Awdeh, Z. L., Williamson, A. R., & Askonas, B. A. (1970) *Biochem. J. 116*, 241.
Bilezekian, S. B., Nossel, H. L., Butler, V. P., Jr., & Canfield, R. E. (1975) *J. Clin. Invest. 56*, 438.
Blombäck, B. (1967) in *Blood Clotting Enzymology* (Seegers, W. H., Ed.) p 143, Academic Press, New York.
Blombäck, B., Blombäck, M., Edman, P., & Hessel, B. (1966) *Biochim. Biophys. Acta 115*, 1966.
Blombäck, B., Hessel, B., Hogg, D., & Therkildsen, L. (1978) *Nature (London) 275*, 501.
Briles, D. E., & Davie, J. M. (1975) *J. Immunol. Methods 8*, 363.
Butler, V. P., Jr., Weber, D. A., Nossel, H. L., LaGamma, K. S., & Canfield, R. E. (1978) *Circulation, Suppl. 58*, 461.
Canfield, R. E., Dean, J., Nossel, H. L., Butler, V. P., Jr., & Wilner, G. D. (1976) *Biochemistry 15*, 1203.
Civin, C. I., Levine, H. B., Williamson, A. R., & Schlossman, S. F. (1976) *J. Immunol. 116*, 1400.
Doolittle, R. F. (1975) *Plasma Proteins 2*, 109.
Eisen, H. N. (1964) *Methods Med. Res. 10*, 106.
Furie, B., Schechter, A. N., Sachs, D. H., & Anfinsen, C. B. (1974) *Biochemistry 13*, 1561.
Furie, B., Provost, K. L., Blanchard, R. A., & Furie, B. C. (1979) *J. Biol. Chem. 254*, 8980.
Haber, E., Richards, F. F., Spragg, J., Austen, K. F., Vallotton, M., & Page, L. B. (1967) *Cold Spring Harbor Symp. Quant. Biol. 32*, 299.
Huseby, R. M. (1973) *Physiol. Chem. Phys. 5*, 1.
Keck, K., Grossberg, A. L., & Pressman, D. (1973) *Immunochemistry 10*, 331.
Kipps, T. J., Benacerraf, B., & Dorf, M. E. (1978) *Proc. Natl. Acad. Sci. U.S.A 75*, 2914.
Maron, E., Shiozawa, C., Arnon, R., & Sela, M. (1971) *Biochemistry 10*, 763.
Nicolotti, R. A., Briles, D. E., Schroer, J., & Davie, J. M. (1979) in *Electrofocus '78*, American Elsevier, New York, (in press).
Nossel, H. L., Butler, V. P., Jr., Wilner, G. D., Canfield, R. E., & Harfenist, E. J. (1976) *Thromb. Haemostasis 35*, 101.
Sachs, D. H., Schechter, A. N., Eastlake, A., & Anfinsen, C. B. (1972a) *J. Immunol. 109*, 1360.
Sachs, D. H., Schechter, A. N., Eastlake, A., & Anfinsen, C. B. (1972b) *Biochemistry 11*, 4268.
Sachs, D. H., Schechter, A. N., Eastlake, A., & Anfinsen, C. B. (1972c) *Proc. Natl. Acad. Sci. U.S.A. 69*, 3790.
Shainoff, J. R., & Dardik, B. N. (1979) *Science 204*, 200.
Wilner, G. D. (1978) *Prog. Hemostasis Thromb. 4*, 211.
Wilner, G. D., Nossel, H. L., Canfield, R. E., & Butler, V. P., Jr. (1976) *Biochemistry 15*, 1209.

Human Factor $\bar{D}$ of the Alternative Complement Pathway. Physicochemical Characteristics and N-Terminal Amino Acid Sequence†

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ABSTRACT: Factor $\bar{D}$ is a plasma serine protease which is required for normal complement activation via the alternative pathway. Factor $\bar{D}$ was isolated from human plasma by sequential CM-Sephadex C-50, Sephadex G-75, and heparin–Sepharose chromatography. Isolated factor $\bar{D}$ (20 μg) showed a single protein band (molecular weight 25000) in the presence of 2-mercaptoethanol, when stained with either Coomassie Brilliant Blue or periodic acid–Schiff's reagent. Isoelectric focusing of purified factor $\bar{D}$ demonstrated two protein bands with isoelectric points at pH 7.0 and pH 6.6. Both bands displayed factor $\bar{D}$ hemolytic activity and both were also detected in normal human serum. On agarose gel electrophoresis, factor $\bar{D}$ (both purified and in serum) was of γ electrophoretic mobility in the presence of 1.8 mM calcium. In the presence of EDTA (5 mM), isolated factor $\bar{D}$ was of β mobility, while factor $\bar{D}$ in serum was of α_1-α_2 mobility. Eleven synthetic ester and nitroanilide substrates were evaluated for hydrolysis by factor $\bar{D}$. Among these, only the factor X_a substrate *N*-benzoylisoleucylglutamylarginine-*p*-nitroanilide hydrochloride was hydrolyzed by factor $\bar{D}$. Minimum molecular weight was 22000; no unusual characteristics in amino acid composition were revealed. Amino-terminal amino acid sequence was determined by manual Edman degradation. The amino-terminal sequence of factor $\bar{D}$ is Ile-Leu-Gly-Gly-Arg-Glx-Ala-Glx-Ala–. Factor $\bar{D}$, therefore, is distinct from, but homologous with, other plasma serine proteases, including thrombin, $C1\bar{r}$, $C1\bar{s}$, coagulation factors X_a and $X1_a$, and plasmin.

