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Research Article

Human activated protein C (APC) is a plasma serine protease that possesses amidolytic and anticoagulant activity. The rate at which the amidolytic and anticoagulant activity of APC was neutralized in normal plasma was essentially identical to that observed in plasma obtained from four individuals with combined Factor V/VIII deficiency disease. Incubation of radioiodinated APC with either normal human plasma or the combined Factor V/VIII-deficient plasmas resulted in the formation of a stable complex (Mr = 96,000) of the enzyme and a plasma protein as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Pretreatment of the radiolabeled APC with diisopropyl fluorophosphate prevented the formation of the enzyme-protein complex. On the basis of its ability to form a complex with radiolabeled APC, the APC-binding protein was purified to homogeneity from normal human plasma by ammonium sulfate fractionation, heparin-agarose chromatography, and QAE-Sephadex A-50 chromatography. The APC-binding protein (Mr = 54,000) is a glycoprotein, and possesses an amino-terminal sequence of Gly-Arg-Thr-Cys-Pro-Lys-Pro-Asp. The amino-terminal sequence of the APC-binding protein exhibited considerable homology with bovine colostrum inhibitor and pancreatic trypsin inhibitor, but no apparent sequence homology with the plasma serine protease inhibitors. Affinity-purified antibody against APC-binding protein immunoprecipitated a complex of radiolabeled APC and native APC-binding protein from normal human plasma. Complex formation was virtually eliminated in plasma immunodepleted of the APC-binding protein. Quantitative electroimmunoassay indicated essentially […]

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Evidence of Normal Functional Levels of Activated Protein C Inhibitor in Combined Factor V/VIII Deficiency Disease

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ABSTRACT Human activated protein C (APC) is a plasma serine protease that possesses amidolytic and anticoagulant activity. The rate at which the amidolytic and anticoagulant activity of APC was neutralized in normal plasma was essentially identical to that observed in plasma obtained from four individuals with combined Factor V/VIII deficiency disease. Incubation of radioiodinated APC with either normal human plasma or the combined Factor V/VIII-deficient plasmas resulted in the formation of a stable complex ($M_r = 96,000$) of the enzyme and a plasma protein as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Pretreatment of the radiolabeled APC with diisopropyl fluorophosphate prevented the formation of the enzyme-protein complex. On the basis of its ability to form a complex with radiolabeled APC, the APC-binding protein was purified to homogeneity from normal human plasma by ammonium sulfate fractionation, heparin-agarose chromatography, and QAE-Sephadex A-50 chromatography. The APC-binding protein ($M_r = 54,000$) is a glycoprotein, and possesses an amino-terminal sequence of Gly-Arg-Thr-Cys-Pro-Lys-Pro-Asp. The amino-terminal sequence of the APC-binding protein exhibited considerable homology with bovine colostrum inhibitor and pancreatic trypsin inhibitor, but no apparent sequence homology with the plasma serine protease inhibitors. Affinity-purified antibody against APC-binding protein immunoprecipitated a complex of radiolabeled APC and native APC-binding protein from normal human plasma. Complex formation was virtually eliminated in plasma immunodepleted of the APC-binding protein. Quantitative electroimmunoassay indicated essentially equal levels of APC-binding protein antigen in normal plasma compared with plasma from four patients with combined Factor V/VIII deficiency disease.

INTRODUCTION

Protein C is a vitamin K-dependent glycoprotein that circulates in blood in a zymogen form (1-3). Purified preparations of both human and bovine protein C are readily converted to a serine protease called activated protein C (APC).[1] This reaction is catalyzed by trace amounts of α-thrombin (3, 4). Recently, this reaction has been shown to be greatly accelerated by a cofactor isolated from endothelial cells (5-7).

Previous work from our laboratory, as well as several others, has clearly demonstrated that APC exhibits anticoagulant activity through its selective inactivation of Factor Va and Factor VIIIa by limited proteolysis (4, 8, 12). Furthermore, recent studies suggest a physiological role for APC in promoting fibrinolysis (13). Thus, protein C apparently plays a critical role in blood coagulation, and its congenital deficiency, hypothetically, would result in a hypercoagulable state with episodes of thrombosis. Indeed, Griffin and his colleagues (14) have identified and characterized a

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family with a recurring thrombotic disease associated with an apparent plasma protein C deficiency.

Although a considerable amount of information has accumulated on the mechanism of action of APC, relatively little is known regarding the mechanism whereby APC is regulated in plasma. Recently, Marlar and Griffin (15) proposed that the molecular basis of the combined Factor V/VIII deficiency disease was due to a congenital deficiency of a plasma inhibitor to APC. In preliminary experiments, we observed no significant difference in the rate at which the anticoagulant activity of human APC was neutralized in plasma obtained from normal individuals and two unrelated patients with combined Factor V/VIII deficiency disease (16). We now have expanded this study to include four patients, and in this article we present evidence that the functional levels of the putative APC inhibitor in normal and combined factor V/VIII-deficient plasma samples are essentially identical.

METHODS

Sepharose 4B, QAE-Sephadex A50, SP-Sephadex C-50, Sephadex G-150, Sephadex G-50, Sephadex G-25, activated CH-Sepharose, protein A-Sepharose CL-4B, and low molecular weight sodium dodecyl sulfate (SDS) electrophoresis standard proteins were products of Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, NJ. Electrophoresis-purity acrylamide, bisacrylamide, N,N,N',N'tetramethylenediamine, ammonium persulfate, and agarose (standard low-m_r) were obtained from Bio-Rad Laboratories, Richmond, CA. Kaolin (acid-washed) was purchased from Fisher Scientific Co., Fair Lawn, NJ. Benzamidine hydrochloride, diisopropyl fluorophosphate (DFP), and 4-vinyl pyridine were purchased from Aldrich Chemical Co., Inc., Milwaukee, WI. SDS was obtained from Gallard-Schlesinger, Carle Place, NY. Lithium heparin (150 U. S. Pharmacopeia U/mg) was obtained from Riker Laboratories, Inc., Northridge, CA. D-Phenylalanyl-L-pipecolyl-L-arginyl-p-nitroanilide (S-2238) was obtained from Ortho Diagnostics, Inc., Raritan, NJ. Freund's incomplete and complete adjuvant were purchased from Difco Laboratories, Detroit, MI. Carrier-free sodium ^{125}I in dilute NaOH (506 mCi/ml) was purchased from New England Nuclear, Boston, MA. Sequenator reagents (Sequanol grade) and 1,3,4,6-tetrachloro-3α,6α-diphenyl glycoluril (Iodo-gen) were supplied by Pierce Chemical Co., Rockford, IL. Heparin-Sepharose was prepared as described (17). Human brain cephalin was prepared according to Bell and Alton (18). Purified rabbit IgG was purchased from Pel-Freeze Biologicals, Rogers, AR. Pansorbin, and rabbit antisera directed against human α_1-antitrypsin, antithrombin III, α_2-macroglobulin, C1 inactivator, and inter-α-trypsin inhibitor were purchased from Calbiochem-Behring Corp., American Hoechst Corp., La Jolla, CA. Rabbit antiserum against human α_2-antiplasmin was kindly provided by Dr. Nubuo Aoki, Jichi Medical School, Tochigi-ken, Japan. Plasma was prepared from normal individuals and combined Factor V/VIII-deficient patients by withdrawing nine parts of whole blood from the antecubital vein into polyethylene tubes containing one part 3.8% sodium citrate, followed by centrifugation at 2,500 g for 15 min at room temperature. Citrated plasma

from five normal individuals was pooled and served as a normal reference plasma. Plasma samples from clinically-defined, combined Factor V/VIII-deficient patients were obtained through the courtesy of Dr. P. M. Mannucci (patient A), Dr. Maribel Clements (patient B), and Dr. Kenneth Smith (patients C and D). Dr. Smith provided plasma samples from two combined Factor V/VIII-deficient siblings (patient C and D). In the four samples of Factor V/VIII-deficient plasma obtained, the Factor V activity ranged from 13- to 15% of normal, whereas the Factor VIII:C activity ranged from 8 to 16% of normal. Factor VIII-related antigen levels in these plasma samples were 70–90% normal. The plasma Factor VIII coagulant antigen (VIII:CAg) levels of the combined Factor V/VIII-deficient patients were measured in the laboratory of Dr. Inga-Marie Nilsson (19), and were found to be 7–14% of normal. In addition, the Factor V antigen levels of these patients' plasma, as determined by radioimmunoassay (20), ranged from 9 to 27% of normal. All plasma samples used in this study were tested for inhibitor activity within 6 mo after venipuncture. In one case (patient B), the plasma was tested within 12 h after venipuncture.

