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Evidence That Functional Subunits of Antihemophilic Factor (Factor VIII) Are Linked by Noncovalent Bonds

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Partially purified human antihemophilic factor (AHF, factor VIII), when treated with high concentrations of salt, has been shown to dissociate into two components: one, of relatively low molecular weight, possesses procoagulant activity, and the other, of higher molecular weight, forms precipitates with heterologous antiserum against AHF and supports ristocetin-induced platelet aggregation. The ease of separation suggests that the two components in the native state might be held together by noncovalent bonds. Earlier observations do not exclude the possibility that the subunits may be covalently bonded in nature but might be severed by plasma proteolytic enzymes during laboratory manipulation. This issue was examined by preparing partially purified AHF from fresh human plasma in the presence of protease inhibitors, including benzamidine, soybean trypsin inhibitor, epsilon-aminocaproic acid, heparin, and hirudin. Under these conditions, gel filtration in the presence of 0.25 *M* calcium chloride and 0.001 *M* benzamidine resulted in its separation into two components, having properties identical to those separated in the absence of these protease inhibitors. The inhibitor mixture blocked generation and action of streptokinase- and kaolin-activated plasmin from plasma, and protected both plasma AHF and partially purified AHF from the action of thrombin. Surface-induced activation of PTA (factor XI) was partially inhibited, and that of Christmas factor (factor IX) was completely inhibited. This observation provides further evidence that in the native state the high- and low-molecular-weight components of preparations of antihemophilic factor are held together by noncovalent bonds.

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ANTIHEMOPHILIC FACTOR (AHF, factor VIII) is a macromolecular glycoprotein with a molecular weight that may be greater than 2 million.[1] It possesses procoagulant activity correcting the defective coagulation of hemophilic plasma, it forms precipitates with specific heterologous antiserum,[2] and it supports ristocetin-induced platelet aggregation.[3] These three properties are associated with a protein fraction of plasma excluded by agarose gels.

Weiss et al.[4] and Owen et al.[5] showed that partially purified AHF could be separated into two components when agarose gel filtration was performed in the presence of high concentrations of sodium or calcium chloride. A high-molecular-weight (HMW) component excluded by the gel contained the bulk of protein,[4,5] precipitated specific heterologous antiserum,[6] and supported ristocetin-induced platelet aggregation,[7] but possessed little or no procoagulant activity. Another, low-molecular-weight (LMW) component was retarded by

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the gel and possessed the bulk of procoagulant activity[4,5] in a form that could be further activated by thrombin[8,9] but was relatively poor in proteins, did not possess precipitating antigens,[6] and did not appreciably support ristocetin-induced platelet aggregation.[7] That the properties of the AHF preparation could be separated into two components by salts at high concentration suggested that in the native state these may be held together by noncovalent bonds. These studies did not exclude the possibility that the components were held together by covalent bonds that were severed by plasma proteases during laboratory manipulations. The experiments described herein did not support this possibility and provided further evidence that the HMW and LMW components are joined by noncovalent links.

MATERIALS AND METHODS

A standard pool of normal citrated plasma was prepared and stored as described previously.[2] This pool was said to contain 1 U/ml each of procoagulant AHF activity, precipitating antigens, and platelet-aggregating activity (sometimes called the von Willebrand factor), as measured by aggregation of washed platelets by ristocetin. The similarity in unitage does not necessarily imply that all circulating AHF possesses all three properties.