Factor $\bar{D}$ is a plasma protease which catalyzes the proteolysis of factor B and is required for normal complement activation via the alternative pathway (Alper & Rosen, 1971; Götze & Müller-Eberhard, 1971; Müller-Eberhard & Götze, 1972; Hunsicker et al., 1973). It consists of a single polypeptide chain with a molecular weight of 25000 (Fearon et al., 1974; Götze, 1976; Dieminger et al., 1976; Brade et al., 1974a,b; Volanakis et al., 1977; Lesavre & Müller-Eberhard, 1978; Götze, 1975). The results of various studies differ regarding the electrical charge of factor $\bar{D}$, whether determined by electrophoresis or isoelectric focusing (Fearon & Austen, 1975; Müller-Eberhard & Götze, 1972; Hunsicker et al., 1973; Lesavre & Müller-Eberhard, 1978; Konno et al., 1978; Martin et al., 1976; Götze & Müller-Eberhard, 1977; Davis et al., 1978). Factor B and C3b, in the presence of magnesium ions, form a reversible complex; this results in a conformational alteration of factor B, which allows its cleavage by factor $\bar{D}$

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(Medicus et al., 1976; Vogt et al., 1975, 1974). This limited proteolysis of factor B results in two fragments, Ba (molecular weight 30 000) and Bb (molecular weight 70 000). The Bb fragment, with C3b, forms the alternative pathway C3 and C5 cleaving enzymes (C3 and C5 convertases) C3bBb and $C3b_nBb$, respectively (Müller-Eberhard & Götze, 1972; Fearon et al., 1973; Medicus et al., 1976; Daha et al., 1976).

Factor D̄ is inactivated by diisopropylphosphorofluoridate (DFP)(at relatively high concentrations) and is therefore considered to be a serine protease (Fearon et al., 1974; Lesavre & Müller-Eberhard, 1978; Götze, 1975). Covalent binding of DFP to factor D̄ has not, however, been described. Factor D̄ has an N-terminal isoleucine, as do many other serine proteases (Volanakis et al., 1977). An arginyl–lysine peptide bond in factor B is cleaved by factor D̄, which is also consistent with the suggestion that factor D̄ is a serine protease (Lesavre and Müller-Eberhard, personal communication). Hydrolysis of synthetic ester substrates has been suggested in some reports (Dieminger et al., 1976; Volanakis et al., 1977), while a lack of activity has been reported by others (Lesavre & Müller-Eberhard, 1978). A zymogen form of factor D̄, which is resistant to DFP, has been isolated from human plasma by Fearon et al. (1974). The zymogen was activated by incubation with trypsin. The physiologic mechanism leading to activation has not been defined. Other data have suggested that only enzymatically active factor D̄ is present in plasma (Lesavre & Müller-Eberhard, 1978). Factor D̄, unlike other complement proteins, does not become physically incorporated into the alternative pathway convertases (Lesavre & Müller-Eberhard, 1978).

We have previously shown that thrombin has factor D̄ functional activity in an assay system utilizing factor D̄ depleted serum and that factor D̄ and thrombin share antigenic determinants (Davis et al., 1978). We suggested, therefore, that either factor D̄ and thrombin were closely related proteases or factor D̄ might be a fragment of thrombin.

The present studies were undertaken to clarify the physicochemical characteristics of purified factor D̄ and to begin to precisely define its relationship with other plasma proteases by determination of its amino acid sequence.

Experimental Procedures

Materials

Fresh frozen human plasma was obtained from American Red Cross Blood Services, Northeast Region. CM-Sephadex C-50, Sephadex G-75, and Sepharose 6B were purchased from Pharmacia Fine Chemicals, Piscataway, NJ. Amicon positive-pressure ultrafiltration cells and PM10 ultrafiltration membranes were purchased from Amicon Corp., Lexington, MA. Heparin–Sepharose (Rosenberg & Damus, 1973) was kindly provided by Dr. Robert Rosenberg. Agarose was obtained from Marine Colloids, Rockland, ME. Acrylamide, *N,N'*-methylenebis(acrylamide), and *N,N,N',N'*-tetramethylethylenediamine were purchased from Eastman Kodak Co., Rochester, NY. Ampholines and sodium dodecyl sulfate were purchased from Bio-Rad Laboratories, Richmond, CA. Molecular weight markers for sodium dodecyl sulfate–polyacrylamide gel electrophoresis were as follows: insulin, purchased from Sigma Chemical Co., St. Louis, MO; BDH cross-linked markers (molecular weight range 14 300–71 500), which were obtained from BDH Chemicals Ltd., Pool BH124NN, England.

