sodium acetate buffer (pH 5.0), containing 0.1 M NaCl for 0 (top) and 60 min (bottom) at 37 °C. The reaction was stopped by raising pH to 8. The gel was fixed and stained with Coomassie brilliant blue.

TABLE V: Physical-Chemical Properties of Fragments Derived from Factor B.

	Source of fragments[a]				
	Serum	Urine[b]		Trypsin	
	B	A	B	A	B
Sedimentation constant, $s^0_{20,w}$ (S)	4.4	2.4	4.3	2.5	4.3
Stokes' molecular radius[c] (Å)	34	25	34	26	34
Diffusion constant, $D_{20,w}$[c]	6.3	7.9	6.3	7.7	6.3
Frictional ratio (f/f_0)	1.32	1.34	1.30	1.33	1.30
Molecular weight					
Sedimentation-equilibrium	61 000			28 000	60 000
Sedimentation-Stokes' radius[d]	60 000	26 000	59 000	28 000	59 000
		27 000			
Polyacrylamide gel electrophoresis[e]	63 000	21 000	60 000	29 000	63 000
		26 000			
Gel chromatography[f]	63 000	21 000	62 000	29 000	62 000

[a] A and B denote acidic and basic fragments, respectively. [b] Since scarcity of material precluded the isolation of these components, all analyses were performed with radioimmunological detection methods. Sedimentation constants were estimated in linear sucrose gradients. [c] Estimated by analytical gel chromatography. The diffusion constant is given as 10^{-7} cm^2 s^{-1}. [d] The partial specific volume for all components was arbitrarily chosen to be 0.72 mL g^{-1}. [e] Determined in 0.1% sodium dodecyl sulfate. [f] Determined in 6 M guanidine hydrochloride on reduced and alkylated material.

Since factor B comprises a single polypeptide chain, it appears likely that the nongenetic charge heterogeneity is of posttranslational type. Several attempts were made to elucidate its nature. Reduction and alkylation of factor B in the presence of 8 M urea did not change the charge heterogeneity appreciably. Maleylation of factor B with ^{14}C-labeled maleic anhydride suggested that there was no apparent variation in the content of available lysine residues between the differently charged forms. Treatment of factor B with 1 M acetic acid, 8 M urea, or 6 M guanidine hydrochloride to remove any noncovalently bound charged prosthetic groups did not meet with success. Attempts to determine the presence of γ-carboxyglutamic acid were negative.

Naturally Occurring Fragments of Factor B in Serum. During the isolation of factor B it was noted that some preparations contained immunologically active material which was more basic than genuine factor B. This component could easily be isolated by gel chromatography (see Experimental Procedure). The yield of the basic material varied considerably from preparation to preparation.

The isolated, basic material was apparently homogeneous with regard to size and charge. The component displayed slight cathodal mobility on agarose electrophoresis at pH 8.6. Polyacrylamide gel electrophoresis at pH 4.5 revealed a single protein zone. However, a few preparations, which were homogeneous on agarose electrophoresis and gel chromatography, resolved into two components on polyacrylamide gel electrophoresis at low pH. The extra protein zone had a higher mobility than the main component.

Some physical data for the basic fragment of factor B are given in Table V. The molecular weight of the fragment, as determined by several methods, is approximately 63 000. The frictional ratio, 1.32, is compatible with a globular structure for the basic protein assuming a "normal" hydration.

One preparation of the basic material, which on acid polyacrylamide gel electrophoresis resolved into two components, was analyzed by gel chromatography in 6 M guanidine hydrochloride. The extra protein displayed an apparent molecular weight of 47 000. This was the only component present in the material except the dominating 63 000-dalton species. Immunological analyses revealed that the 63 000- and 47 000-dalton polypeptide chains were related (see below).