Amino acid analyses were carried out by standard procedures (21–23). Neuraminic acid was determined according to Warren (24).

S-Pyridylethylated derivatives of the human APC-binding protein were prepared according to Friedman et al. (25). Salt and excess reagents were removed by gel filtration on a Sephadex G-50 column (2.6 × 50 cm) equilibrated with 5% formic acid followed by lyophilization.

Automated Edman degradations were performed with a Beckman sequencer (model 890C; Beckman Instruments, Inc., Fullerton, CA) by a modification of the technique described by Edman and Begg (26). For amino-terminal analyses, 2.8-mg quantities (50 nmol) of the S-pyridylethylated derivative of the APC-binding protein were used. The analysis was carried out on two different binding protein preparations. Dilute Quadrol (0.2 M) was employed as the coupling buffer and pretreated polybrene (3 mg) was added to the sample to enhance its adsorption to the sequencer cup (27). Phenylthiohydantoin amino acids were identified and quantitated by high pressure liquid chromatography (28, 29).

SDS-polyacrylamide gel electrophoresis (PAGE) was performed in glass tubes (6 × 100 mm) as previously described (2). The tube gels were run at room temperature at a constant current of 5 mA/gel. The gels were stained for protein and carbohydrate as described (2). The distribution of radioactivity in tube gels was determined by slicing the gel into 1-mm sections (Joyce-Loebl gel slicer) followed by counting each slice in a Beckman model 4000 gamma counting spectrometer (Beckman Instruments, Inc.). Discontinuous SDS-electrophoresis was performed in slab gels according to Laemmli (30) by using 10% polyacrylamide-resolving gels and 3% polyacrylamide-concentrating gels. Alternatively, continuous SDS-PAGE was carried out in 7.5% polyacrylamide slab gels equilibrated with 0.1 M Tris-H₃PO₄ (pH 7):0.1% SDS. The slab gels were initially fixed for 30 min in 10% TCA:50% CH₃OH and stained in a solution containing 0.2% Coomassie Blue G-250:50% CH₃OH:10% acetic acid. The gels were destained in 7.5% acetic acid:5% CH₃OH and dried in a Bio-Rad model 224 slab dryer (Bio-Rad Laboratories). Molecular weights were estimated by interpolation from a linear semilogarithmic plot of molecular weight vs. migration distance with the following protein standards: phosphorylase b (94,000), bovine serum albumin (BSA) (67,000), ovalbumin (43,000), carbonic anhydrase (29,000), soybean trypsin inhibitor (20,000), and α-lactalbumin (14,400).

Quantitative immunoelectrophoresis was performed ac-

cording to Laurell (31) in 1% agarose containing 50 mM barbital buffer (pH 8.6):10 mM EDTA:0.02% NaN₃. Electrophoresis was conducted for 10 h at 10 V/cm in a Bio-Rad model 1400 electrophoresis cell (Bio-Rad Laboratories) with 10% ethylene glycol coolant (0–2°C) continuously circulating through the plate. Before electrophoresis, protein samples were carbamylated essentially as described by Weeke (32). Briefly, one volume of protein solution was mixed with an equal volume of a fresh solution of 2 M KCNO, incubated at 45°C for 30 min, and immediately subjected to electrophoresis after appropriate dilution in the electrophoresis buffer. Crossed immunoelectrophoresis was performed according to Ganrot (33).

Autoradiography was performed essentially as described by Swanstrom and Shank (34). Kodak X-Omat AR film (Eastman Kodak Co., Rochester, NY) was inserted between the dried slab gle and a DuPont Cronex Lightning-Plus intensifying screen (DuPont Co., Instrument Products Div., Wilmington, DE) in a Kodak x-ray exposure holder. Films were exposed for 1–4 h at −70°C, and developed at room temperature according to the manufacturer.

Radioiodination was performed according to a slight modification of the technique described by Salacinski et al. (35). 1,3,4,6-tetrachloro-3α,6α-diphenyl glycoluril (1 mg) was dissolved in 25 ml of dichloromethane, and 50 µl of that solution was evaporated to dryness under nitrogen in a polypropylene iodination vial. Tris-HCl buffer (1.0 M, 5 µl, pH 8.0) was added to the iodination vial, followed by Na ^{125}I (0.5 mCi, 5 µl) and 100–150 µl of Tris-buffered saline (0.05 M Tris-HCl [pH 7.5]:0.1 M NaCl) (TBS) containing ~50 µg of protein. The iodination mixture was incubated at room temperature for 5 min, and subsequently gel-filtered in a Sephadex G-25 column (1.5 × 8 cm) previously treated with 500 mg of BSA to prevent adsorption of the radiolabeled protein. Equilibration and elution of this column was performed with 10 mM sodium borate (pH 8.2):140 mM NaCl. 1-ml fractions were collected in polystyrene tubes containing 1 mg of BSA in 10 µl of TBS. Assuming there is complete recovery of protein mass after gel filtration, this procedure resulted in radiolabeled proteins with a specific radioactivity in the range of 0.5 to 2.0 × 10⁶ cpm/µg protein. Greater than 95% of the radioactivity in these preparations was precipitable by either 5% TCA or an affinity-purified antibody raised against the unlabeled protein. Furthermore, in the case of radioiodinated human protein C and APC, >95% of the radioactivity was adsorbed to barium citrate. In spite of its ability to bind readily to barium citrate and its respective antibody, substantial amounts of APC-amidolytic and -anticoagulant activities were lost during iodination. Assuming there is complete recovery of ^{125}I-APC from the Sephadex G-25 step, the ^{125}I-APC-amidolytic- and ^{125}I-APC anticoagulant-specific activities were ~60 and 50%, respectively, of the unlabeled preparation. Once radioiodinated, however, the amidolytic activity of ^{125}I-APC was quite stable when stored at 4°C in the presence of BSA (1 mg/ml).

Proteins. Human protein C and human α-thrombin were prepared as previously described (3). Human APC was prepared as follows. 300 µg of human α-thrombin (in 200 µl of TBS) was added to 5 ml of TBS containing 5 mg of human protein C. The mixture was incubated in a plastic tube for 3 h at room temperature. The incubation mixture was chilled to 4°C and applied to a column of SP-Sephadex C-50 (1.6 × 10 cm) previously equilibrated at 4°C with TBS. After sample application, the column was eluted with TBS at a rate of 0.5 ml/min. Under these conditions, human APC eluted in the unadsorbed fraction, while thrombin remained tightly bound to the resin. Aliquots of the human APC pool

were stored at −70°C until further use. 100 µl of the APC pool (33 µg), when incubated at 37°C with 300 µl of 0.4% fibrinogen in TBS, failed to produce a clot in 3 h, indicating the absence of thrombin. The isolated APC, however, possessed potent anticoagulant activity as judged by its ability to prolong the kaolin-cephalin clotting (KCCT) time of human plasma. In a typical experiment, 1 µg of purified human APC increased the KCCT of normal plasma from ~50 s to >1,500 s. As observed in previous experiments, the anticoagulant activity of the isolated human APC was totally inhibited by DFP (3).