N-Acetyl-L-arginine methyl ester hydrochloride (AcArgOMe) and N^{α}-acetylglycyl-L-lysine methyl ester acetate (AcGlyLysOMe) were purchased from Cyclochemical Division of Travenol Laboratories, Inc., Los Angeles, CA. *N*-Acetyl-L-tyrosine ethyl ester (AcTyrOEt) was obtained from Pierce Chemical Co., Rockford, IL. *p*-Tosyl-L-arginine methyl ester hydrochloride (TsArgOMe) was purchased from Mann Research Laboratories, NY. N^{α}-Benzoyl-DL-arginine-*p*-nitroanilide hydrochloride (BzArgPNA) and N^{α}-benzoyl-L-arginine ethyl ester hydrochloride (BzArgOEt) were from Aldrich Chemical Co., Milwaukee, WI. *N*-(Carbobenzyloxy)-L-tyrosine *p*-nitrophenyl ester (ZTyrONp) was purchased from Nutritional Biochemical Corp., Cleveland, OH. N^{α}-(Carbobenzyloxy)-L-lysine *p*-nitrophenyl ester (ZLysONp) was purchased from Sigma. *N*-Benzoylphenylalanylvalylarginine-*p*-nitroanilide hydrochloride (S2160), D-phenylalanylpiperazinylarginine-*p*-nitroanilide hydrochloride (S2238) and *N*-benzoylisoleucylglutamylglycylarginine-*p*-nitroanilide hydrochloride (S2222) were products of Kabi Diagnostic, Stockholm, Sweden. Bovine trypsin was from Sigma. Human thrombin was a gift from Dr. John Fenton, II (New York State Department of Health, Albany, NY). $C1\bar{r}$ and $C1\bar{s}$ were kindly provided by Dr. Judith Andrews (Assimeh et al., 1974; Taylor et al., 1977). Human factor X deficient plasma was purchased from George King Bio-Medical, Inc., Overland Park, KS.

Methods

Protein Purification. Factor B and cobra venom factor were purified by published methods (Kerr & Porter, 1978; Ballow & Cochrane, 1969). Factor D̄ was isolated from 4 L of fresh frozen human plasma by chromatography on a 2-L column of CM-Sephadex C-50 equilibrated with 0.05 M sodium phosphate, 0.01 M disodium ethylenediaminetetraacetate (EDTA), and 0.2 M NaCl, pH 6.0. Factor D̄ was eluted with an 8-L linear concentration gradient to 2.0 M NaCl. Factor D̄ containing fractions were concentrated by positive-pressure ultrafiltration and applied to an 8 × 90 cm Sephadex G-75 column equilibrated in 0.05 M barbital, 0.3 M NaCl, and 0.01 M EDTA, pH 7.4. Final purification was obtained by further gel filtration on a 2.5 × 190 cm Sephadex G-75 column. When necessary, factor D̄ preparations were further subjected to chromatography on heparin–Sepharose equilibrated in 0.05 M imidazole hydrochloride and 0.0025 M $CaCl_2$, pH 6.0. Under these conditions, factor D̄ binds to heparin–Sepharose and, after being washed with 10 column volumes of starting buffer, is eluted with a linear NaCl concentration gradient to 0.5 M. Protein concentration of isolated factor D̄ was determined by the technique of Lowry et al. (1951).

Factor D̄ Functional Assays. Factor D̄ activity was monitored during purification by the lysis of guinea pig erythrocytes in the presence of factor D̄ depleted serum in agarose gels (Martin et al., 1976). Factor D̄ depleted serum was prepared by gel filtration of normal human serum on Sephadex G-75 (Martin et al., 1976). Functional activity of isolated factor D̄ was confirmed by demonstration of cleavage of isolated factor B in the presence of cobra venom factor by crossed immunoelectrophoresis. Quantitative factor D̄ hemolytic titrations were performed by incubation of 0.1-mL dilutions of factor D̄ containing samples with 10^7 C3b-coated sheep erythrocytes (Lachmann & Hobart, 1978) for 30 min at 30

[1] Abbreviations used: DFP, diisopropylphosphorofluoridate; NaDodSO$_4$, sodium dodecyl sulfate; EDTA, disodium ethylenediaminetetraacetate; AcArgOMe, *N*-acetyl-L-arginine methyl ester hydrochloride; BzArgOEt, N^{α}-benzoyl-L-arginine ethyl ester hydrochloride; AcGlyLysOMe, N^{α}-acetylglycyl-L-lysine methyl ester acetate; AcTyrOEt, *N*-acetyl-L-tyrosine ethyl ester; TsArgOMe, *p*-tosyl-L-arginine methyl ester hydrochloride; BzArgPNA, N^{α}-benzoyl-DL-arginine-*p*-nitroanilide hydrochloride; ZTyrONp, *N*-(carbobenzyloxy)-L-tyrosine *p*-nitrophenyl ester; ZLysONp, N^{α}-(carbobenzyloxy)-L-lysine *p*-nitrophenyl ester.

Table I: Synthetic Substrates

N-acetyl-L-arginine methyl ester (AcArgOMe)
N^{α}-benzoyl-L-arginine ethyl ester (BzArgOEt)
N^{α}-acetylglycyl-L-lysine methyl ester (AcGlyLysOMe)
N-acetyl-L-tyrosine ethyl ester (AcTyrOEt)
p-tosyl-L-arginine methyl ester hydrochloride (TsArgOMe)
N-(carbobenzyloxy)-L-tyrosine *p*-nitrophenyl ester (ZTyrONp)
N^{α}-(carbobenzyloxy)-L-lysine *p*-nitrophenyl ester (ZLysONp)
N^{α}-benzoyl-DL-arginine-*p*-nitroanilide hydrochloride (BzArgPNA)
N-benzoylisoleucylglutamylglycylarginine-*p*-nitroanilide hydrochloride (S2222)
D-phenylalanylpiperazinylarginine-*p*-nitroanilide hydrochloride (S2238)
N-benzoylphenylalanylvalylarginine-*p*-nitroanilide (S2160)

°C, in the presence of excess purified factor B and partially purified C3 nephritic factor (Fearon et al., 1973). Incubations were performed in half isotonic barbital buffered saline containing 2.5% dextrose, 0.1% gelatin, 0.5 mM $MgCl_2$, and 0.15 mM $CaCl_2$, pH 7.5. Alternative pathway convertase sites were then developed by incubation for 60 min at 37 °C with 0.3 mL of rat serum diluted 1:20 in isotonic barbital buffered saline containing 2.5% dextrose, 0.1% gelatin, and 0.04 M EDTA. The average number of hemolytic sites per cell was calculated from the extent of hemolysis as previously described (Rapp & Borsos, 1970; Fearon et al., 1973).