Partially purified AHF was prepared by the method of Zimmerman et al.[2] modified by the addition of protease inhibitors.* Typically, 40 ml of venous blood of a donor who had fasted overnight was drawn through an 18-gauge needle into a silicone-coated polypropylene syringe, and immediately transferred into a silicone-coated polypropylene tube (30 × 105 mm) containing 1/50 volume of a mixture of 0.5 *M* sodium citrate buffer, pH 5.0, 0.05 *M* benzamidine hydrochloride hydrate (Aldrich Chemical Co., Milwaukee, Wis.), 500 mg/liter crystalline soybean trypsin inhibitor (SBTI, a preparation inhibiting 1.58 mg trypsin/mg; Worthington Biochemical Corp, Freehold, N.J.), 100,000 U/liter sodium heparin (Upjohn, Kalamazoo, Mich.), 250 g/liter ε-aminocaproic acid (EACA, Nutritional Biochemicals Corp., Cleveland, Ohio), and 10,000 antithrombin units (ATE) hirudin/liter (4080 ATE U/mg protein, Iveb Arzneimittelwerk, Dresden, Democratic Republic of Germany). After mixing, the plasma was separated by centrifugation at 2700 *g* for 15 min at 2°C, and transferred to an uncoated 30 × 105 mm polypropylene tube. Sufficient 53.3% ethanol containing 0.001 *M* benzamidine, 10 mg of SBTI, 2000 units heparin, 5 g EACA, and 200 ATE units hirudin/liter was added to bring the final ethanol concentration to 3%. After incubation with occasional mixing in a −3°C ice bath for 30 min, the precipitate was sedimented by centrifugation at 2700 *g* for 15 min at −3°C. The cryoethanol precipitate was dissolved in 1.2 ml of a mixture of 0.02 *M* imidazole (Matheson, Coleman and Bell, Norwood, Ohio) and saline (0.14 *M* sodium chloride) buffer, pH 6.5, 0.001 *M* benzamidine, 10 mg SBTI/liter, 2000 units heparin/liter, 5 g EACA/liter, and 200 ATE units hirudin/liter. After sedimenting undissolved particles by centrifugation at 2700 *g* for 10 min at room temperature, 1 ml of the supernatant crude AHF concentrate was filtered at room temperature through a 0.9 × 51-cm plastic column of 4% agarose (Biogel A1.5M, 100–200 mesh, BioRad Laboratories, Richmond, Calif.), equilibrated and eluted with the imidazole–saline–inhibitor mixture at a constant pressure at the outflow of 30 cm of water, collecting 0.9 ml fractions. The fractions were immediately assayed for AHF procoagulant activity in the presence of protamine sulfate (see below). AHF-rich fractions excluded from the gel (usually fractions 13–15) contained a total of 50–80 μg of protein and 2.4–3.0 units of AHF procoagulant activity. These fractions were pooled and concentrated to about 0.6–0.8 ml by negative pressure dialysis at 4°C through cellophane tubing (1/4-inch diameter, Fisher Scientific Co., Pittsburgh, Pa.) against a mixture of 0.02 *M* imidazole buffer (pH 6.5), 0.14 *M* sodium chloride, 0.001 *M* benzamidine, and 5 g/liter EACA. Heparin, SBTI, and hirudin were coconcentrated with AHF

*The inhibitors selected and the concentrations used were suggested by Dr. Earl W. Davie, University of Washington, Seattle, Wash.

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in the process, and thus the protein content of the concentrate attributable to AHF could not be determined.

In some experiments, hirudin was omitted entirely, and the AHF purification steps were carried further. The cryoethanol precipitate, dissolved in 5 ml of imidazole-saline-inhibitor mixture (without hirudin), was mixed at room temperature with 5 ml of 20% polyethylene glycol (MW 6000–7500, Matheson, Coleman and Bell), dissolved in the same imidazole-saline-inhibitor mixture without hirudin. The precipitate was separated by centrifugation at 2700 *g* for 10 min at room temperature, and redissolved in 1.2 ml of the imidazole-saline-inhibitor mixture (without hirudin). Subsequent purification by gel-filtration in 4% agarose was carried out similarly without hirudin in the buffer.

Separation of partially purified AHF into two components was conducted essentially by the method of Owen et al.[5] One-half milliliter of the concentrated preparation of partially purified AHF (containing a total of 0.75–1.25 units of AHF procoagulant activity) prepared in the presence of protease inhibitors was applied to a 0.9 × 24.5-cm plastic column of 8% agarose (Biogel A1.5 M, 200–400 mesh, BioRad Laboratories) equilibrated and eluted with Tris-$CaCl_2$ buffer (0.05 *M* Tris (hydroxymethyl) aminomethane, Sigma Chemical Co., St. Louis, Mo., 0.25 *M* $CaCl_2$, pH 7.35) containing 0.001 *M* benzamidine and 0.15 *M* β-alanine (A-grade, Calbiochem, Los Angeles, Calif.) at a constant pressure at the outflow of 30 cm of water, and collected in 0.9-ml fractions at room temperature. The fractions were immediately assayed for AHF procoagulant activity in the presence of protamine sulfate. Recovery of AHF activity after passage through the column varied from 27% to 40%. Individual fractions were then exhaustively dialyzed against barbital-saline buffer for determination of antigen activity, platelet aggregating activity, and for measurement of procoagulant AHF.

