Monoclonal Antibodies to Human Vitamin K–Dependent Protein S

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Monoclonal antibodies to human protein S have been prepared using established hybridoma technology. One antibody was isolated that binds protein S only when Ca\(^{2+}\) is present; others bind antigen equally well in the presence or absence of EDTA. Other antibodies display a diminished affinity for protein S in the presence of EDTA. Purified immunoglobulins from cell lines displaying Ca\(^{2+}\) dependence were immobilized and used to purify protein S from fractions obtained by barium precipitation of citrated plasma, ammonium sulfate fractionation, and chromatography on diethylaminoethanol (DEAE)-Sephadex and dextran sulfate agarose. Essentially homogeneous protein S was isolated from the barium-citrate–adsorbed, 35% ammonium sulfate fraction by a partially Ca\(^{2+}\)-dependent antibody and were eluted partially with EDTA and NaCl and finally with NaSCN. The largest and most abundant of the high mol wt materials is likely protein S–complement C4b-binding protein complex. The immunoaffinity-isolated protein S was bound from plasma. To demonstrate that immunoaffinity-isolated protein S is free and complexed forms.

MATERIALS AND METHODS

Materials. Some of the protein S used in these studies was generously provided by Dr S. P. Bajaj. Other vitamin K-dependent proteins were isolated in our laboratory by conventional methods. Radioiodination of proteins used in screening assays was done with Bolton-Hunter reagent or with Iodogen (Pierce Chemical Co). Human protein C was activated with thrombin as described by Kisiel et al. Human factor V was isolated by a modification of the Katzmann procedure. Fresh frozen plasma was thawed, and benzamidine–HCl, heparin, and soybean trypsin inhibitor were added to final concentrations of 10 mmol/L, 2 U per milliliter and 20 μg per milliliter, respectively. The plasma was stirred at 4°C, and 1.0 mol/L of BaCl\(_2\) was added (80 μL per liter). After 15 minutes of stirring, solid polyethylene glycol was added to a final concentration of 4% (wt/vol). Following an additional 30 minutes of stirring, the sample was centrifuged at 6,000 g at 4°C for 30 minutes. Equal volumes of water and 0.025 mol/L of Tris-HCl, 5.0 mmol/L of CaCl\(_2\), 1.0 mmol/L of benzamidine–HCl, pH 7.5 were added to the supernatant. Packed QAE cellulose which had been equilibrated

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with 0.025 mol/L of Tris-HCl, 5.0 mmol/L of CaCl₂, and 1.0 mmol/L of benzamidine-HCl, pH 7.5, was added (200 mL per liter of plasma), and the suspension was stirred for 30 minutes at 4 °C. The resin was then transferred to a sintered glass funnel for batch chromatography at room temperature. The resin was washed with 0.02 mol/L of Imidazole, 5.0 mmol/L of NaCl, pH 6.5, followed by a wash with the same buffer containing 0.1 mol/L of NaCl. Following the second wash, the factor V was eluted with the same buffer containing 0.3 mol/L of NaCl. The eluate containing factor V was applied to a column (2.5 x 10 cm) of immobilized antibody to human factor V. After the sample was applied, the column was washed with 0.02 mol/L of Imidazole, 5.0 mmol/L of CaCl₂, and 0.3 mol/L of NaCl, pH 6.5, until the absorbance (280 nm) was <0.010. The factor V was then eluted with the same buffer, but 1.5 mol/L in NaCl. Fractions containing factor V were pooled, chilled to 4 °C and concentrated by precipitation with solid ammonium sulfate (0.48 g per milliliter) at 4 °C. To prepare factor Va, a solution of factor V (0.5 mg per milliliter) in 0.02 mol/L of Tris–HCl and 0.15 mol/L of NaCl, pH 7.4, was treated with thrombin for 15 minutes at 37 °C at a final concentration of 6 NIH U per milliliter. The factor Va was then diluted with dapsylarginine-N-(3-ethyl-1,5-pentanediyl)amide (DAPA) at a final concentration of 17 nm. The Va was then diluted with 0.02 mol/L of Tris–HCl and 0.15 mol/L of NaCl, pH 7.4, and CaCl₂ was added to give a final solution of 0.76 μmol/L of Va, containing 2 mmol/L of CaCl₂. The Va was then stored on ice and used within five hours. Human factor X was activated to Xα according to a method described for bovine factor X.34 Thrombin was prepared as described by Lundblad et al.36 Phospholipid vesicles (PCPS) of 75% phosphatidylcholine and 25% phosphatidylserine were prepared by the procedure of Barenholz and colleagues38 as described by Higgins and Mann.39 DAPA was prepared as described by Nesheim and co-workers.40 Other reagents were obtained commercially and were of analytical quality.