Electrophoresis and *Isoelectric Focusing.* Twelve percent $NaDodSO_4$–polyacrylamide gel electrophoresis was performed according to the method of Laemmli (1970). Agarose gel electrophoresis was performed in 0.8% agarose 0.05 M barbital buffer, pH 8.6, containing either 1.8 mM calcium lactate or 5 mM EDTA. Isoelectric focusing in thin-layer polyacrylamide gel was performed as previously described (Audeh et al., 1968). Two percent ampholines in a ratio of three parts pH 5–7, three parts pH 7–9, and one part pH 3–10 were incorporated in 5% polyacrylamide gel with 0.2 M taurine.

Esterolytic Assays. Factor $\bar{D}$ was analyzed for the ability to hydrolyze each of the synthetic substrates listed in Table I. Hydrolysis of AcArgOMe, BzArgOEt, AcGlyLysOMe, and AcTyrOEt was analyzed by the colorimetric method of Roberts (1958). Each ester was used at a concentration of 0.01 M in 0.1 M Tris-HCl and 1 mM $CaCl_2$, pH 8.2. Factor $\bar{D}$, trypsin, thrombin, $C1\bar{r}$, and $C1\bar{s}$ were incubated with each ester in a volume of 1.025 mL for 60 min at 37 °C. Hydrolysis of TsArgOMe was determined by spectrophotometric assay (Walsh, 1970). Assays with ZTyrONp and ZLysONp were performed by spectrophotometric determination of released *p*-nitrophenol (Andrews et al., 1978). ZTyrONp was used at a concentration of 0.03 mM in a final volume of 1.0 mL in 0.05 M Tris–acetate and 0.1 M NaCl, pH 8.1. ZLysONp was used at the same concentration and volume in 0.1 M acetate, pH 6.0. The rate of hydrolysis was monitored on a Gilford 240N spectrophotometer with continuous recording on a Heath SR-255B recorder. The release of *p*-nitrophenol was monitored at 410 nm for ZTyrONp and at 340 nm for ZLysONp. Hydrolysis of the nitroanilide substrates BzArgPNA, S2222, S2238, and S2160 was evaluated in 0.05 M Tris-HCl and 0.227 M NaCl, pH 8.3, at substrate concentrations of 0.05 mM for BzArgPNA, 0.1 mM for S2160 and S2238, and 1 mM for S2222. Assays were performed by spectrophotometric determination at 410 nm of liberated *p*-nitroaniline during incubation with enzyme at 23 °C in a Cary Model 118C spectrophotometer (Varian Associates). All the described esterolytic assays were performed with isolated factor $\bar{D}$ (and control proteases) at a concentration of 0.5 mM. Assay for factor X activity was performed by reconstitution of the Quick prothrombin time of factor X deficient plasma.

Amino Acid Analysis. Samples were hydrolyzed in 6 N HCl at 110 °C for 24 h under vacuum (Moore & Stein, 1963). Amino acids were identified on a Beckman 121 MB amino acid analyzer equipped with a system AA integrator (Spectro-Physics), which monitored at 570 and 400 nm at 0.1 OD maximum sensitivity. A high-speed 60-min single-column (AA10 resin) program was followed. No corrections were made for destruction of threonine, serine, and tyrosine. Tryptophan and cysteine were not determined.

Amino Acid Sequence Analysis. Amino-terminal sequence analysis was performed by a three-stage manual Edman degradation essentially as described by Schlessinger & Hay (1977). Back-hydrolysis of thiazolinone amino acids was carried out according to the procedure of Mendez & Lai (1975) using 1% $SnCl_2$ in constant boiling HCl under vacuum for 18–24 h at 150 °C. With these methods, the first nine residues were determined with 10 nmol of isolated factor $\bar{D}$. The sequence was confirmed with two additional purified factor $\bar{D}$ preparations.

Results

Factor $\bar{D}$ Purification. The purification procedure described resulted in the isolation of 1–1.5 mg of factor $\bar{D}$ from 4 L of starting plasma. Yields were thus 12–18%, assuming a normal plasma concentration of 2 μg/mL (Lesavre & Müller-Eberhard, 1978). Yields, as determined by hemolytic assay, ranged from 15 to 20%; specific activity of purified factor $\bar{D}$ averaged 15 000 effective hemolytic molecules per μg. The use of fresh frozen plasma without prior dialysis together with only one ion-exchange resin allowed the entire purification to be completed in 5–6 days. The use of more than one ion-exchange column frequently resulted in apparent aggregation of purified factor $\bar{D}$ as judged by isoelectric focusing. These preparations did not focus but showed numerous bands of factor $\bar{D}$ activity near the point of application on the polyacrylamide gel (data not shown). Prolongation of the time required for purification resulted in contamination by proteins (or protein fragments) with molecular weights in the range of 10 000–15 000 (as determined by 12% $NaDodSO_4$–polyacrylamide gel electrophoresis). These low molecular weight contaminants were removed by chromatography on heparin–Sepharose as described under Methods. This final procedure was required with approximately one in every five preparations.