Functional AHF was measured by an earlier method.[10] To overcome the inhibitory effects of heparin, samples in the imidazole-saline-inhibitor mixture were diluted 20-fold in barbital-saline buffer containing protamine sulfate (0.002 mg/ml final concentration, Eli Lilly and Co., Indianapolis, Ind.) before AHF assay. At a dilution of 1:20 or more with respect to the sample, the concentration of the other four inhibitors, benzamidine, SBTI, EACA, and hirudin, alone or in combination, did not appreciably affect the assay system. The sample eluted from the 8% agarose column with Tris-$CaCl_2$ buffer containing benzamidine and β-alanine was assayed by a modification of the method. Each sample was diluted tenfold in barbital-saline buffer containing protamine sulfate (0.004 mg/ml final concentration) to neutralize heparin coconcentrated with the sample. One-tenth milliliter of warm barbital-saline buffer was added to a mixture of 0.1 ml hemophilic plasma and 0.1 ml kaolin-Centrolex "O" that had been incubated at 37°C for 8 min, and then 0.1 ml of the diluted test sample, which now contained 0.025 *M* $CaCl_2$, was added. The clotting time of this mixture was compared to those obtained with serial dilution in barbital-saline buffer of pooled plasma that had first been incubated for 2 min in an ice bath with the Tris-$CaCl_2$ buffer containing 0.001 *M* benzamidine and 0.15 *M* β-alanine. The volume of Tris-$CaCl_2$ buffer-benzamidine-β-alanine added to the pooled plasma was one that would give a final concentration of 0.025 *M* $CaCl_2$ after dilution with barbital-saline buffer.

Antiserum to AHF for quantitative immunoelectrophoresis was prepared in a female "grade" goat and rendered monospecific by absorption with a fraction of plasma soluble in 3% ethanol but insoluble in 8% ethanol as described earlier.[10] The crude IgG fraction containing precipitating antibodies to AHF was separated by a minor modification[11] of the method of Steinbuch et al.[12]

Precipitating antigen related to AHF was measured by an earlier adaptation[2] of Laurell's[13] method of quantitative immunoelectrophoresis in which an appropriate amount of the monospecific goat antibodies against AHF was embedded in 1% agarose (Matheson, Coleman and Bell). The test was sensitive to 0.025–0.05 U/ml of AHF-related antigen.

Aggregation of platelets by ristocetin was measured by addition of samples to suspensions of washed normal platelets. Venous blood from a normal donor was drawn into 1/50 vol of 0.5 *M* sodium citrate buffer (pH 5.0) and centrifuged at 150 *g* for 10 min at room temperature. The platelets were separated and washed according to the albumin density gradient separation method of Walsh.[14] Twelve milliliters of platelet-rich plasma (PRP) was layered onto a 1.5-ml layer of 45% bovine serum albumin (Armour Pharmaceutical Co., Chicago, Ill.) in a 15 × 100-mm polypropylene tube. A gradual continuous density gradient of albumin was produced by mixing the interface between PRP and albumin before sedimenting the platelets by centrifugation at 900 *g*

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for 10 min at room temperature. After removing the supernatant platelet-poor plasma, the platelets, which now formed a layer on the albumin cushion, were resuspended in 10 ml calcium-free Tyrode's solution. The platelets were then similarly resedimented and washed six times. The final platelet suspension contained 200×10^9 platelets/liter. A volume of 0.05 ml of the test sample was incubated with 0.4 ml of platelet suspension for 1 min at 37°C in 8 × 50-mm flat bottomed silicone-coated glass tubes, and stirred magnetically. Five-hundredths of a milliliter of ristocetin (10 mg/ml in barbital-saline buffer, a gift of Abbott Laboratories, North Chicago, Ill.) was then added, and the change in optical transmittance was measured with a platelet aggregation Profiler (Model PAP-2A, Bio/Data Corp., Hatboro, Pa.). A reference curve was obtained by a modified method of Weiss et al.[3], in which the logarithm of the concentration of pooled plasma diluted in barbital-saline buffer was plotted against the logarithm of the change in percentage in transmittance 1 min after the addition of ristocetin. A linear plot was obtained between 1 U/ml and 0.05 U/ml standard pooled plasma concentration, with a test sensitivity of 0.03 U/ml. The concentration of platelet-aggregating activity in the test sample was computed by interpolation of the per cent change in transmittance induced by the sample in comparison to the linear portion of the reference curve.