In the hemolytic diffusion plate assay, the isolated 63 000-dalton fragment was inactive. Götze and Müller-Eberhard (1971) have shown that a basic 60 000-dalton fragment, obtained after inulin activation of the alternate pathway, retains the C3-converting property of factor B. To relate the isolated 63 000-dalton component to that obtained after inulin activation of the alternate pathway, 200 mL of serum was subjected to incubation with inulin. A basic 60 000-dalton fragment was isolated by successive chromatographies on Sephadex G-200 and DEAE-Sephadex. During the isolation procedure, the chromatograms were tested for factor B activity in the hemolysis assay. In no case did the observed activity coincide with the elution position for the basic, factor B derived fragment. Likewise, the highly purified fragment did not display any biological activity even at high concentrations.

Rabbit antisera were prepared against the genuine factor B and against the 63 000-dalton fragment isolated from serum. The antisera were used to compare the immunological characteristics of factor B in serum, isolated factor B, and the 63 000-dalton component. Figure 4 shows that the tested proteins are antigenically indistinguishable when the antiserum against the 63 000-dalton component was used. However, when the antiserum against intact factor B was used it was apparent that the 63 000-dalton component was antigenically deficient compared with the intact factor B (Figure 4). Isolated factor B and factor B of fresh serum gave rise to reactions of complete immunological identity.

Naturally Occurring Fragment of Factor B in Urine. Urine of patients suffering from tubular proteinuria contains large amounts of low molecular weight plasma proteins which are present in serum only in trace quantities (see Peterson and Berggård, 1971). Therefore, tests were made to examine the occurrence of molecules in urine which were immunologically related to factor B. Figure 5 shows the result of a separation of urinary macromolecules on a column of Sephadex G-100. With use of an antiserum against factor B, it could be shown that factor B related material occurred mainly at two elution positions. Due to the small amounts of material available, no attempts were made at a chemical isolation of the components. However, some information was obtained using radioimmunological techniques.

Fractions comprising A and B in Figure 5 were separately pooled and concentrated. Aliquots from each fraction were

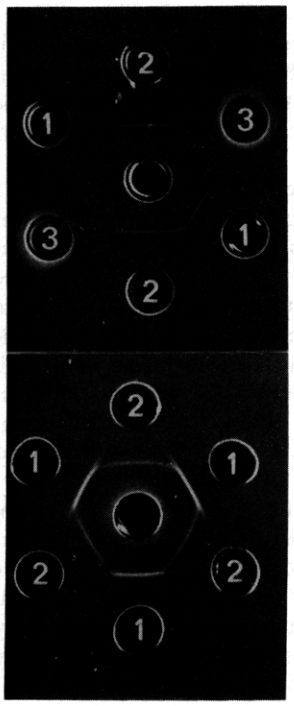

FIGURE 4: Ouchterlony immunodiffusion analysis of the highly purified factor B (1), the 63 000-dalton component (2), and normal human serum (3). (Upper) An antiserum raised against the 63 000-dalton component (center well) was used. (Lower) An antiserum against intact factor B (center well) was employed.

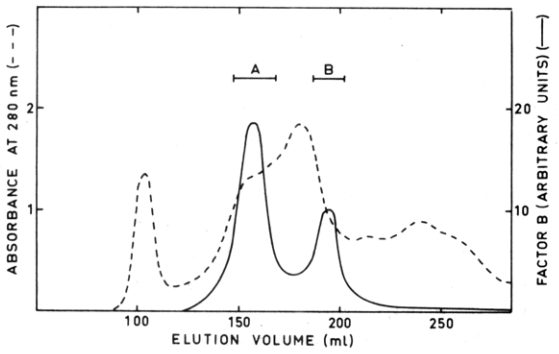

FIGURE 5: Chromatography on Sephadex G-100 of concentrated protein from pooled urine of patients with tubular proteinuria. The column (140 × 1.7 cm) was equilibrated with 0.02 M Tris-HCl buffer, pH 8.0, containing 0.15 M NaCl. Fractions of 2.2 mL were collected at 15-min intervals. The distribution in the effluent of factor B reacting material was determined by a single radial immunodiffusion technique. These estimations do not represent absolute quantities as no corrections were made for diffusion differences due to variation in the size of the reactive proteins. The eluted protein was combined into fractions A and B as denoted by the solid bars.