Physicochemical Characteristics of Isolated Factor $\bar{D}$. When subjected to 12% $NaDodSO_4$–polyacrylamide gel electrophoresis, isolated factor $\bar{D}$ (20 μg) showed greater than 90% of the Coomassie Brilliant Blue stainable protein within a single band (Figure 1). Molecular weight, determined on 12% $NaDodSO_4$–polyacrylamide gel electrophoresis, was 25 000, both in the presence and in the absence of 2.5% mercaptoethanol. This molecular weight is in agreement with that determined by amino acid analysis (see below). Factor $\bar{D}$, following $NaDodSO_4$–polyacrylamide gel electrophoresis, was weakly stained by periodic acid–Schiff's reagent (Zacharius et al., 1969), indicating the presence of a small amount of carbohydrate. Purified factor $\bar{D}$ subjected to isoelectric focusing in polyacrylamide slab gel demonstrated two stainable protein bands; a major band was present at pH 7.0 and a less intensely staining band was present at pH 6.6. Each band had factor $\bar{D}$ activity when the gel was overlayed with agarose gel containing guinea pig erythrocytes and factor $\bar{D}$ depleted serum (Figure 2) (Martin et al., 1976). Both bands of factor $\bar{D}$ activity were also detected in normal human serum (Figure 2).

Figure 3 shows a total protein stain of purified factor $\bar{D}$ compared with normal serum following agarose gel electro-

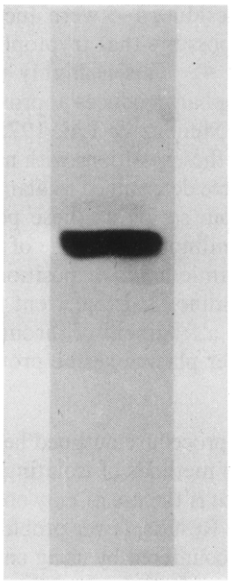

FIGURE 1: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of human factor D̄. Twenty micrograms of factor D̄ was applied to the gel without reducing agent. Electrophoresis was performed in 12% polyacrylamide gel at 30 mA/gel. The anode was to the bottom.

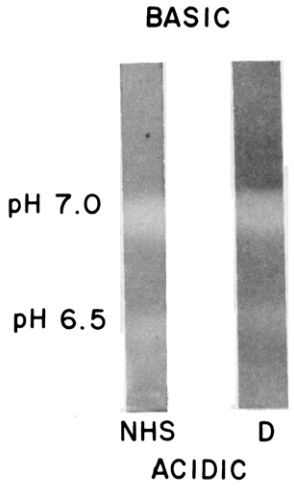

FIGURE 2: Normal human serum and isolated factor D̄ were subjected to isoelectric focusing in 4% polyacrylamide gel with a pH range of 3–10. Following isoelectric focusing, the gel was overlayed with 0.8% agarose gel, 1.7% guinea pig erythrocytes, and 5% factor D̄ depleted serum in barbital-buffered saline containing 5 mM $MgCl_2$ and 10 mM ethylenebis(oxyethylenenitrilo)tetraacetic acid, pH 7.3. Gels were then incubated for 1–2 h at 37 °C until development of the factor D̄ dependent bands of lysis. The anode was to the bottom.

phoresis in the presence and absence of calcium. In Figure 4A, hemolytic activity of isolated factor D̄ was plotted following elution from fractionated agarose gels. As shown, factor D̄ hemolytic activity corresponds in electrophoretic mobility to the stained protein bands shown in Figure 3. In the presence of 1.8 mM calcium, isolated factor D̄ is of γ electrophoretic mobility; while in the presence of 5 mM EDTA, it is of β mobility. Factor D̄, therefore, appears to bind calcium ions. Normal human serum was also subjected to preparative agarose gel electrophoresis (Figure 4B). As shown, in the presence of calcium, factor D̄ activity in serum has the same electrophoretic mobility as does isolated factor D̄. However, in the presence of EDTA, factor D activity in serum is more anodal than that of isolated factor D̄. Isolated factor D̄, when added to factor D̄ depleted serum, exhibited the same more anodal electrophoretic mobility (data not shown) as did factor D̄ in serum.

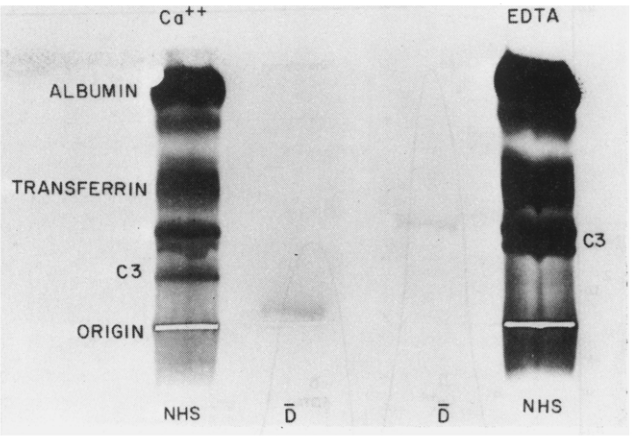

FIGURE 3: Agarose gel electrophoresis of normal human serum and isolated factor D̄ in 0.05 M barbital buffer, pH 8.6, containing either 1.8 mM calcium lactate or 5 mM EDTA. Gels were stained with Coomassie Brilliant Blue. The anode was to the top.