Human antithrombin III was purified by the procedure described by Mahoney et al. (36). The antithrombin III was homogeneous as judged by SDS-PAGE (unreduced M_r = 58,000) and readily neutralized the amidolytic and coagulant activity of α-thrombin.

Preparation of affinity-purified antibodies. Three New Zealand rabbits were each immunized with 0.5–1 mg of purified APC-binding protein. For the initial injection, antigen was emulsified in an equal volume of Freund's complete adjuvant, and injected intradermally in multiple sites on the animal's back. Two booster injections, with Freund's incomplete adjuvant, were administered at 2-wk intervals after the initial injection. After the second booster shot, blood was collected from the central artery of the ear (37). The blood was clotted at 4°C for 15 h in 1 × 15 cm tubes, and the sera obtained by centrifugation. Antisera were stored at 4°C with 0.02% sodium azide present. Monospecific antiserum against human protein C was raised in a goat by essentially the same immunization protocol. Blood was collected from the external jugular vein of the animal, clotted at 4°C, and the sera collected by centrifugation.

Affinity-purified antibody against human protein C and the APC-binding protein were prepared by affinity chromatography of the sera in Sepharose columns containing the respective antigen covalently linked to the resin. Protein C-Sepharose and APC-binding protein-Sepharose were prepared by coupling the antigen to activated CH-Sepharose exactly as recommended by the manufacturer (Pharmacia Fine Chemicals). Approximately 5 ml of each affinity resin was prepared containing 20 mg of coupled antigen. In preparation for affinity chromatography, the antisera were treated with an equal volume of a neutral saturated solution of ammonium sulfate, centrifuged, and the pellet redissolved in a volume of TBS one-half that of the original serum volume. The antibody was dialyzed extensively against TBS at 4°C, and clarified by centrifugation. The supernatant was warmed to room temperature and applied to the affinity resin, previously equilibrated at room temperature with TBS. After sample application, the column was eluted with ten column-volumes of TBS:1 M NaCl. The antibody was eluted from the affinity column with one column-volume of 0.1 M glycine-HCl (pH 2.5). The affinity-purified antibody was collected in plastic tubes containing an appropriate amount of 0.5 M Tris-HCl (pH 8.5) that immediately adjusted the pH of the eluant to 7.5. The affinity-purified antibodies were dialyzed at 4°C against TBS:0.02% NaN₃ and stored at 4°C.

Neutralization of APC in various plasmas. The inhibition of APC in normal plasma and combined Factor V/VIII-deficient plasmas was assayed by measuring the ability of the plasma to neutralize the amidolytic and anticoagulant activity of the enzyme. In the amidolytic assay, 250 µl of plasma, 25 µl of APC (33 µg/ml in TBS:0.1% BSA), and 250 µl of TBS were incubated in a plastic tube at 37°C. A control incubation mixture, containing 25 µl of TBS in place of the enzyme, was run in parallel to the test mixture. At selected

times, 100 μl of each incubation mixture (test and control) was added to 1.0 ml of 0.1 mM S-2238 in 0.05 M Tris-HCl (pH 8.3):0.1 M NaCl. The hydrolysis of the S-2238 by each aliquot at 37°C was monitored at 405 nm in a Gilford spectrophotometer (model 2220; Gilford Instrument Laboratories Inc., Oberlin, OH) equipped with a strip chart recorder (model 6050) and a constant temperature circulator (Polytemp model 80; PolyScience Corp., Niles, IL). Under these conditions, little, if any, hydrolysis of S-2238 occurred in the control incubation mixture throughout the incubation period. Inclusion of heparin (10–50 U. S. Pharmacopeia U/ml final concentration) in the incubation mixtures, however, dramatically increased the background amidolytic activity of plasma proteases. This was found to be the case with normal plasma, combined Factor V/VIII-deficient plasma, and human plasma deficient in either Factor XII, Factor XI, Factor IX, high molecular weight kininogen, or prekallikrein.

In the anticoagulant assay, 1 ml of plasma and 50 μl of APC (50 ng/μl of TBS:0.1% BSA) were incubated at 37°C in a plastic tube. At various times, a 100 μl-aliquot was removed and added to a borosilicate glass tube (10 × 75 mm) containing 100 μl of normal plasma and 100 μl of kaolin-cephalin (5 mg kaolin/ml cephalin) previously incubated at 37°C for 4.75 min. 15 s later, 100 μl of 25 mM CaCl₂ was added and the clotting time recorded. The amount of APC activity remaining in the incubation mixture at any point was estimated from a log-log plot relating KCCT to added APC. The standard curve was constructed as follows. In a borosilicate glass tube (10 × 75 mm), 100 μl of normal plasma and 100 μl of kaolin-cephalin were mixed and incubated at 37°C. At 4.5 min into the incubation, 100 μl of normal plasma (warmed to 37°C) was added to the tube. 15 s later, 1–20 μl of APC (containing 25–1,000 ng enzyme dissolved in TBS:0.1% BSA) was added to the incubation mixture. 15 s later, 100 μl of 25 mM CaCl₂ was added to initiate clot formation and the KCCT was recorded. Assays were performed in duplicate. The KCCT increased linearly with 25–500 ng of APC in the assay system, but increased exponentially when greater amounts of enzyme were added to the assay. In addition, the precision between duplicate assays decreased substantially when 500–1,000 ng of APC was included in the assay. Accordingly, the initial concentration of APC in the various plasma samples tested (2,500 ng/ml) was selected to yield a clotting time in the linear portion of the standard curve when 100 μl of the incubation mixture was added to the assay.

Demonstration of complex formation between APC and a plasma protein in various plasma samples. Covalent complex formation of APC and a plasma protein was demonstrated by SDS-PAGE of plasma-¹²⁵I-APC incubation mixtures. Two different approaches were used to prepare the samples for electrophoresis. In the first approach, 225 μl of either normal plasma or combined Factor V/VIII-deficient plasma was incubated at 37°C in a 1.4-ml snap-cap centrifuge tube with 5 μl heparin (10 U/ml final concentration) and 25 μl of ¹²⁵I-APC (14.3 ng ¹²⁵I-APC/μl). At various times after the addition of the radiolabeled enzyme, 25 μl-aliquots were removed, added to 25 μl of 8 M urea:10% SDS, and incubated at 100°C for 2 min. A portion of the sample (10–15 μl) was subjected to SDS-electrophoresis in 7.5% polyacrylamide gels as described.

Although this method adequately demonstrated complex formation, it nonetheless often resulted in skewed, unsymmetrical radioactive peaks due to the substantial load of protein applied to the gel. Furthermore, subsequent studies revealed that the mobility of the complex, and hence its

molecular weight value, was affected by the large amount of protein applied to the gel. To overcome these problems, a second approach was devised whereby the radiolabeled enzyme and the enzyme-binding protein complex was immunoprecipitated from plasma with affinity-purified goat anti-human protein C. In this method, the various plasma samples were adsorbed beforehand with the appropriate amount of protein A-Sepharose and barium sulfate to remove endogenous IgG and protein C, respectively. Preliminary experiments demonstrated that plasma treated in this manner completely retained its ability to neutralize the amidolytic and anticoagulant activities of APC, as well as form a complex with radiolabeled APC by using the procedure described above. Thus, in a typical immunoprecipitation experiment, 200 μl of adsorbed plasma, 10 μl of ¹²⁵I-APC, and 2 μl of heparin (10 U/ml final concentration) were incubated in a 1.4-ml centrifuge tube at 37°C for 3 h. At this time, 10 μl of goat anti-human protein C (30 μg IgG) was added, and the mixture incubated at 4°C for 15 h. The mixture then was treated with 100 μl of a 10% suspension of 5×-washed *Staphylococcus aureus* cells (Pansorbin), incubated at 22°C for 15 min, and centrifuged (Eppendorf microfuge model 5412). The pellet was resuspended in 500 μl of 25 mM Tris-HCl (pH 7.5):50 mM NaCl and recentrifuged. Finally, the washed pellet was resuspended in 50 μl of 5% SDS:25 mM Tris-HCl (pH 7.0), and incubated at 100°C for 2 min. The pellet was removed by centrifugation and the supernatant subjected to SDS-gel electrophoresis. The SDS-protein sample generally contained 90–95% of the radioactivity added to the incubation mixture.