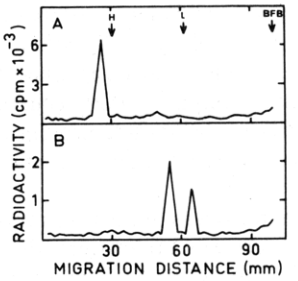

FIGURE 6: Polyacrylamide gel electrophoresis in sodium dodecyl sulfate of factor B related material in urine. The factor B containing fractions (A and B in Figure 5) were separately labeled with ^{125}I and factor B related protein was recovered from each fraction by indirect immunoprecipitation and after reduction was subjected to electrophoresis. Normal rabbit serum did not precipitate significant amounts of radioactivity. (A) Material from fraction A is depicted; (B) material from fraction B (see Figure 5) is shown. In each run, ^{131}I-labeled marker immunoglobulin heavy (H) and light (L) chains were included. BFB denotes the position of the tracking dye bromphenol blue.

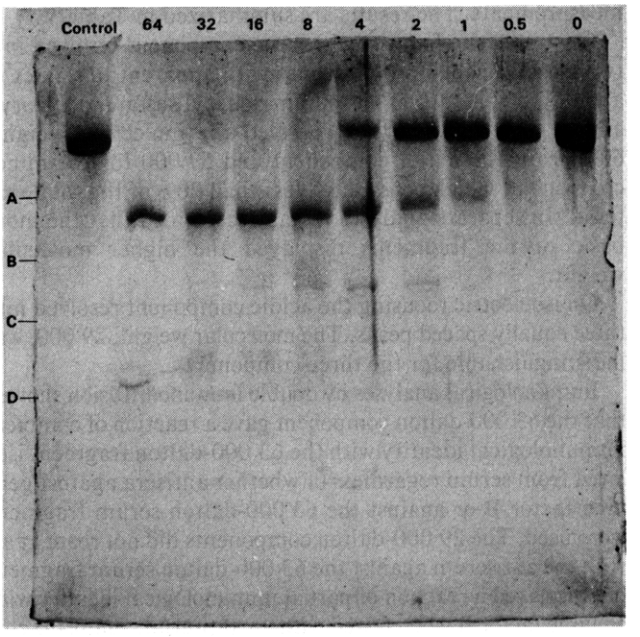

FIGURE 7: Polyacrylamide gel electrophoresis in sodium dodecyl sulfate of factor B after trypsin digestion. Samples of factor B, 1 mg/ml in 0.2 M $NaHCO_3$ (pH 8.0), were incubated for various times at 37 °C with trypsin, 4 μg/mL. The digestions were stopped by adding soybean trypsin inhibitor to the incubations and the material was subjected to electrophoresis. The figures denote incubation time in minutes. A control sample was incubated for 64 min without enzyme. The letters indicate the position of radiolabeled markers: (A) albumin (mol wt 69 000); (B) ovalbumin (43 000); (C) retinol binding protein (21 000); and (D) β_2-microglobulin (12 000).

labeled with ^{125}I and molecules reactive with antiserum against factor B or the 63 000-dalton component were separately precipitated and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The factor B related molecules in fraction A reacted similarly with either antiserum and displayed an apparent molecular weight of 60 000 (see Figure 6). Isoelectric focusing in 8 M urea revealed a single protein component with a p*I* of about 7 to 8.

No material in fraction B reacted with the antiserum against the 63 000-dalton component. The antiserum against intact factor B precipitated molecules with apparent molecular weights of 27 000 and 21 000. Both types of molecules displayed low p*I* values as evidenced by isoelectric focusing in 8 M urea. By this method each fragment resolved into at least three components, demonstrating an extensive charge heterogeneity.