Table II: Factor D̄ Amino Acid Analysis[a]

	residues/molecule	residues/100 residues: this study	residues/100 residues: Volanakis et al. (1977)
Asp	18–19	9.1	7.4
Thr	8	4.1	4.3
Ser	14–15	7.1	7.6
Glu	18	9.1	9.8
Pro	14	7.1	8.7
Gly	21	10.7	9.8
Ala	21	10.7	5.8
Val	16	8.1	6.8
Met	3	1.5	1.4
Ile	5	2.5	6.0
Leu	23	11.7	6.9
Tyr	4	2.0	1.1
Phe	1	0.5	2.0
Lys	7	3.6	7.1
His	8	4.1	7.0
Arg	16	8.1	5.5

[a] Tryptophan and cysteine were not determined.

Esterolytic Activity of Factor D̄. Among the 11 synthetic substrates tested (Table I), factor D̄ was active only against the coagulation factor X_a nitroanilide substrate, S2222 (Aurell et al., 1977). Factor D̄, at a concentration of 0.15 mM, hydrolyzed 670 mol of substrate per mol of factor D̄ per min. Since this small amount of hydrolysis could be due to minor contamination of factor D̄ with factor X_a, isolated factor D̄ (20 μg) was tested for factor X_a activity. No factor X or X_a activity could be detected. In addition, factor D̄ (20 μg) did not induce fibrinogen clotting in normal plasma when incubated for as long as 24 h.

Amino Acid Analysis and N-Terminal Amino Acid Sequence. Amino acid analysis of isolated factor D̄ is shown in Table II, in comparison with that reported by Volanakis et al. (1977). Several significant differences in amino acid content between the two analyses are apparent. The reasons for these differences are not clear. Amino acid analysis has been performed on three different factor D̄ preparations, and no significant differences were noted. Minimum molecular weight calculated from the amino acid composition was 22 000.

The N-terminal amino acid sequence of factor D̄ is shown in Table III, in comparison with several other complement and coagulation proteases. These data were obtained with 10 nmol of starting material. Yields obtained at each step (in number of nanomoles) following back-hydrolysis were 8, 8, 9, 8, 5, 5, 6, 4, and 4. Background amino acids at each step included

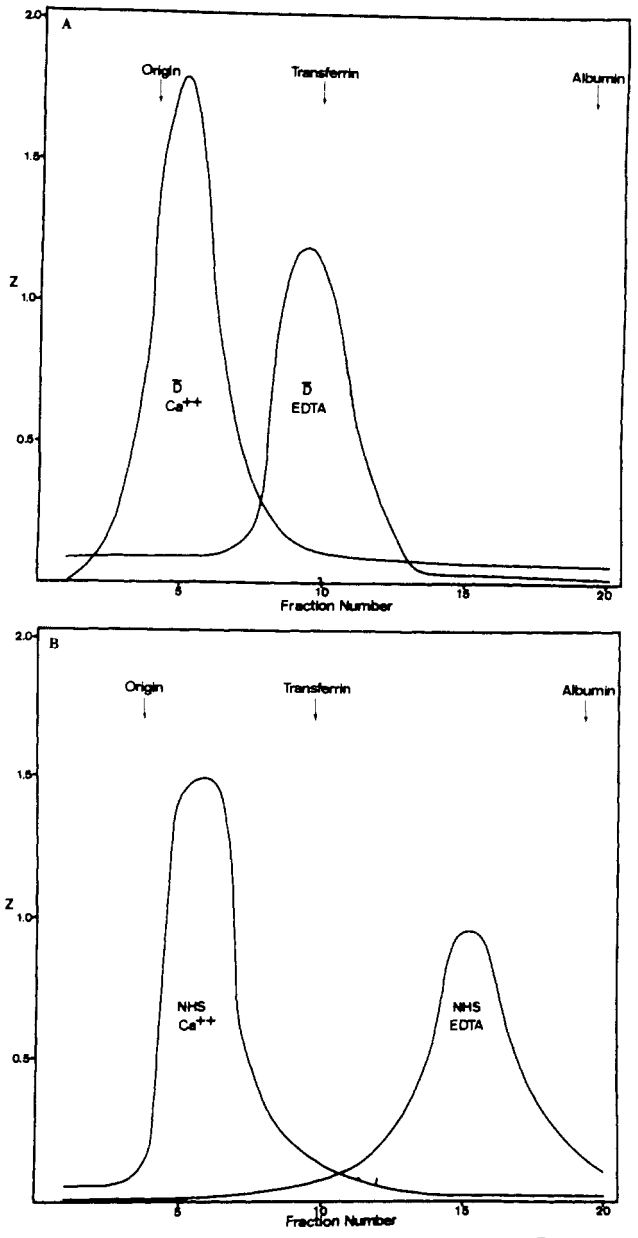

FIGURE 4: Agarose gel electrophoresis of isolated factor D̄ (A) and normal human serum (NHS) (B) in the presence and absence of calcium. Conditions were the same as those described in the legend of Figure 3. Following electrophoresis, gels were sliced into 2.5-mm fractions, and protein was eluted by freezing and thawing. Eluates were tested for factor D̄ activity as described under Methods, and the number of effective hemolytic molecules per cell (Z) was calculated. Positions of the origin, transferrin, and albumin are indicated by arrows. The anode was to the right.