**Protein concentrations.** The mol wts and extinction coefficients (ε₂₈₀, 280 nm) of the respective proteins were taken as follows: factor Vα, 330,000, 9.6; prothrombin,41 70,000, 13.8; factor Xa, 46,000, 11.6; activated protein C42, 60,000, 14.5; protein S,70,000, 10.0.

**Solid-phase radioimmunoassay.** The assay used to screen culture supernatants has been described previously. In brief, 0.2 mL of supernatant was added to polystyrene tubes that had been coated with rabbit IgG anti-mouse immunoglobulin. After incubation, the tubes were washed three times with 0.02 mol/L of Tris–HCl, 0.10 mol/L of NaCl, and 0.02% NaN₃, pH 7.3, and ¹²⁵I-protein was added in the same buffer made 0.1% with BSA; in some instances, the buffer was made 2 mol/L with CaCl₂ or with EDTA. After incubation, the tubes were washed three times with 0.02 mol/L of Tris–HCl, 0.10 mol/L of NaCl, 0.02% NaN₃, and 0.1% BSA, pH 7.3, and were counted in a Beckman gamma counter.

**Immunization, cell fusion, and cloning.** BALB/c mice were initially immunized by intraperitoneal injection of 40 μg of protein S in complete Freund’s adjuvant. Six months later, a second injection of 40 μg of protein S in 0.01 mL of sodium phosphate, 0.15 mol/L of NaCl, pH 7.4, was given. Immunized spleen cells were fused with murine NS-1 myeloma cells in the presence of polyethylene glycol and distributed into the wells of our 24-well culture plates.24 Cells in positive wells selected for further study were subcloned by limiting dilution.35 Those yielding cloned cells were subsequently injected intraperitoneally into BALB/c mice for the production of antibody-rich ascites fluid.37

**Polyacrylamide gel electrophoresis.** Slab gel electrophoresis was run in the presence of sodium dodecyl sulfate (SDS) by the method of Laemmli.36 Proteins were visualized with Coomassie brilliant blue R-250 and destained by diffusion in 18% methanol-9% acetic acid. ¹²⁵I-Labeled proteins were visualized by autoradiography.

**HPLC.** The HPLC system consisted of two Waters 6000 pumps, a Water U6K injector, a Waters 660 programmer, a Waters 441 detector equipped with a zinc lamp, a 214-nm filter, and a Beckman Ultraport RPSC column. Solvent A was 0.05% trifluoroacetic acid (Pierce Chemical Co) in water processed through a Millipore Norganic cartridge. Solvent B was 0.05% TFA in HPLC grade acetonitrile. Proteins were generally injected in 5 to 50 μL of 0.2 mol/L Tris-HCl, 0.15 mol/L of NaCl, pH 7.4, made up to ~100 μL with HPLC buffer A. Elution was achieved with a 15-minute linear gradient from 30% buffer B to 60% buffer B; flow rate was 1.0 mL per minute. Spectra were recorded on a Bascom-Turner series 8000 recorder and in some instances were electronically reduced to fit the format of the figure.

**Amino acid analysis.** Alkaline hydrolysis was done by the procedure of Price.45 Analyses were performed by postcolumn derivitization with o-phenaldehyde using an Interaction AA911 column and elution with a linear gradient from 0.2 N sodium citrate, pH 3.28, to 0.15 N NaCl, 0.05 N NaOH, and 0.15 N boric acid, pH 9.8.