Immunodepletion of APC-binding protein from normal human plasma. APC-binding protein was removed from normal plasma using *S. aureus* cells (Pansorbin), previously loaded with affinity-purified antibody against the binding protein, as a solid phase immunoadsorbent (38). The cells were loaded with antibody as follows. Two 500-μl quantities of Pansorbin (10% suspension) were each mixed with 3 ml of 10 mM sodium phosphate (pH 7.5):100 mM NaCl:1 mM EDTA:0.1% Triton X-100, and centrifuged. The supernatants were separated from the pellet by aspiration, and the cells resuspended in 500 μl of the above buffer. 1 ml of affinity-purified anti-binding protein (1.1 mg IgG) was added to one tube while the other was treated with 1.0 ml of rabbit IgG (1 mg/ml in TBS) to serve as a control immunoadsorbent. Both mixtures were incubated at 37°C for 30 min. The mixtures were diluted with 3 ml of the above buffer, centrifuged, and the supernatants aspirated. The pellets were washed once with the above buffer, once with TBS:0.02% NaN₃, and stored at 4°C.

To each of the pellets was added 500 μl of normal human plasma, previously adsorbed with protein A-Sepharose and barium sulfate, and 5 μl of ¹²⁵I-APC-binding protein (4,000 cpm/μl). The pellets were resuspended in the plasma, incubated at 4°C for 15 h, and centrifuged. Each supernatant was counted for radioactivity. Approximately 95% of the radioactivity was adsorbed to the pellet loaded with antibody against the binding protein, whereas ~95% of the counts remained in the supernatant of the control immunoadsorbent.

Purification of APC-binding protein. All steps in the purification procedure were performed at 4°C. The elution position of the APC-binding protein in column effluents was determined by SDS-gel autoradiography after incubation of the column fraction with ¹²⁵I-APC in the presence of heparin. The presence of heparin was found to be essential as little, if any, complex was observed in its absence. Routinely, 1 ml of column fraction, 10 μl of ¹²⁵I-APC (125,000 cpm), and 5

μl heparin (10 U/ml final concentration) were incubated at 37°C for 3 h. At this point, 15 μl of affinity-purified goat anti-human protein C (30 μg of IgG) was added to the mixture, and incubation continued for 4 h at 4°C. Pansorbin (150 μl; 5×-washed) was added to the mixture and incubated for 30 min at room temperature. The cells were pelleted by centrifugation, resuspended in 50 μl of 5% SDS:10% mercaptoethanol:25 mM Tris-HCl (pH 7):10% Ficoll, and incubated at 100°C for 2 min. The suspension was centrifuged, and the supernatant subjected to SDS-gel electrophoresis in a 10% polyacrylamide slab gel followed by autoradiography. In the isolation procedure, 50 ml of 1 M $BaCl_2$ were added to 1 liter of human citrated cryosupernatant, and the mixture stirred for 15 min. The precipitate was removed by centrifugation (5,000 g, 30 min). The supernatant was brought to 40% saturation with ammonium sulfate by the slow addition of salt. The mixture was stirred for 1 h and the precipitate was removed by centrifugation and discarded. The supernatant was adjusted to 70% saturation with ammonium sulfate by the slow addition of the salt. The precipitate was collected by centrifugation (5,000 g; 30 min) and dissolved in 250 ml of 50 mM Tris-HCl (pH 7.5). The protein solution was dialyzed against 20 liters of this buffer for 15 h. A precipitate that developed during dialysis was removed by centrifugation (18,000 g, 10 min).

The protein solution was applied to a heparin-agarose column (2.6 × 25 cm) previously equilibrated with 50 mM Tris-HCl (pH 7.5). The column was washed with 150 ml of equilibrating buffer containing 0.075 M NaCl. The column was then eluted with a linear gradient of NaCl generated from 250 ml of 50 mM Tris-HCl (pH 7.5):0.075 M NaCl and 250 ml of 50 mM Tris-HCl (pH 7.5):0.4 M NaCl. After the gradient, the column was eluted with 250 ml of 50 mM Tris-HCl (pH 7.5):1 M NaCl. The flow rate was 2 ml/min and 10-ml fractions were collected in plastic tubes. Those fractions containing protein that formed a complex with radiolabeled APC were pooled and dialyzed against two 4-liter quantities of 25 mM Tris-HCl (pH 8.0) for 15 h.

The retentate (70 ml) was applied to a QAE-Sephadex A-50 column (2.6 × 35 cm) previously equilibrated with 25 mM Tris-HCl (pH 8.0). After sample application, the column was washed with 200 ml of equilibrating buffer. The inhibitor was eluted from the column with a linear gradient of NaCl consisting of 600 ml of equilibrating buffer and 600 ml of equilibrating buffer containing 0.4 M NaCl. The flow rate was 1.5 ml/min and 10-ml fractions were collected in plastic tubes. Fractions from the QAE-Sephadex column containing protein that reacted with radiolabeled APC were subjected to SDS-PAGE. Only those fractions exhibiting electrophoretic homogeneity were pooled and stored at 4°C in a plastic container.

RESULTS

Inhibition of APC by normal and combined Factor V/VIII-deficient plasma. The inhibition of APC by normal plasma and combined Factor V/VIII-deficient plasma was measured in two different assays: (a) an amidolytic assay employing S-2238 as the substrate for APC, and (b) a standardized coagulant assay designed to measure the anticoagulant activity of APC.

In the anticoagulant assay, a reproducible relationship was observed between the logarithm of the clotting time and the logarithm of the APC added to the assay (Fig. 1). Using the linear portion of this curve,
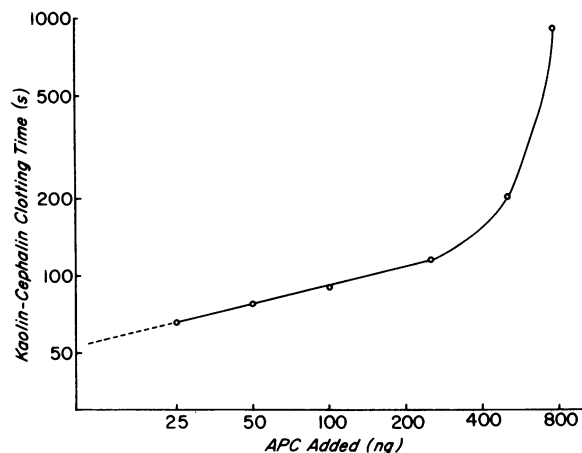

FIGURE 1 APC anticoagulant standard curve. The logarithm of the KCCT is plotted against the logarithm of added APC. Assays were performed in duplicate as described in Methods.

we were able to determine semiquantitatively on a temporal basis the APC-anticoagulant activity after its addition to various plasma samples. The incubation mixture consisted of citrated plasma and APC at a concentration of 2.5 μg APC/ml plasma. Under these conditions, plasma from three unrelated patients with a combined Factor V/VIII deficiency (patients A–C) inhibited the anticoagulant activity of APC at a rate indistinguishable from that observed using normal pooled plasma (Fig. 2). Furthermore, plasma from a
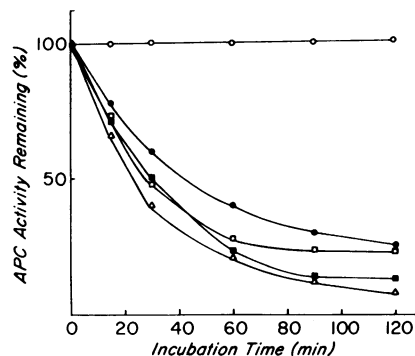