Table III: Factor D̄ Amino Terminal Sequence

	1 2 3 4 5 6 7 8 9 10
factor D̄	Ile-Leu-Gly-Gly-Arg-Glx-Ala[a]-Glx-Ala[a]
thrombin[b]	Ile-Val-Glu-Gly-Ser-Asn-Ala - Glu-Ile - Gly
factor X_a[c]	Ile-Val-Gly-Gly-Glu-Glu-Cys - Lys-Asp-Gly
factor $X1_a$[d]	Ile-Val-Gly-Gly-Thr-Val
C1r̄ b chain[e]	Ile-Ile-Gly-Gly-Glu-Lys-Ala - Lys-Met-Gly
C1s̄ b chain[e]	Ile-Ile-Gly-Gly-Ser-Asp-Ala - Asp-Ile - Lys

[a] Or serine. [b] Elion et al. (1977). [c] Di Scipio et al. (1977). [d] Kurachi & Davie (1977). [e] Sim et al. (1977).

only those amino acids which are present in the largest quantities in the protein (glycine, alanine, valine, aspartic acid, glutamic acid, leucine, and proline). No greater than 1 nmol of any background amino acid was detected at any position. Identical results have been obtained with three different factor D̄ preparations. Residues 1–5 were unequivically identified. It is theoretically possible that tryptophan could be present at positions 3 and 4. This is highly unlikely since back-hydrolysis of tryptophan produces approximately 70% glycine and 30% alanine (Mendez & Lai, 1975); only glycine was detected at each of these positions with no increase in alanine. Positions 7 and 9 were determined as alanine, but alanine could also be derived from serine at these positions, since back-hydrolysis of the anilinothiazolinone of serine gives alanine. Likewise, the glutamic acids at positions 6 and 8 could be derived from glutamine. It is apparent from these data that factor D̄, while not a fragment of thrombin, definitely is homologous with other plasma serine proteases.

Discussion

The purification procedure outlined here is similar in many respects to previous methods of isolating factor D̄. The most significant difference is the use of only one ion-exchange resin. As presented under Results, fewer problems with aggregation of factor D̄ were encountered by using only the CM-Sephadex and avoiding the use of further ion-exchange steps. In addition, both yield and purity were improved by completing the purification as rapidly as possible. The yields obtained with the purification procedure described here (1–2 mg/4 L of plasma) are also roughly equivalent to yields obtained with previously described methods. Volanakis et al. (1977) report that 0.646 mg were obtained from 2.8 L of plasma while Lesavre & Müller-Eberhard (1978) obtained 0.5 mg from 1.6 L. In general, the physicochemical characteristics of factor D̄ reported here are similar to those from previous reports. The fact that factor D̄ is a glycoprotein as determined by positive PAS staining has not been previously reported. In addition, the isoelectric point which we have determined for both isolated factor D̄ and for factor D̄ in serum is somewhat lower than previous determinations have suggested (Martin et al., 1976; Fearon & Austen, 1975). These differences are probably due to methodological differences in pH determination of the isofocused gels.

Binding of calcium by factor D̄ has not been noted previously. Earlier reports have commented upon the apparent incompatibility of the isoelectric point of factor D̄ with its electrophoretic mobility (Martin et al., 1976; Götze & Müller-Eberhard, 1977). Also, one previous study has pointed out that isolated factor D̄ differs in electrophoretic mobility from factor D̄ in serum (Konno et al., 1978). Our results confirm this finding and also demonstrate that this difference only occurs in the absence of calcium. As indicated by the change in electrophoretic mobility, factor D̄ binds calcium. In other experiments (data not shown), $MgCl_2$ (5 mM) had precisely the same effect upon the electrophoretic mobility of isolated factor D̄ as did calcium. In the absence of calcium and magnesium, factor D̄ in serum (or factor D̄ added to D̄ depleted serum) probably binds to an as yet unidentified protein (Konno et al., 1978).

Factor D̄ does not hydrolyze any of the seven synthetic ester substrates tested, which confirms the previous results of Lesavre & Müller-Eberhard (1978) and differs from those of Volanakis et al. (1977). However, among the four nitroanilides evaluated, the factor X_a substrate, *N*-benzoylisoleucylglutamylglycylarginine-*p*-nitroanilide hydrochloride, was hydrolyzed, although at a very low initial rate. The hydrolysis was not due to any identifiable contamination with coagulation factor X_a or thrombin. The sequence of amino acids surrounding the arginyl–lysine bond in factor B, which is cleaved by factor D̄, was recently reported to be Gln-Lys-Arg-Lys-Ile-Val (Lesavre and Müller-Eberhard, personal communica-

tion). This sequence has a glutamine in the same relative position as the glutamic acid in the S2222 substrate. This structural similarity may be related to the stability of factor $\bar{D}$ to hydrolyze this substrate, although there are obvious size and charge differences between the two sequences. Resolution of this problem will require the use of synthetic peptide substrates which more closely mimic the sequence of factor B.

We have previously found that factor $\bar{D}$ and human β/γ thrombin (but not α thrombin) cross-react immunochemically by using antisera to both factor $\bar{D}$ and to prothrombin (Davis et al., 1978). This finding, combined with the finding that thrombin in high concentrations had factor $\bar{D}$ like activity, prompted us to suggest the possibility that factor $\bar{D}$ might be a thrombin fragment or that they might be closely related proteases. Lesavre & Müller-Eberhard (1978), using antiserum to factor $\bar{D}$, found no cross-reaction between $\bar{D}$ and α thrombin. Similar cross-reaction between thrombin and factor X_a has been previously reported (Fujikawa et al., 1974). The amino acid sequence data reported here demonstrate that factor $\bar{D}$ is not a fragment of thrombin. However, as shown in Table III, factor $\bar{D}$ shows definite homology with the other plasma proteases listed. On the basis of their analogous functions in the classical and alternative pathways, $C1\bar{s}$ and factor $\bar{D}$ might be expected to be highly homologous. Further sequence data are required before the degree of homology between these complement and coagulation proteases can be determined.