**Conventional isolation of protein S from plasma.** The vitamin K-dependent proteins in citrated plasma were adsorbed to barium citrate and fractionated with ammonium sulfate. After dialysis against 0.025 mol/L of citrate, 0.10 mol/L of NaCl, and 1 mmol/L of benzamidine–HCl, pH 6, the sample was applied to a DEAE-Sephadex column equilibrated with dialysis buffer. Proteins were eluted by a linear gradient from 0.1 mol/L of NaCl to 0.6 mol/L of NaCl in equilibration buffer.46 Fractions were pooled based on the elution profile obtained when A₂₈₀ was plotted against fraction number. Pooled fractions were then made 1 mol/L with DFP, and 5 mmol/L with EDTA, and dialyzed against 20 mmol/L of 2-(N-morpholino)-ethane sulfonic acid-Tris, pH 6, containing 1 mmol/L of benzamidine-HCl. The dialysate was made 2.5 mol/L with CaCl₂ and chromatographed on a dextran sulfate agarose column equilibrated with dialysis buffer made 2.5 mol/L with CaCl₂. Proteins were eluted by a linear gradient to 1 mol/L NaCl in the same buffer,47 with protein S eluting just ahead of the protein C activity.

**Isolation of immunoglobulin fraction of murine ascites fluids.** The immunoglobulin fraction of murine ascites fluids were isolated by gel filtration chromatography on Ultrogel AcA34 (LKB Instruments) at 24 °C in 0.01 mol/L of sodium phosphate, 0.15 mol/L of NaCl, 0.02% of NaN₃, pH 7.4. The immunoglobulins were precipitated by addition of ammonium sulfate to 70% saturation, collected by centrifugation, and stored in a 1:1 mixture of glycerol and elution buffer.

**Purification of protein S with immobilized antibody.** Purified anti-protein S IgG was reacted with CNBr-activated Sepharose 4B at 1 mg IgG per milliliter of resin using 0.2 mol/L citrate, pH 6.5, as coupling buffer. Eluate from a dextran sulfate agarose column that contained protein S was dialyzed against 0.02 mol/L of Tris–HCl, and 0.15 mol/L NaCl, pH 7.4, and applied to the antibody-Sepharose column that had been equilibrated in the same buffer. If the sample volume was less than the void volume of the column, flow was stopped for one hour after the sample was applied. Otherwise, the flow rate was slowed to about 0.3 mL per minute. The column was washed with sample buffer until a constant A₂₈₀ was achieved (≤0.005) and then eluted with one or more of the following buffers: 0.01 mol/L of EDTA; 0.02 mol/L of Tris–HCl; 0.15 mol/L NaCl, pH 7.4; 0.02 mol/L of Tris–HCl; 1.2 mol/L of NaCl, pH 7.4; 3 mol/L of Na₂SCN; 0.02 mol/L of Tris–HCl; 0.15 mol/L of NaCl, pH 7.4; or with 0.1 mol/L of glycine and 2 mol/L of NaCl, pH 2.8. After use, resins
were stored in 0.02 mol/L of Tris-HCl; 0.15 mol/L of NaCl; and 0.02% NaN₃, pH 7.4.

Isolation of protein S from barium citrate-adsorbed, vitamin K-dependent proteins. Citrated plasma was precipitated with barium chloride; the precipitate was suspended in 35% ammonium sulfate, 0.05 mol/L of benzamidine-HCl and centrifuged. After dialysis against 0.02 mol/L of Tris-HCl, 0.15 mol/L of NaCl, pH 7.4, the supernatant was applied to an anti-protein S-Sepharose column that had been equilibrated with dialysis buffer. Flow rate was maintained at about 0.3 mL per minute, and the column was eluted to a stable baseline with 0.02 mol/L of Tris-HCl and 0.15 mol/L of NaCl, pH 7.4, with 10 mmol/L of EDTA in the same buffer.