FIGURE 2 Inactivation of human APC in normal plasma and plasma from three unrelated patients with combined Factor V/VIII deficiency disease. Plasma (1 ml) was incubated with APC (2.5 μg/ml final concentration) at 37°C. At selected times, 0.1 ml was withdrawn and added to a glass tube containing 0.1 ml of normal plasma and 0.1 ml of kaolin-cephalin previously incubated at 37°C for 4.75 min. 15 s later, 0.1 ml of 25 mM $CaCl_2$ was added and the KCCT recorded. The amount of APC remaining in the incubation mixture was estimated from the APC standard curve. ■, normal pooled plasma; ●, patient A; □, patient B; △, patient C; ○, control sample consisting of APC (2.5 μg/ml final concentration) incubated in TBS:1% BSA at 37°C.

fourth patient with a combined Factor V/VIII deficiency (patient D, a sibling of patient C) inhibited APC activity at essentially the same rate as patient C (data not shown). Approximately 50% of the APC activity was inhibited by the various plasma samples in 30 min. A control sample of APC (plasma substituted with TBS:1% BSA) exhibited no apparent loss of anticoagulant activity throughout the incubation period (Fig. 2).

In the amidolytic assay for APC, slightly higher ratios of APC to plasma (3.3 μg APC:ml plasma) were necessary in the incubation mixture to produce a measurable hydrolytic rate in the spectrophotometric assay. The slight difference in APC concentration notwithstanding, both normal and combined Factor V/VIII-deficient plasma neutralized the amidolytic activity of APC at essentially the same rate as that observed in the anticoagulant assay, i.e., ~50% inhibition at 30 min (data not shown). As in the anticoagulant assay, a control sample of APC retained full amidolytic activity throughout the incubation period.

As noted earlier, heparin was not included in either of our incubation systems as it (a) produced substantial increases in the background hydrolysis rate of S-2238 in the amidolytic assay, and (b) totally inhibited the anticoagulant assay at concentrations as low as 0.1 U/ml heparin in the incubation mixture. Interestingly, the stimulation of the background S-2238 hydrolysis was observed in every human plasma sample that we tested, including prekallikrein-deficient plasma.

Complex formation of ^{125}I-APC and a plasma-binding protein in various plasma samples. When ^{125}I-APC ($M_r = 60,000$) was incubated with either normal plasma or plasma with a combined deficiency of Factor V/VIII, ~40% of the radioactivity was incorporated into a stable complex at essentially the same rate. The apparent molecular weight of the unreduced complex was 96,000 as determined by SDS-PAGE (Fig. 3A). After reduction of the sample with mercaptoethanol, the molecular weight of the complex decreased to 80–82,000 (Fig. 3B), suggesting that the plasma-binding protein was interacting with the heavy chain of ^{125}I-APC. Rate studies of reduced samples subjected to SDS-PAGE indicated that as radioactivity was incorporated into the 82,000-M_r complex, the radioactivity of the ^{125}I-APC heavy chain ($M_r = 40,000$) decreased proportionally. The radioactivity of the light chain of ^{125}I-APC ($M_r = 22,000$) remained unchanged during complex formation.

Incubation of ^{125}I-protein C with plasma did not result in complex formation, indicating that complex formation was specific for APC. To determine if the plasma-binding protein was interacting with the active site of APC, the ^{125}I-APC was treated with 5 mM DFP and the resulting diisopropyl (DIP)-^{125}I-APC incu-
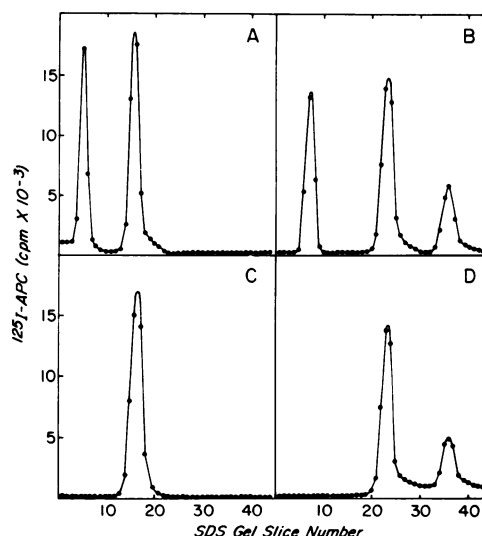

FIGURE 3 The radioactivity profile of ^{125}I-APC and ^{125}I-DIP-APC after incubation with plasma. Normal plasma (or Factor V/VIII-deficient plasma) was incubated with ^{125}I-APC (A and B) or ^{125}I-DIP-APC (C and D) for 2 h at 37°C. Radioactivity was immunoprecipitated with affinity-purified antihuman protein C, and adsorbed to *S. aureus* cells. The radioactivity was eluted from the cells with SDS buffer and subjected to SDS-PAGE in tube gels as described in Methods. The gels were sliced (1 mm) and the slices counted in a gamma counter. (A, C are unreduced samples; B, D are reduced samples).

bated with plasma. Under these conditions, no complex formation was observed (Fig. 3, C and D); this supports the premise that ^{125}I-APC was forming a complex with a plasma protein through its serine residue in the active site.

In this study, the amount of radioactivity incorporated into complex was related to the ratio of ^{125}I-APC to plasma volume, but it never exceeded 40% of the total radioactivity in the sample at saturating levels of all plasma samples tested. This was also found to be the case when using plasma previously treated with barium sulfate and protein A-Sepharose. The degree of complex formation did not appear to be influenced by the electrophoretic system employed (continuous electrophoresis at pH 7 vs. discontinuous electrophoresis at pH 9) as was reported for complexes of antithrombin III with either Factor Xa or thrombin (39). Furthermore, the extent of complex formation as assessed by gel filtration of ^{125}I-APC-plasma incubation mixtures in Sephadex G-150 appeared to be ~40–50%, suggesting that the degree of complex formation observed in SDS-PAGE in all likelihood did not occur as a result of dissociation during the denaturation with SDS. The reason for the low conversion of ^{125}I-APC into enzyme-inhibitor complex is unknown at this point. Conceivably, the reduced intrinsic activity of

the ^{125}I-APC for protein substrates, as noted in the KCCT assay, is one possible explanation for this behavior. Additional studies, however, are needed to clarify this issue.

Complex formation of ^{125}I-APC and a plasma binding protein in normal plasma and plasma with a combined Factor V/VIII deficiency was found to be accelerated by the addition of heparin to the incubation mixture. The amount of radioactivity incorporated into complex was proportional to the heparin concentration up to 10 U/ml final concentration. With this concentration of heparin in the incubation mixture, the rate of complex formation was enhanced roughly fivefold over that observed in an incubation mixture without added heparin. The rate of complex formation in heparinized incubation mixtures did not appear to be affected by the addition of calcium ions and phospholipid, either alone or in combination.

The observation that heparin augmented complex formation of ^{125}I-APC with its binding protein in plasma prompted us to reinvestigate the possibility that the plasma-binding protein of APC was either antithrombin III or a previously unrecognized derivative of antithrombin III. Earlier work from our laboratory indicated that human antithrombin III, in the presence of heparin, had no apparent effect on the amidolytic activity of APC (3). Accordingly, ^{125}I-APC was incubated at 37°C with purified human antithrombin III (inhibitor to enzyme ratios of 100–1,000:1) in the presence of TBS and heparin (10 U/ml final concentration). Under these conditions, no radioactive complex was observed by SDS-PAGE after a 3-h incubation, confirming our earlier observation. This provides strong evidence for the concept that antithrombin III was not involved in complex formation with ^{125}I-APC.