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References

Alper, C. A., & Rosen, F. S. (1971) *Adv. Immunol. 18*, 251.
Andrews, J. M., Roman, D. P., Jr., Bing, D. H., & Cory, M. (1978) *J. Med. Chem. 21*, 1202.
Assimeh, S. N., Bing, D. H., & Painter, R. H. (1974) *J. Immunol. 113*, 225.
Audeh, Z. L., Williamson, A. R., & Askonas, B. A. (1968) *Nature (London) 219*, 66.
Aurell, L., Friberger, P., Karlsson, G., & Claeson, G. (1977) *Thromb. Res. 11*, 595.
Ballow, M., & Cochrane, C. G. (1969) *J. Immunol. 103*, 944.
Brade, V., Lee, G. D., Nicholson, A., Shin, H. S., & Mayer, M. M. (1974a) *J. Immunol. 111*, 1389.
Brade, V., Nicholson, A., Lee, G. D., & Mayer, M. M. (1974b) *J. Immunol. 112*, 1845.
Daha, M. R., Fearon, D. T., & Austen, K. F. (1976) *J. Immunol. 117*, 630.
Davis, A. E., III, Rosenberg, R. D., Fenton, J. W., II, Bing, D. H., Rosen, F. S., & Alper, C. A. (1978) *J. Immunol. 120*, 1771.
Dieminger, L., Vogt, W., & Lynen, R. (1976) *Z. Immunitaetsforsch., Exp. Klin. Immunol. 152*, 231.
Di Scipio, R. G., Hermodson, M. A., & Davie, E. W. (1977) *Biochemistry 16*, 5253.
Elion, J., Downing, M. R., Butkowski, R. J., & Mann, K. G. (1977) in *Chemistry and Biology of Thrombin* (Lundblad, R. L., Fenton, J. W., II, & Mann, K. G., Eds.) p 97, Ann Arbor Science Publishers, Ann Arbor, MI.
Fearon, D. T., & Austen, K. F. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3220.
Fearon, D. T., Austen, K. F., & Ruddy, S. (1973) *J. Exp. Med. 138*, 1305.
Fearon, D. T., Austen, K. F., & Ruddy, S. (1974) *J. Exp. Med. 139*, 355.
Fujikawa, K., Coan, M. H., Enfield, D. L., Titani, K., Ericsson, L. H., & Davie, E. W. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 427.
Götze, O. (1975) in *Proteases and Biological Control* (Reich, E., Rifkin, D. B., & Shaw, E., Eds.) p 255, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
Götze, O. (1976) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 35*, 269.
Götze, O., & Müller-Eberhard, H. J. (1971) *J. Exp. Med. 134*, 905.
Götze, O., & Müller-Eberhard, H. J. (1977) *Adv. Immunol. 24*, 251.
Hunsicker, L. G., Ruddy, S., & Austen, K. F. (1973) *J. Immunol. 110*, 128.
Kerr, M. A., & Porter, R. R. (1978) *Biochem. J. 171*, 99.
Konno, T., Katsuno, Y., & Hirai, H. (1978) *J. Immunol. Methods 21*, 325.
Kurachi, K., & Davie, E. W. (1977) *Biochemistry 16*, 5831.
Lachmann, P. J., & Hobart, M. J. (1978) in *Handbook of Experimental Immunology* (Weir, D. M., Ed.) Vol. 1, p 5A.10, Blackwell Scientific Publications, Oxford, England.
Laemmli, U. K. (1970) *Nature (London) 227*, 680.
Lesavre, P. H., & Müller-Eberhard, H. J. (1978) *J. Exp. Med. 148*, 1498.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265.
Martin, A., Lachmann, P. J., Halbwachs, L., & Hobart, M. J. (1976) *Immunochemistry 13*, 317.
Medicus, R. G., Götze, O., & Müller-Eberhard, H. J. (1976) *J. Exp. Med. 144*, 1076.
Mendez, E., & Lai, C. Y. (1975) *Anal. Biochem. 68*, 47.
Moore, S., & Stein, W. H. (1963) *Methods Enzymol. 6*, 819.
Müller-Eberhard, H. J., & Gotze, O. (1972) *J. Exp. Med. 135*, 1003.
Rapp, H. J., & Borsos, T. (1970) in *Molecular Basis of Complement Action*, p 111, Appleton-Century-Crofts, New York.
Roberts, P. S. (1958) *J. Biol. Chem. 23*, 285.
Rosenberg, R. D., & Damus, P. S. (1973) *J. Biol. Chem. 248*, 6490.
Schlessinger, D. H., & Hay, D. I. (1977) *J. Biol. Chem. 252*, 1689.
Sim, R. B., Porter, R. R., Reid, K. B. M., & Gigli, I. (1977) *Biochem. J 163*, 219.
Taylor, P. A., Fink, S. Bing, D. H., & Painter, R. H. (1977) *J. Immunol. 118*, 1722.
Vogt, W., Dieminger, L., Lynen, R., & Schmidt, G. (1974) *Hoppe-Seyler's Z. Physiol. Chem. 355*, 171.
Vogt, W., Schmidt, G., Dieminger, L., & Lynen, R. (1975) *Z. Immunitaetsforsch., Exp. Klin. Immunol. 149*, 440.
Volanakis, J. E., Schrohenloher, R. E., & Stroud, R. M. (1977) *J. Immunol. 119*, 337.
Walsh, K. A. (1970) *Methods Enzymol. 19*, 41.
Zacharius, R. H., Zell, T. E., Morrison, J. H., & Woodlock, J. J. (1969) *Anal. Biochem. 30*, 148.