Isolation of high mol wt material from plasma. Plasma from blood drawn into citrate, benzamidine-HCl, soybean trypsin inhibitor anticoagulant was applied directly to an antibody-Sepharose column that had been equilibrated with 0.02 mol/L Tris-HCl and 0.15 mol/L of NaCl, pH 7.4. Flow rate was maintained at about 0.3 mL per minute, and the column was washed with equilibration buffer until a stable baseline was achieved (ΔA₀.₀₁₀ ≤ 0.010). The column was then eluted successively with 10 mmol/L of EDTA and 0.02% NaN₃, pH 7.4, with 0.5 to 2.0 mol/L of NaCl in three increments and 3 mol/L of NaSCN in the same buffer.

Recovery of dilute protein samples. When required prior to analysis, protein samples were concentrated in Centricon 30 microconcentrators (Amicon). Typically, 60% to 70% of the protein was recovered when 8 to 10 mL of sample was concentrated to ~0.25 mL.

Activated protein C and cofactor protein S assay. A two-stage assay was used to measure the cofactor activities of both conventionally isolated protein S, and immunoaffinity-isolated protein S. The factor Va inactivation mixture (final vol 0.056 mL) contained factor Va (13.6 nmol/L), PCPS (10.4 μmol/L), and CaCl₂ (2 mmol/L), in 0.020 mol/L of Tris-HCl, 0.15 mol/L of NaCl, pH 7.4, and was briefly incubated at 22 °C in the presence or absence of protein S (38 mmol/L). The reaction was initiated by the addition of APC (7.4 mmol/L). Aliquots (20 μL) were withdrawn at various times after incubation at 37 °C, to determine the amount of factor Va activity remaining by the assay previously described. The assay mixture contained prothrombin (1.49 μmol/L), factor Xa (3.5 mmol/L), CaCl₂ (2 mm), PCPS (23 μmol/L) and DAPA (3 μmol/L). The factor Xa concentration was adjusted to a 26-fold molar excess over the highest potential factor Va concentration to assure that Va was limiting and that the assay was directly proportional to Va concentration.

RESULTS

Fifty of the initial 96 wells were determined to be positive for antibody by the solid phase RIA. Eleven showed a varying dependence on Ca²⁺ for the binding of ¹²⁵I-protein S. The reactivity patterns of the 12 supernatants selected for further study are shown in Fig 1. Of the 12 selected for subcloning, six showed Ca²⁺ dependence (wells 12, 15, 19, 26, 71, and 76), four showed no Ca²⁺ dependence (wells 14, 27, 50, and 87), and two showed a slight increase in binding of ¹²⁵I-protein S in the presence of EDTA (wells 22 and 48). Seven of the hybrids did not yield cloned cells (wells 12, 14, 22, 26, 27, 48, and 76). Those that gave clones exhibited reactivity patterns as ascites fluids similar to those found in the initial screen; this is apparent in the titration curves shown in Fig 2.

Three of the ascites fluids were checked for cross-reactivity with human vitamin K-dependent proteins prothrombin, factor IX, factor X, and protein C; again ¹²⁵I-labeled proteins were used in the solid-phase RIA. Results are summarized in Table 1. Only when protein C was added were the cpm bound significantly above background. By dissolving the residues in...
the tubes in 0.0625 mol/L of Tris-HCl, 0.2% and SDS, pH 6.8, separating the \(^{125}\)I-proteins electrophoretically, and visualizing them with autoradiography, it was determined that the counts being bound were due to contaminating protein S in the protein C preparation (data not shown).

Immunoglobulins were isolated from ascites fluids 1b and 2a by gel filtration, and the isolated immunoglobulins were immobilized on Sepharose 4B. Ascites fluid 1b yielded 3 mg IgG per milliliter of fluid and was coupled at 0.7 mg IgG per milliliter of resin. Ascites fluid 2a yielded 6 mg IgG per milliliter of fluid and was coupled at 0.9 mg IgG per milliliter of resin. Both immobilized antibodies were used to purify protein S, which had been isolated from plasma by barium sulfate and then coupled at 0.9 mg IgG per milliliter of resin. Ascites fluid 2a yielded 6 mg IgG per milliliter of fluid and was coupled at 0.7 mg IgG per milliliter of resin. Ascites fluid 1b yielded 3 mg IgG per milliliter of resin. Both immobilized antibodies were used to purify protein S, which had been isolated from plasma by barium sulfate and then coupled at 0.7 mg IgG per milliliter of resin.