Some physical-chemical data for the urinary fragments were determined and are summarized in Table V.

Limited Proteolysis of Factor B. Factor B was subjected to limited proteolytic digestion with trypsin to examine if fragments similar to those occurring naturally in serum and urine could be generated. Figure 7 shows that factor B is very sensitive toward digestion with trypsin. After a short period of exposure to the enzyme, two main fragments were generated with apparent molecular weights of about 60 000 and 30 000,

as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. On prolonged digestion the 30 000-dalton fragment disappeared gradually and a new fragment with the apparent molecular weight 17 000 occurred (see Figure 7). From the intensity of the stained protein zones, the 60 000-dalton fragment appeared highly resistant toward further proteolysis.

A sample of factor B was treated with trypsin for 8 min and the digestion mixture was exhaustively dialyzed against 0.01 M sodium acetate buffer, pH 6.0, containing 0.2 M NaCl and 5 mM EDTA. The mixture was applied on a column of sulfoethyl-Sephadex and eluted with a 200-mL linear NaCl gradient from 0.2 M to 1.5 M NaCl. As revealed by immunological analyses, factor B related material occurred at three elution positions. The major amounts of material emerged in the break-through fraction and at an apparent NaCl concentration of about 0.9 M. Minor amounts of material, representing intact factor B, appeared in the middle of the chromatogram. The acidic and basic components were separately pooled, concentrated, and subjected to some physical–chemical measurements. The results are summarized in Table V.

On isoelectric focusing the basic component resolved into two closely spaced protein zones with apparent p*I* values of about 7 to 8. Analyses on sodium dodecyl sulfate–polyacrylamide gel electrophoresis revealed the molecular weights 63 000 for the major component and 59 000 for the minor component, which constituted less than 20% of the total material. In separate analyses it was ascertained that the more basic of the fragments displayed the higher molecular weight.

On isoelectric focusing the acidic component resolved into three equally spaced peaks. The molecular weight, 29 000, was indistinguishable for the three components.

Immunological analyses by double immunodiffusion showed that the 63 000-dalton component gave a reaction of complete immunological identity with the 63 000-dalton fragment isolated from serum regardless of whether antisera against genuine factor B or against the 63 000-dalton serum fragment were used. The 29 000-dalton components did not react at all with the antiserum against the 63 000-dalton serum fragment but displayed a reaction of partial immunological identity with genuine factor B when tested with an antiserum against factor B.

NH_2 terminal amino acid analyses were performed on the 63 000-dalton fragment. Several dansylated amino acids occurred and they varied somewhat from preparation to preparation. Thus, the data suggest that the 63 000-dalton fragment is frayed in its NH_2 terminus. In contrast, all preparations of the 29 000-dalton components gave unambiguously proline as the only dansylated amino acid in addition to dansyl-ε-lysine and dansyl-*O*-tyrosine. This suggests that the 29 000-dalton fragment is derived from the NH_2-terminal portion of factor B and it appears that this region of the molecule comprises most of the charge heterogeneity.

The biological activity of the isolated fragments was assessed. Neither the basic nor the acidic components could fulfill the functions of intact factor B in the assay employed. Mixtures of the two types of fragments were also inactive.

Discussion

Factor B was isolated and obtained in highly purified form. Most of the chemical and physical–chemical data determined in this study agree well with those earlier reported (Boenisch and Alper, 1970b) but the carbohydrate composition differs significantly. Boenisch and Alper (1970b) determined the carbohydrate content to 10.2% whereas in this study the value 7.3% was obtained. The most striking difference resides in the values for sialic acid which are twice as high in our determinations. Part of the discrepancies may reside in the differences in the techniques employed but it should be noted that the figures for neutral hexoses and hexoseamines are concordant.