Estimation of molecular weight (MW) in fractions eluted from an 8% agarose column in Tris-$CaCl_2$ buffer containing benzamidine and β-alanine was made according to the method of Andrews.[15] The nonenzyme protein markers used were human fibrinogen (fraction 1-4 of Blombäck et al.,[16] MW 340,000), human IgG (MW 160,000, Miles Laboratories, Inc., Kankakee, Ill.), bovine serum albumin (MW 69,000, Miles Laboratories, Inc.), and SBTI (MW 21,500). The void volume was determined with blue dextran 2000 (Pharmacia, Uppsala, Sweden; MW > 2,000,000 according to manufacturer).

The effects of the inhibitor mixture on thrombin action, plasmin generation and action, and surface-induced PTA (factor XI) and Christmas factor (factor IX) generation were studied in the absence or presence of inhibitors at a final concentration in the incubation mixture of benzamidine 0.001 *M*, heparin 2000 U/liter, SBTI 10 mg/liter, EACA 5 g/liter, and hirudin 200 ATE U/liter.

The effect of the inhibitor mixture to block thrombin action on AHF was tested by incubating at 37°C, in 10 × 75-mm polystyrene tubes, equal volumes of various concentrations of bovine thrombin (topical thrombin, Parke Davis and Co., Detroit, Mich.) and plasma or partially purified AHF prepared in the absence of inhibitors. At time intervals, an aliquot was removed, diluted 50-fold with barbital-saline buffer and assayed for AHF activity. Where necessary, the diluting buffer contained protamine sulfate (0.0008 mg/ml) to neutralize the effect of heparin. The inhibitor mixture protected AHF from the activation-inactivation action of thrombin at a final concentration of 2.5 NIH U/ml for plasma AHF and 0.25 NIH U/ml for partially purified AHF. In the absence of the inhibitors, thrombin activated and then inactivated both plasma and partially purified AHF at a concentration as low as 0.05 NIH U/ml, and caused clotting of plasma at 0.15 NIH U/ml within 3 min.

The ability of the inhibitor mixture to block streptokinase-induced plasmin generation was performed by an earlier method.[17] A mixture of 0.1 ml plasma diluted 30-fold in barbital-saline buffer, 0.2 ml bovine fibrinogen, 3 mg/ml (fraction I, Nutritional Biochemical Corp., Cleveland, Ohio), 0.1 ml streptokinase, 1000 Christian U/ml (high purity, the gift of Lederle, American Cyanimid Co., New York, N.Y.), and 0.1 ml bovine thrombin (topical thrombin, Parke Davis and Co.), 50 NIH U/ml was incubated at 37°C in 10 × 75-mm polystyrene tubes, and the time between addition of thrombin and lysis of a fibrin clot was noted. Clot lysis was complete in 8 min in the absence of inhibitors, but it did not occur during incubation for 48 hr when the inhibitors were present.

The effect of the inhibitor mixture on the action of plasmin generated by kaolin from acidified plasma in low-ionic-strength buffer was tested by an earlier method.[17] The euglobulin precipitate containing kaolin after incubation at 37°C for 1 hr of 0.5 ml normal plasma, 0.25 ml kaolin suspension (8 mg/ml barbital-saline buffer), and 9.25 ml 0.01 *M* sodium acetate buffer, pH 4.8, was resuspended in 0.5 ml imidazole-saline buffer. Duplicates of 0.2 ml of this suspension were incubated at 37°C in 10 × 75-mm polystyrene tubes with 0.1 ml bovine fibrinogen (3 mg/ml), and 0.1 ml bovine thrombin (50 NIH U/ml) in the presence or absence of the five inhibitors. The time between addition of thrombin and lysis of fibrin clot was noted. In the ab-

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sence of inhibitors, the clot lysed in 13 min, whereas no lysis occurred within 48 hr in the presence of inhibitors.