The radioactive complex of ^{125}I-APC and APC-binding protein was immunoprecipitated from various plasma samples by affinity-purified antibody to human protein C. This technique was found to be quite helpful in reducing the amount of protein applied to SDS-polyacrylamide gels. The relatively large amounts of protein applied to the gel from a plasma-^{125}I-APC incubation mixture (250–400 μg) greatly affected the symmetry and mobility of the radioactive protein bands, particularly in unreduced samples. Inasmuch as human plasma contains 8–17 mg/ml IgG and 1–1.2 μg/ml human protein C,[2] plasma samples were adsorbed with protein A-Sepharose (1 ml gel/ml plasma) and barium sulfate (40 mg/ml) to remove these proteins. Plasma adsorbed in this manner was as effective in neutralizing the amidolytic and anticoagulant ac-

tivities of APC as untreated plasma. Furthermore, adsorbed plasma formed a complex with ^{125}I-APC at a rate indistinguishable from that observed with untreated plasma. In the immunoprecipitation procedure, substitution of the antihuman protein C with 100 μl of rabbit antisera directed against either human antithrombin III or α_1-antitrypsin failed to precipitate the complex of ^{125}I-APC and APC-binding protein. This observation suggested, but did not prove, that the APC-binding protein was not immunologically related to either α_1-antitrypsin, antithrombin III, or a derivative of antithrombin III.

Purification and properties of the APC-binding protein. The APC-binding protein was isolated from human plasma by a three-step procedure that included ammonium sulfate fractionation, heparin-agarose chromatography, and QAE-Sephadex chromatography. Approximately 20 mg of the purified protein was obtained from 1 liter of plasma.

In the isolation procedure, the APC-binding protein concentrations were determined qualitatively, based on its ability to form a covalent complex with ^{125}I-APC as monitored by SDS-gel autoradiograms. After isolation to electrophoretic homogeneity, the APC-binding protein had no measurable effect on APC-amidolytic and -anticoagulant activity at a protein-to-enzyme molar ratio of 100:1. Furthermore, no apparent complex was observed by SDS-PAGE when equimolar amounts of unlabeled APC were incubated with the APC-binding protein at 37°C in the presence or absence of heparin.

The inability of the purified APC-binding protein to neutralize APC activity, or form a complex with unlabeled preparations of APC, casts doubt as to whether any relationship exists between this protein and that responsible for the inhibition of APC in plasma. We are, by definition, assuming that the protein in plasma that forms a covalent complex with ^{125}I-APC is a serine protease inhibitor. On the basis of evidence to be presented below, we believe the APC-binding protein isolated in this study is identical to the protein that forms a covalent complex with ^{125}I-APC in plasma incubation mixtures. However, in the absence of functional activity after purification, we are tentatively referring to this protein throughout this article as an APC-binding protein until further studies can unequivocally demonstrate inhibitory activity in purified preparations.

In the isolation procedure, the APC-binding protein was initially resolved from a substantial amount of protein by heparin-agarose column chromatography (Fig. 4). Furthermore, the APC-binding protein was resolved from antithrombin III by this step, antithrombin III consistently eluted from the heparin-agarose in the 1 M NaCl elution buffer.

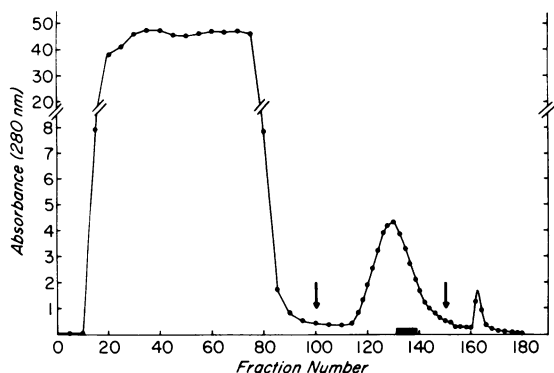[2] As determined by radioimmunoassay (Canfield, W. M., and W. Kisiel, manuscript in preparation).

FIGURE 4 Elution pattern of the APC-binding protein from heparin-agarose. APC-binding protein was eluted from the column (2.6 × 25 cm) with a linear gradient of 250 ml of 50 mM Tris-HCl (pH 7.5) containing 0.075 M NaCl and 250 ml of 50 mM Tris-HCl (pH 7.5) containing 0.4 M NaCl. The first arrow indicates initiation of the gradient. After the gradient, the column was eluted with 50 mM Tris-HCl (pH 7.5) containing 1 M NaCl (second arrow). Fractions that were pooled for APC-binding protein are indicated by the bar.

The APC-binding protein was purified to electrophoretic homogeneity by QAE-Sephadex A-50 column chromatography (Fig. 5). A single protein band was observed by SDS-PAGE for the APC-binding protein (Fig. 6) and a molecular weight of 47,000 was estimated by this technique for the unreduced protein. After reduction with 20% 2-mercaptoethanol, the apparent molecular weight of the APC-binding protein increased to 54,000. A single protein and carbohydrate-staining band was observed in SDS-PAGE when as much as 100 μg of the APC-binding protein was applied to the gel, attesting to its physical homogeneity. Furthermore, SDS-gel autoradiograms of radioiodinated preparations of the purified APC-binding
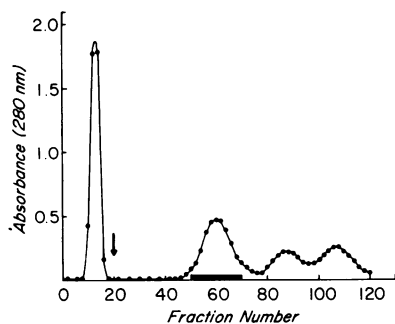

FIGURE 5 Elution pattern of the APC-binding protein from QAE-Sephadex. Protein was eluted from the column (2.6 × 35 cm) with a linear gradient of 600 ml of 25 mM Tris-HCl (pH 8.0) and 600 ml of 25 mM Tris-HCl (pH 8.0) containing 0.4 M NaCl. Fractions (10 ml) were collected at a flow rate of 1.5 ml/min. Fractions that were pooled are indicated by the bar.

protein revealed a single, intense radioactive band. Under reducing conditions routinely used in this laboratory (5–10% mercaptoethanol, 37°C, 30 min), the APC-binding protein appeared as a doublet in SDS-PAGE with apparent molecular weight values of 54,000 and 51,000. Under more rigorous reducing conditions (20% mercaptoethanol, 60°C, 30 min), the protein migrated as a single band (M_r = 54,000). In addition, the S-pyridylethylated derivative of the APC-binding protein migrated as a single band with an apparent molecular weight of 54,000.

When the APC-binding protein was subjected to alkaline disc gel electrophoresis (40), a single band was observed in 7.5% polyacrylamide gels. The mobility of the protein was remarkably low (R_f = 0.14 relative to bromophenol blue). Crossed immunoelectrophoresis of plasma and purified APC-binding protein yielded a single, coincident peak when subjected to electrophoresis in the second dimension in agarose gels containing affinity-purified antibody raised against the purified APC-binding protein. The electrophoretic mobility of the APC-binding protein clearly distinguishes it from the plasma protease inhibitors of comparable molecular weight, such as antithrombin III and α_1-antitrypsin, which migrate with relatively high mobilities (41).