Based on the electrophoretic heterogeneity of factor B, a subunit structure comprising different types of polypeptide chains was proposed (Alper et al., 1972). The present results seem to rule out this possibility. Thus, molecular weight determinations of fully reduced and alkylated factor B by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and by gel chromatography in 6 M guanidine hydrochloride demonstrated that factor B most probably consists of a single polypeptide chain. Moreover, the finding that proline apparently is the only NH_2-terminal residue argues against the existence of dissimilar subunits.

A fragment of factor B, which was occasionally encountered in serum, was isolated. It displayed characteristics similar to those described for the glycine-rich γ-glycoprotein (Boenisch and Alper, 1970a). In contrast to intact factor B, the 60 000-dalton fragment was homogeneous by electrophoresis and it had a much slower mobility. This fragment also displayed characteristics similar to those noted for the larger of the two fragments generated on activation of factor B (Götze and Müller-Eberhard, 1971). Another fragment of factor B with the apparent molecular weight 47 000 was also noted in serum. Its size and charge were very similar to those of the 60 000-dalton fragment. Although the amounts available of the minor fragment precluded detailed analyses, information about its antigenicity suggests that the 47 000-dalton component most probably had arisen by further proteolytic cleavage of the 60 000-dalton fragment.

The possible existence of a fragment constituting the remaining portion of factor B on generation of the 60 000-dalton fragment was not apparent in serum. Since such a fragment, at the most, should have a molecular weight of about 30 000 its occurrence in urine was examined. It is well known that most low molecular weight plasma proteins are catabolized in the kidney proximal tubuli (see Peterson and Berggård, 1971). Therefore, an impaired tubular reabsorption of protein greatly enhances the urinary excretion of low molecular weight plasma proteins. As expected, urine of patients suffering from tubular proteinuria contained several fragments related to factor B. One of the fragments was apparently similar to the fragments isolated from serum. However, two other low molecular weight fragments were also observed. These fragments, with the apparent molecular weights 27 000 and 21 000, respectively, did not share antigenic sites with the 60 000-dalton fragment. The 27 000-dalton fragment, which in contrast to the 60 000- and 47 000-dalton fragments displayed a considerable charge heterogeneity, was acidic rather than basic in its electrophoretic behavior. This component most probably represents the second fragment which arises when factor B is cleaved to yield the 60 000-dalton species.

On activation factor B is cleaved by a serine esterase, factor D (Fearon et al., 1973), and the resulting products are a basic 60 000-dalton component and an acidic 20 000-dalton fragment (Götze and Müller-Eberhard, 1971). The present data show that limited digestion of factor B with trypsin generates similar type fragments. However, two types of basic fragments with the apparent molecular weights 63 000 and 59 000 were generated. Each basic fragment appeared relatively homogeneous by isoelectric focusing. The acidic 30 000-dalton fragment was, however, heterogeneous and resolved into three distinct components on charge separation. NH_2-terminal

amino acid analyses suggested that the 30 000-dalton fragment represents the NH_2-terminal portion of factor B. In contrast to the 60 000-dalton fragment, which appeared highly resistant toward proteolysis, the 30 000-component seemed to be further degraded by trypsin to yield a 17 000-dalton fragment. Although formal evidence is lacking, these data seem to suggest that factor B is composed of two domains of tightly folded polypeptide portions which are linked by an extended stretch of the polypeptide chain. This extended stretch of the chain is highly susceptible to proteolytic attack. It appears likely that trypsin as well as factor D preferentially cleave this part of the factor B molecule although hydrolysis may not occur at exactly the same peptide bonds. The naturally occurring fragments of factor B present in serum and urine bear a striking resemblance to those produced by limited proteolysis. Thus, it is likely that the naturally occurring fragments result from the in vivo activation of factor B.