Generation of activated PTA (factor XI) was measured by incubating 0.1 ml Christmas factor (factor IX)-deficient plasma and 0.1 ml imidazole-saline buffer at 37°C in 10 × 75-mm glass tubes. At time intervals, a 0.02-ml aliquot of the incubation mixture was removed and mixed in a 10 × 75-mm polystyrene tube with 0.08 ml barbital-saline buffer (or barbital-saline buffer containing protamine sulfate 0.04 mg/ml when the incubation mixture contained inhibitors) and 0.1 ml soybean phosphatides (Centrolex "O" 0.1% in 0.15 *M* sodium chloride, the gift of Central Soya Co., Chicago, Ill.). One-tenth milliliter of PTA-deficient plasma was then added, and the mixture was incubated for 1 min at 37°C. Thereafter, 0.1 ml 0.025 *M* calcium chloride was added and the clotting time measured. In the absence of inhibitors, the clotting time shortened from 310 sec, after 10 sec of incubation, to 220 sec, after 15 min incubation; in the presence of inhibitors, the clotting time shortened from 314 to 269 sec. When the initial incubation mixture without inhibitors was mixed in a polystyrene tube and immediately assayed for active PTA activity as described, the clotting time was 400 sec.

Active Christmas factor generation was measured by an earlier method.[18] One-tenth milliliter of classic hemophilic plasma was incubated at 37°C in 10 × 75-mm glass tubes with 0.05 ml of 0.05 *M* $CaCl_2$ in the presence or absence of the five inhibitors. At intervals 0.02 ml aliquots were removed and mixed in a 10 × 75-mm polystyrene tube with 0.08 ml barbital-saline-citrate buffer (85 parts barbital-saline buffer mixed with 15 parts of 0.13 *M* trisodium citrate) and 0.02 ml barbital-saline buffer with or without protamine sulfate (0.04 mg/ml). After addition of 0.1 ml Centrolex "O" and 0.1 ml Christmas factor-deficient plasma, the mixture was then incubated at 37°C for 1 min. One-tenth milliliter 0.05 *M* calcium chloride was then added, and the clotting time was measured. In the absence of inhibitors, the clotting time shortened from 720 sec at 10 sec incubation to 320 sec after incubation for 5 min. In the presence of inhibitors, the clotting time remained at about 1000 sec throughout the incubation. When the initial incubation mixture without inhibitors was mixed in a polystyrene tube and immediately assayed as described, the clotting time was 950 sec.

Barbital-saline buffer was composed of 0.025 *M* sodium barbital and 0.125 *M* sodium chloride at pH 7.5. All buffers used for column chromatography contained sodium azide at a concentration of 200 mg/liter. Protein was measured by the method of Lowry et al.[19] Centrifugations were performed at 2°C in an International PR-2 centrifuge, and at room temperature in an International UV centrifuge.

RESULTS

Two components were separated from partially purified AHF prepared in the presence of protease inhibitors (heparin, benzamidine, SBTI, EACA, and hirudin) and gel-filtered in the presence of 0.25 *M* $CaCl_2$ and benzamidine (Fig. 1). A HMW protein peak was eluted at the void volume that was relatively poor in clot-promoting activity, but contained material that formed precipitates with heterologous antiserum to whole AHF and supported ristocetin-induced aggregation of washed platelets. A later LMW fraction, retarded by the gel, contained procoagulant activity but little protein, AHF-like antigen, and platelet-aggregating activity. A third protein peak was eluted at a later point than AHF at a volume corresponding to that of a marker of SBTI. Similar results were obtained in seven separate experiments, both with or without the presence of hirudin. In four experiments, using the same column, the molecular weight of the procoagulant activity peak, as determined by comparing its elution volume with those of the nonenzyme marker proteins, was estimated to be about 230,000. This was in good agreement with results obtained by SDS-polyacrylamide gel electrophoresis of a LMW fraction containing procoagulant activity separated in the absence of protease inhibitors.[10]

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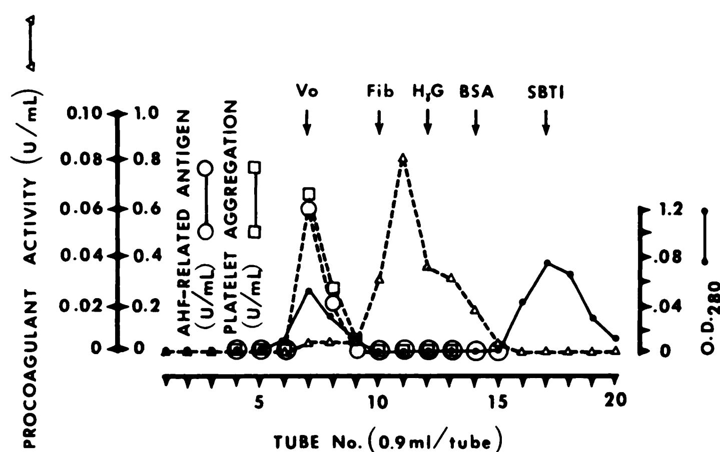