The amino acid composition of the APC-binding protein is presented in Table I. The most notable features of the amino acid composition are the relatively high quantities of proline, half-cystine, and lysine. The
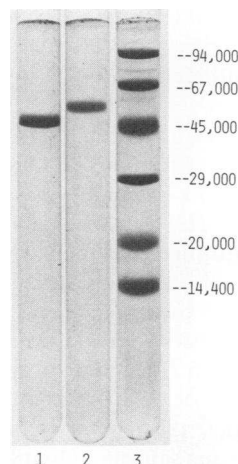

FIGURE 6 SDS-PAGE of the APC-binding protein. Sample 1 contained 20 μg of unreduced binding protein; sample 2, 20 μg of reduced binding protein; and sample 3, a mixture of reduced standard proteins that includes phosphorylase b (94,000), BSA (67,000), ovalbumin (45,000), carbonic anhydrase (29,000), soybean trypsin inhibitor (20,000), and α-lactalbumin (14,400). Electrophoresis was carried out as in Methods.

TABLE I
Amino Acid Composition of The APC Binding Protein

Amino acid	Mol
	%
Aspartic acid	8.39
Threonine	6.97
Serine	6.31
Glutamic acid	7.64
Proline	9.36
Glycine	7.51
Alanine	5.21
Half-cystine*	5.36
Valine	6.15
Methionine	1.24
Isoleucine	3.79
Leucine	5.36
Tyrosine	4.10
Phenylalanine	5.87
Histidine	1.70
Lysine	8.90
Arginine	3.21
Tryptophan‡	2.91

* Determined as cysteic acid according to Hirs (23).
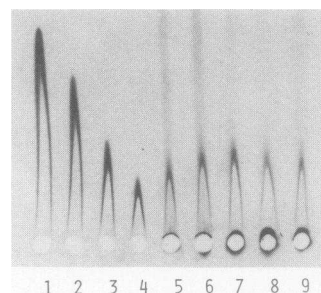
‡ Determined by the method of Hugli and Moore (22).

FIGURE 7 Quantitative electroimmunoassay of the APC-binding protein in various plasma samples. Wells 1–4 contained 5 μl of serial dilutions of purified inhibitor (1, 63 μg/ml; 2, 42 μg/ml; 3, 25 μg/ml; 4, 13 μg/ml). Well 5 contained 5 μl of a 1:4 dilution of normal pooled plasma, and wells 6–9 contained 5 μl of a 1:4 dilution of combined Factor V/VIII-deficient plasma (patients A–D). Agarose contained 2% (vol/vol) monospecific antiserum. 10 V/cm was applied for 10 h, anode is at the top.

relatively high half-cystine content may explain, in part, the resistance of the protein to reduction. In addition, the high content of basic residues (arginine, lysine, and histidine) relative to the acidic residues (aspartic acid and glutamic acid) probably accounts for the low electrophoretic mobility of the protein observed in alkaline disc gels. The APC-binding protein is glycosylated and contains ~7% neuraminic acid by weight.

Purified preparations of the APC-binding protein did not form a precipitin band in Ouchterlony double-diffusion experiments with antisera raised against human antithrombin III, α_1-antitrypsin, α_2-antiplasmin, inter-α-trypsin inhibitor, Cl-inactivator, and α_2-macroglobulin. Each of these antisera, however, formed a single precipitin band in agarose with undiluted plasma. Rabbit antisera raised against the purified APC-binding protein formed a single precipitin band when allowed to react with either purified APC-binding protein, normal plasma, or combined Factor V/VIII deficient-plasma samples. Quantitative electroimmunoassay for the APC-binding protein in normal and Factor V/VIII-deficient plasma samples indicated that its concentration was essentially the same in all plasma samples tested (Fig. 7). By this technique, the APC-binding protein antigen concentration was determined to be 70–80 μg/ml plasma. On the basis of these studies, the APC-binding protein did not appear to be im-

munologically related to any of the well-characterized plasma protease inhibitors.

The nonidentity of the APC-binding protein with the known plasma protease inhibitors was established unequivocally by amino-terminal sequence analyses of the S-pyridylethylated derivative of the APC-binding protein. Glycine was identified as the amino-terminal residue of the protein, and no other amino acids were detected. The amino-terminal sequence of the first 22 residues of the APC-binding protein is shown in Fig. 8. No apparent homology was discernible between the amino-terminal sequence of APC-binding protein and the corresponding sequence in human antithrombin III, α_1-antitrypsin, and α_2-antiplasmin (Fig. 8). However, considerable homology (25–32%) was noted between APC-binding protein and bovine colostrum inhibitor and pancreatic trypsin inhibitor (Fig. 8). Of particular interest is the lysine residue in position 19 of the APC-binding protein, as this residue constitutes the reactive site in the colostrum and pancreatic trypsin inhibitor (42). An inspection of current supplements to the Atlas of Protein Sequence data base (45) revealed that the APC-binding protein isolated in this study represented a unique, uncharacterized human plasma protein.

Immunoprecipitation of the complex of ^{125}I-*APC and APC-binding protein by antibody raised against the purified APC-binding protein.* As noted earlier, affinity-purified antihuman protein C immunoprecipitated both free ^{125}I-APC and a complex of ^{125}I-APC and the APC-binding protein. When antihuman protein C was replaced with an equal amount of affinity-purified anti-APC-binding protein, ~35–40% of the radioactivity added was immunoprecipitated from the incubation mixture and adsorbed to *S. aureus* cells. Analysis of the plasma supernatant and the SDS eluate

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Human Activated Protein C
  Binding Protein            Gly Arg Thr Cys Pro Lys Pro Asp Asp Leu Pro Phe Ser Thr Val Val Pro Leu Lys Thr Phe Tyr

Bovine Colostrum Inhibitor    Phe Gln Thr Pro Pro Asp Leu Cys Gln Leu Pro  -  Gln Ala Arg Gly Pro Cys Lys

Pancreatic Trypsin Inhibitor          Arg Pro Asp Phe Cys Leu Glu Pro Pro Tyr Thr  -  Gly Pro Cys Lys Ala

Antithrombin III              His Gly Ser Pro Val Asp Ile Cys Thr Ala Lys Pro Arg Asp Ile Pro Met Asn Pro Met Cys Ile

α1-Antitrypsin                Glu Asp Pro Gln Gly Asp Ala Ala Gln Lys Thr Asp Thr Ser His His Asp Gln Asp His Pro Thr

α2-Antiplasmin                Asn Gln Glu Gly
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FIGURE 8 Amino-terminal sequence of the APC-binding protein and various protease inhibitors. Amino acid residues in the APC-binding protein that are identical with the protease inhibitors are shown in blocks. Dashes refer to spaces that have been inserted to bring the proteins into alignment for better homology. Sequence data for the proteinease inhibitors were taken from Laskowski et al. (42), Leicht et al. (43), and Wiman and Collen (44).

from the cells by SDS-PAGE revealed that essentially all the radioactivity in the plasma supernatant represented free ^{125}I-APC ($\sim$5% complex), whereas immunoprecipitated radioactivity represented exclusively the enzyme-binding protein complex. Fig. 9 shows the radioactivity profile of the anti-APC-binding protein immunoprecipitate before and after reduction with 10% mercaptoethanol. In the unreduced sample (Fig. 9A), a single peak of radioactivity was observed with a corresponding molecular weight of 96,000. After reduction (Fig. 9B), two peaks were observed with apparent molecular weight values of 82,000 and 22,000. These peaks represent the co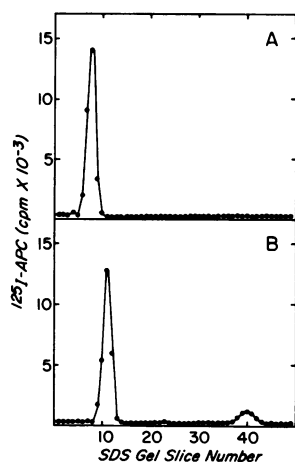mplex of binding protein-APC heavy chain and the APC light chain, respectively. The identity of each of these radioactive peaks was determined through comparison of SDS gels of the antihuman protein C immunoprecipitate run in parallel. Thus, antibody raised against the APC-binding protein isolated in this study recognized and immunoprecipitated the ^{125}I-APC in complex with its plasma binding protein, and strongly supports the contention that the protein isolated in this study is indeed the plasma protein that forms a covalent complex with ^{125}I-APC in plasma incubation mixtures.