Immobilized antibody 2a bound about 250 \(\mu\)g of protein S/mg IgG (255 \(\mu\)g per milliliter of resin) and, in contrast with antibody 1b, bound material was totally eluted with 0.01 mol/L of EDTA, 0.02 mol/L of Tris-HCl, and 0.15 mol/L of NaCl, pH 7.4. After reequilibration with 0.02 mol/L of Tris-HCl, and 0.15 mol/L of NaCl, pH 7.4, columns of immobilized antibody 2a were reused repeatedly.

Immobilized antibody 2a bound about 250 \(\mu\)g of protein S/mg IgG (255 \(\mu\)g per milliliter of resin). Bound protein S was partially eluted with 0.01 mol/L of EDTA; 0.02 mol/L of Tris-HCl, and 0.15 mol/L of NaCl, pH 7.4; or with 0.02 mol/L of Tris-HCl, and 0.12 mol/L of NaCl, pH 7.4, and was totally eluted with 3.0 mol/L of NaSCN, 0.02 mol/L of Tris-HCl, and 0.15 mol/L of NaCl, pH 7.4; or with 0.10 mol/L of glycine and 2.0 mol/L of NaCl, pH 2.8. After reequilibration with 0.02 mol/L of Tris-HCl, and 0.15 mol/L of NaCl, pH 7.4, columns of immobilized antibody 2a were reused repeatedly.

The HPLC analysis of an experiment in which partially purified protein S (in 13 mL of 0.02 mol/L of Tris-HCl and 0.15 mol/L of NaCl, pH 7.4, \(A_{280} = 0.298\)) was applied to a 9-mL column of immobilized antibody 2a is shown in Figs 3 and 4. The migration of four vitamin K-dependent proteins is shown in the bottom profile (Fig 3); in this system, factor VII coeluted with prothrombin in protein C eluted just after factor IX. The major component in the sample (middle profile, Fig 3) comigrated with protein S; however, the sample was significantly contaminated with materials that comigrated with factor X, prothrombin, and factor IX, and with a material that eluted just ahead of protein S. In the flow-through from the column (top profile, Fig 3), the protein S peak was greatly diminished relative to the other peaks.

The column was eluted with 0.01 mol/L of EDTA, 0.02 mol/L of Tris-HCl, and 0.15 mol/L of NaCl, pH 7.4; or with 3.0 mol/L of NaSCN, 0.02 mol/L of Tris-HCl, and 0.15 mol/L of NaCl, pH 7.4; HPLC analysis of the eluted material (Fig 4) showed a single peak that comigrated with authentic protein S. Typical \(\gamma\) carboxyglutamic acid (Gla) analyses of the antibody-purified protein S gave 9.5 to 12.5 Gla per molecule, based on a total of 63 glutamyl residues.

Polyacrylamide gel analysis of material eluted from an antibody 2a column with EDTA is shown in Fig 5. On the nonreduced gel, the antibody-purified material (lane 3) comigrated as a single band with antigen protein S (lane 2). On the reduced gel, the antibody-purified material (lane 6) migrated as a doublet; this observation is consistent with the findings of Dahlbäck, who reported reduced bands with apparent mol wts of 85,000 and 75,000. The ratio of the two reduced forms of protein S varied from preparation to preparation and in some preparations, minor reduced bands in the range of 30,000 to 40,000 mol wt were observed.

Immobilized antibody 1b bound about 100 \(\mu\)g of protein S/mg IgG (70 \(\mu\)g per milliliter of resin) and, in contrast with antibody 2a, bound material was totally eluted with 0.01 mol/L of EDTA, 0.02 mol/L of Tris-HCl, and 0.15 mol/L of NaCl, pH 7.4. After reequilibration with 0.02 mol/L of Tris-HCl, and 0.15 mol/L of NaCl, pH 7.4, immobilized antibody 1b was reused repeatedly.