Fig. 1. Gel filtration of AHF partially purified in the presence of protease inhibitors (heparin, benzamidine, SBTI, and EACA) on an 8% agarose column, 0.9 × 24.5 cm. A 0.5-ml sample (1.6 procoagulant U/ml) was applied and eluted with 0.05 *M* Tris buffer (pH 7.35) containing 0.25 *M* $CaCl_2$, 0.001 *M* benzamidine, and 0.15 *M* β-alanine. Arrows indicate markers: blue dextran (V_o), human fibrinogen (Fib), human γ-globulin (HγG), bovine serum albumin (BSA), and soybean trypsin inhibitor (SBTI).

DISCUSSION

The multifunctional properties of partially purified preparations of human AHF can be dissociated by addition of high concentrations of salt, as first suggested by sucrose gradient centrifugation studies of Thelin et al.[20] and confirmed by gel filtration techniques by Weiss et al.[4] and Owen et al.[5] This dissociation has been repeatedly confirmed,[6,10,21] and the properties of the dissociated components further defined. A HMW component, rich in protein, forms precipitates with heterologous antiserums to whole AHF and supports ristocetin-induced platelet aggregation. In contrast, the LMW component is relatively poor in protein and corrects the clotting defect of hemophilic plasma. Removal of the excess salt has been shown to result in recombination of the two separated components.[22]

The ease with which dissociation of AHF preparations occurs under these conditions suggests that the two components are bound together noncovalently, a view that is not universally accepted.[23,24] An alternative hypothesis, that the components of AHF are bound together covalently, suggests that the components derived from AHF preparations are bonded covalently in the native state, but are severed during the preparative procedures by plasma proteases. A variety of enzymes, including plasma thrombin and plasmin, have been shown to alter either the function or the structure of native AHF, or both.[9,23,24] Indeed, LMW components with procoagulant activity can be separated by treatment of partially purified AHF with low concentration of thrombin[9,25,26] or by clotting of recalcified cryoprecipitates.[26] The properties of these LMW components appear to differ from those separated in the presence of high concentrations of salt. The component produced by treatment with thrombin cannot be further activated upon incubation with additional thrombin,[9] while that produced by

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clotting of recalcified cryoprecipitates does not recombine with its HMW counterpart upon dialysis to remove excess calcium chloride.[26]

In the present study, conducted at the suggestion of Dr. E. W. Davie, only freshly drawn blood was used to prepare AHF. Protease inhibitors, effective both in blocking the generation and action of activated plasmin from plasma, and in protecting plasma AHF and partially purified AHF from the action of additional thrombin, were present throughout the preparative procedures. This inhibitor mixture also completely inhibited surface-induced activation of Christmas factor, while surface-induced activation of PTA and hence of Hageman factor (factor XII) was only partially abolished. Care was taken, however, to avoid surface contact of the plasma and AHF-containing fractions throughout the preparative procedures. Nonetheless, clear separation of these partially purified AHF preparations into two components was demonstrated upon gel filtration of the preparation in the presence of 0.25 M $CaCl_2$. These two components were similar to those separated from preparations made in the absence of protease inhibitors. The LMW component could also be further activated with low concentration of thrombin according to the technique of Rick et al.,[8] but the original activity was too low to allow proper interpretation. The low yield of activity in this experimental system has also precluded performance of recombination experiments. Our data suggested that the separation of partially purified AHF into two components was not a proteolytic artifact but a genuine phenomenon, and provided further evidence that the components are held together by noncovalent bonds in the native state. We could not exclude the possibility that scission of AHF was the result of the action of unrecognized enzymes not inhibited by the agents used in our studies. The present observations do not explain why similar separation cannot be achieved with certain highly purified human[23] and bovine[24] preparations, the latter prepared in the presence of benzamidine, SBTI, heparin, and EACA. In view of the observable differential lability of various functional properties attributed to AHF, perhaps the AHF prepared in these latter studies was altered during the multiple preparative steps necessary to obtain highly purified AHF.