Complex formation of ^{125}I-APC and APC-binding protein in plasma immunodepleted of the APC-binding protein. Incubation of ^{125}I-APC with plasma immunodepleted of the APC-binding protein by the procedure described in Methods, resulted in $\sim$8% of the radioactivity in complex in 3 h incubation time. In contrast, incubation of ^{125}I-APC with plasma previously treated with purified rabbit IgG resulted in $\sim$40% of the counts in complex in this time frame. Thus, an 80% reduction in complex formation was observed in plasma immunodepleted of APC-binding protein. Serial immunodepletions of plasma (three times) with antibody-loaded cells reduced the complex to 3% (a 93% reduction), although complex formation in control plasma remained essentially unchanged. Interestingly, in spite of the serial immunodepletions, a small amount of complex was observed. The reason for this is not readily apparent, but it is not inconceivable that a trace amount of antigen either is not reacting with the antibody or dissociates from the antigen-antibody complex. Assuming 99.9% of the antigen was removed, the binding protein level in plasma would still be 80 ng/ml, and presumably it is this population of antigen that forms a complex with ^{125}I-APC.

FIGURE 9 The radioactivity profile of ^{125}I-APC after incubation in plasma and immunoprecipitation with rabbit anti-APC-binding protein. Normal plasma was incubated with ^{125}I-APC for 2 h at 37°C. Radioactivity was immunoprecipitated with affinity-purified anti-APC-binding protein, and adsorbed to *S. aureus* cells. The radioactivity was eluted from the cells with SDS buffer and subjected to SDS-PAGE in tube gels as described in Methods. The gels were sliced (1 mm) and the slices counted in a gamma counter. A, unreduced SDS sample; B, reduced SDS sample.

DISCUSSION

The results of this study confirm that normal human plasma contains an inhibitor directed against APC, an

observation initially described by Marlar and Griffin (15). These investigators went on to propose that this inhibitor is congenitally deficient in patients with combined Factor V/VIII deficiency disease (15). This proposal was formulated from existing data regarding the in vitro proteolytic specificity of APC, as well as compelling evidence that plasma from four unrelated combined Factor V/VIII-deficient patients failed to inhibit the amidolytic activity of APC (15). In this proposal, Marlar and Griffin (15) suggested that the absence of a plasma inhibitor to APC allowed for the progressive and continuous in vivo proteolytic inactivation of Factors V and VIII in these patients by unopposed APC. Their hypothesis did not include, however, a mechanism for the continuous production of APC, a condition seemingly necessary for this condition to occur, assuming there are normal biosynthetic rates for Factors V and VIII. Furthermore, it is highly interesting and statistically unusual that the four unrelated patients' plasma used in their study possessed inhibitor activity $\leq 2\%$ of normal plasma (15).

The data presented in this article are clearly at variance with that reported by Marlar and Griffin (15). In this study, plasma from four patients with clinically defined combined Factor V/VIII deficiency disease inhibited the anticoagulant and amidolytic activities of human APC at essentially the same rate as that observed with normal pooled plasma. Furthermore, incubation of ^{125}I-APC with either normal or combined Factor V/VIII-deficient plasma resulted in complex formation at essentially the same rate and final extent. Complex formation was evaluated by SDS-PAGE and was assumed to be a stable covalent complex between the radiolabeled enzyme and its putative plasma inhibitor. Complex formation was totally dependent on the integrity of the active site serine residue of APC, as DFP-treated enzyme failed to form a covalent complex in either normal or combined Factor V/VIII-deficient plasma.

The anticoagulant assay for APC provided us with an important degree of specificity not found in the amidolytic assay using S-2238, a substrate designed for thrombin assay. The hydrolysis of S-2238 after the addition of APC to plasma observed in this study, as well as that of Marlar and Griffin (15), may not totally reflect changes in APC activity, but may in part reflect changes in the activity of other proteases or esterases that cleave this substrate. The use of the anticoagulant assay, coupled with the complex formation experiments with ^{125}I-APC, provides two independent and specific lines of evidence that demonstrate that APC is inactivated at essentially the same rate in normal and combined deficiency plasma.

Based on its ability to form a complex with ^{125}I-APC, as judged by SDS-PAGE, a plasma protein was purified to homogeneity by a combination of ammonium sulfate fractionation, heparin-agarose chromatography, and QAE-Sephadex chromatography. The purified protein was chemically, physically, and immunologically distinct from the well-characterized plasma serine protease inhibitors, which included antithrombin III, α_1-antitrypsin, α_2-antiplasmin, α_2-macroglobulin, Cl inactivator, inter-α-trypsin inhibitor, and the recently described heparin cofactor II (46, 47). The purified APC-binding protein, however, did not directly inhibit the anticoagulant or amidolytic activities of APC. Moreover, the purified binding protein failed to form a covalent complex with unlabeled preparations of APC. Our interpretation of these findings was that either the protein that we had purified had lost essentially all of its biological activity during the isolation procedure, or that we had isolated an unknown protein with a trace amount of APC inhibitor present. On the basis of (a) the amino-terminal sequence data, (b) the immunoprecipitation experiments with anti-binding protein, (c) the immunodepletion experiments, and (d) the molecular weight of the APC-binding protein as it relates to the molecular weight of the complex observed, we are reasonably certain that the former interpretation is more consistent with the experimental observations. At this point, however, we cannot categorically rule out the remote possibility that a highly immunogenic contaminant in our final preparation, with a blocked amino-terminus, is responsible for complex formation with ^{125}I-APC. This contaminant would have to constitute $<0.1\%$ of the total protein (as judged by autoradiography of labeled APC-binding protein) and would obviously have to exhibit the same electrophoretic mobility in the presence or absence of SDS as the major protein.

The reason(s) for the complete loss of APC inhibitory activity during isolation was inexplicable. Conceivably, the biological activity of the APC-binding protein is exceedingly sensitive to a particular condition of the isolation procedure, and the final product is rendered inactive. This possibility prompted us to develop a standardized assay for the putative APC inhibitor to evaluate loss of activity during the isolation procedure. As noted earlier, complex formation of ^{125}I-APC with binding protein in column fractions, as monitored by SDS-PAGE, was dependent on the presence of heparin in the incubation mixture. The requirement for heparin all but eliminated the APC anticoagulant assay. Recent preliminary results using the amidolytic assay indicate that the inhibitor activity is greatly diminished after ammonium sulfate precipitation and subsequent dialysis against TBS. It is not inconceivable that, like human antithrombin III (48), the APC inhibitor is highly unstable in dilute buffers with ionic strength levels <0.15 M. This possibility, among others

such as cofactor requirements for the expression of inhibitory activity, is currently under active investigation in our laboratory.

The results of this study, taken collectively, do not support the proposal that a deficiency in APC inhibitor forms the molecular basis of the combined Factor V/VIII deficiency disease, a proposal now generally accepted as fact (49). Our finding of decreased VIII:CAg levels in all of our patients is in agreement with a recent report by Hultin and Eyster (50) who, to our knowledge, were the first to demonstrate a decreased VIII:CAg level in a combined Factor V/VIII-deficient patient. In addition, the Factor V antigen levels of the patients' plasma used in this study all were decreased, and paralleled their Factor V coagulant activity levels. Thus, from our observations using plasma from well-defined combined Factor V/VIII-deficient patients, it would appear that caution should be exercised in concluding that all combined Factor V/VIII-deficient patients are functionally deficient in an inhibitor directed against APC. Whether our four combined Factor V/VIII-deficient patients reflect a unique genotype of this disorder remains to be established. It is hoped that future studies will clarify the relationship between the APC-binding protein described here and the plasma protease inhibitor involved in the physiological regulation of APC.

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