The hemolytic activity of the serum and urinary fragments as well as of those generated by limited trypsin digestion was tested in the hemolytic diffusion plate assay. Whereas intact factor B gave rise to hemolysis none of its degradation products did. The larger of the fragments generated by inulin treatment of serum has been reported to induce cleavage of C3 (Götze and Müller-Eberhard, 1971). This observation could, however, not be corroborated in the present study. Slight differences in the methodology employed may account for the discrepancy but it is also likely that the basic fragment may undergo inactivation due to further proteolysis. This appears reasonable to suggest since several peptide bonds in factor B appear sensitive to proteolysis.

Factor B displays two types of electrophoretic heterogeneity (Alper et al., 1972). The genetic polymorphism most probably resides in the amino acid sequence. The present data suggest that the majority of the amino acid sequence differences should be looked for in the NH_2-terminal portion of factor B since at least the charge heterogeneity seems to be almost exclusively confined to this region of the molecule. The nongenetic polymorphism, which results in several electrophoretic forms of factor B, does not seem to depend on variations in the sialic acid content, as shown here and documented previously (Boenisch and Alper, 1970b). In addition, no noncovalently associated charged prosthetic groups seem to be responsible for the heterogeneity. It is possible that the heterogeneity results from the deamidation of factor B but fresh serum as well as outdated plasma give identical electrophoretic patterns. It is likely that determinations of the primary structure of at least the NH_2-terminal portion of factor B have to be performed to elucidate the origin of the heterogeneity.

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References

Alper, C. A., Boenisch, T., and Watson, L. (1972), *J. Exp. Med. 135,* 68.

Alper, C. A., Goodkofsky, I., and Lepow, I. (1973), *J. Exp. Med. 137,* 424.

Alper, C. A., and Johnson, A. M. (1969), *Vox Sang. 17,* 445.

Berggård, I. (1961), *Ark. Kemi 18,* 291.

Berggård, I., and Bearn, A. G. (1968), *J. Biol. Chem. 243,* 4095.

Boenisch, T., and Alper, C. A. (1970a), *Biochim. Biophys. Acta 214,* 135.

Boenisch, T., and Alper, C. A. (1970b), *Biochim. Biophys. Acta 221,* 529.

Fearon, D., Austen, F., and Ruddy, S. (1973), *J. Immunol. 111,* 1730.

Götze, O., and Müller-Eberhard, H. J. (1971), *J. Exp. Med. 134,* 90.

Gray, W. R. (1967), *Methods Enzymol. 11,* 469.

Haupt, H., and Heide, K. (1965), *Clin. Chim. Acta 12,* 419.

Hunter, W. M., and Greenwood, (1962), *Nature (London) 194,* 495.

Karlsson, F. A., Peterson, P. A., and Berggård, I. (1972), *J. Biol. Chem. 247,* 1065.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.

Mancini, G., Carbonara, A. O., and Heremans, J. F. (1965), *Immunochemistry 2,* 235.

Martin, A., Lachmann, P. J., Halbwachs, L., and Hobart, M. J. (1976), *Immunochemistry 13,* 317.

Müller-Eberhard, H. J. (1960), *Scand. J. Clin. Lab. Invest. 12,* 33.

Müller-Eberhard, H. J. (1971), *Prog. Immunol., 2nd 1,* 173.

Müller-Eberhard, H. J. (1975), *Annu. Rev. Biochem. 44,* 697.

Nilsson, S. F., and Peterson, P. A. (1975), *J. Biol. Chem. 250,* 8543.

O'Farrell, P. H. (1975), *J. Biol. Chem. 250,* 4007.

Östberg, L., Sege, K., Rask, L., and Peterson, P. A. (1976), *Folia Biol. (Prague) 22,* 372.

Peterson, P. A., and Berggård, I. (1971), *J. Biol. Chem. 246,* 25.

Peterson, P. A., Evrin, P. E., and Berggård, I. (1969), *J. Clin. Invest. 48,* 1189.

Scheidegger, J. J. (1955), *Int. Arch. Allergy Appl. Immunol. 7,* 103.

Woods, K. R., and Wang, K.-T. (1967), *Biochim. Biophys. Acta 133,* 369.