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REFERENCES

1. Kass L, Ratnoff OD, Leon MA: Studies on the purification of antihemophilic factor (factor VIII). I. Precipitation of antihemophilic factor by concanavalin A. J Clin Invest 48:351, 1969

2. Zimmerman TS, Ratnoff OD, Powell AE: Immunologic differentiation of classic hemophilia (factor VIII deficiency) and von Willebrand's disease, with observations on combined deficiencies of antihemophilic factor and proaccelerin (factor V) and on an acquired circulating anticoagulant against antihemophilic factor. J Clin Invest 50:244, 1971

3. Weiss HJ, Hoyer LW, Rickles FR, Varma A, Rogers J: Quantitative assay of a plasma factor deficient in von Willebrand's disease that is necessary for platelet aggregation: Relationship to factor VIII procoagulant activity and antigen content. J Clin Invest 52:2708, 1973

4. Weiss HJ, Phillips LL, Rosner W: Separation of sub-units of antihemophilic factor (AHF) by agarose gel chromatography. Thromb Diath Haemorrh 27:212, 1972

5. Owen WG, Wagner RH: Antihemophilic factor: Separation of an active fragment following dissociation by salts or detergents. Thromb Diath Haemorrh 27:502, 1972

6. Rick ME, Hoyer LW: Immunologic studies

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of antihemophilic factor (AHF, factor VIII). V. Immunologic properties of AHF subunits produced by salt dissociation. Blood 42:737, 1973

7. Weiss HJ, Hoyer LW: von Willebrand's factor: Dissociation from antihemophilic factor procoagulant activity. Science 182:1149, 1973

8. Rick ME, Hoyer LW: Activation of low molecular weight fragment of antihaemophilic factor (factor VIII) by thrombin. Nature 252: 404, 1974

9. Cooper HA, Reisner FF, Hall M, Wagner RH: Effects of thrombin treatment on preparations of factor VIII and the Ca^{2+}-dissociated small active fragment. J Clin Invest 56:751, 1975

10. Ratnoff OD, Slover CC, Poon M-C: Immunologic evidence that the properties of human antihemophilic factor (factor VIII) are attributes of a single molecular species. Blood 47:657, 1976

11. Ratnoff OD: Studies on the product of reaction between activated Hageman factor (factor XII) and plasma thromboplastin antecedent (factor XI). J Lab Clin Med 80:704, 1972

12. Steinbuch M, Audran R: The isolation of IgG from mammalian sera with the aid of caprylic acid. Arch Biochem Biophys 134:279, 1969

13. Laurell C-B: Quantitative estimation of protein by electrophoresis in agarose gel containing antibodies. Anal Biochem 15:45, 1966

14. Walsh PN: Platelet washing by albumin density gradient separation (ADGS). Adv Exp Med Biol 34:245, 1972

15. Andrews P: The gel-filtration behavior of proteins related to their molecular weights over a wide range. Biochem J 96:595, 1965

16. Blombäck B, Blombäck M: Purification of human and bovine fibrinogen. Arkiv Kemi 10:415, 1956

17. Ogston D, Ogston CM, Ratnoff OD, Forbes CD: Studies on a complex mechanism for the activation of plasminogen by kaolin and by chloroform: The participation of Hageman factor and additional cofactors. J Clin Invest 48:1786, 1969

18. Ratnoff OD, Davie EW: The activation of Christmas factor (factor IX) by activated plasma thromboplastin antecedent (activated factor XI). Biochem 1:677, 1962

19. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: Protein measurement with the Folin phenol reagent. J Biol Chem 193:265, 1951

20. Thelin GM, Wagner RH: Sedimentation of plasma antihemophilic factor. Arch Biochem 95:70, 1961

21. Austen DEG: Factor VIII of small molecular weight and its aggregation. Br J Haematol 27:89, 1974

22. Cooper HA, Grigg TR, Wagner RH: Factor VIII recombination after dissociation by $CaCl_2$. Proc Natl Acad Sci 70:2326, 1973

23. McKee PA, Andersen JC, Switzer ME: Molecular structural studies of human factor VIII. Ann NY Acad Sci 240:8, 1975

24. Legaz ME, Weinstein MJ, Heldebrant CM, Davie EW: Isolation, subunit structure, and proteolytic modification of bovine factor VIII. Ann NY Acad Sci 240:43, 1975

25. Weiss HJ, Kochwa S: Molecular forms of antihaemophilic globulin in plasma, cryoprecipitate and after thrombin activation. Br J Haematol 18:89, 1970

26. Gordon NR, Shulman NR: The effect of clotting on structure and function of human factor VIII. Ann NY Acad Sci 240:79, 1975

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