Dahlbäck and Stenflo reported that the complement C4b binding protein–protein S complex is precipitated by 35%
ammonium sulfate whereas the bulk of the free vitamin K-dependent proteins are not. In light of this observation, the barium citrate-adsorbed material from 22 mL of plasma was suspended in 35% ammonium sulfate containing benzamidine hydrochloride. After 2 hours at 4°C, the suspension was centrifuged. The supernatant was dialyzed against 0.02 mol/L of Tris-HCl and 0.15 mol/L of NaCl, pH 7.4, and applied to a 4-mL column of immobilized antibody 1b. Bound material was eluted with 0.01 mol/L of EDTA, 0.02 mol/L of Tris-HCl and 0.15 mol/L of NaCl, pH 7.4, and the portion which represented ~3 mL of plasma was subjected to slab gel electrophoresis (Fig 6). On both the nonreduced and reduced gels, the antibody-isolated material (lanes 2 and 4) comigrated with the conventionally isolated protein S (lanes 1 and 3) and was essentially homogeneous.

Whole citrated plasma was applied to immobilized antibody 2a, and the column was eluted with 0.02 mol/L of Tris-HCl and 0.15 mol/L of NaCl, pH 7.4, and then sequentially with the same buffer made 0.01 mol/L with EDTA, 0.5 and 1.0; 2.0 mol/L with NaCl; and finally 3.0 mol/L with NaSCN. When 11 mL of plasma was applied to a 9-mL column of immobilized antibody 2a, 0.06 A₂₈₀ units was eluted with 0.01 mol/L of EDTA, 0.04 A₂₈₀ units was eluted with 0.5 mol/L of NaCl, and 1.09 A₂₈₀ units was eluted with 3 mol/L of NaSCN. The Gla analysis of the NaSCN-eluted material gave 1.3 Gla per 100 glutamyl residues. Gel electrophoretic analysis of the eluted material is shown in Fig 7. The amount of NaSCN-eluted material loaded on the gel (lanes 1 and 5) represents material from ~0.4 mL of plasma; the EDTA-eluted (lanes 3 and 7) and NaCl-eluted (lanes 4 and 8) materials represent material from ~2.5 mL of plasma.

When samples were analyzed without reduction of disulfide bonds, only the NaSCN-eluted material (lane 1) gave a free protein S band on the Coomassie Blue-stained gel. The bulk of the NaSCN-eluted material in nonreduced samples had an apparent mol wt >300,000; EDTA and NaCl-eluted material contained only a trace of material of that size. All immune column eluates, when analyzed with disulfide bonds intact, and two to three components of intermediate mol wt (150,000 to 200,000). The EDTA-eluted material had several components with apparent mol wts that were less than that of protein S.

After reduction of the disulfide bridges, the majority of the NaSCN-eluted material (lane 5) migrated slightly faster than the smaller component protein S (lane 6). This band
Using hybridoma technology, monoclonal antibodies have been raised against human protein S. The affinity of the different antibodies for antigen varies relative to the availability of Ca²⁺. Antibodies 3a and 5a bind antigen equally well in the presence or absence of EDTA. Antibodies 2a and 4a bind antigen significantly less in the presence of EDTA, and antibody 1b does not bind at all in the presence of EDTA. It is likely that protein S, like other vitamin K-dependent proteins, undergoes a conformational alteration that is stabilized by Ca²⁺. The partial Ca²⁺ dependence of antibodies 2a and 4a probably occurs because they react with epitopes that are not Ca²⁺-dependent per se, but that are stabilized by Ca²⁺, whereas the Ca²⁺-dependent antibody 1b probably interacts with a truly Ca²⁺-dependent epitope. Let us suppose that protein S undergoes the transition:

\[ S \rightleftharpoons S^* \]

\[ K_1 = \frac{[S^*]}{[S]} \]

If Ca²⁺ interacts preferentially with S*, then:

\[ S^* + n\text{Ca}^{2+} \rightleftharpoons S^*(\text{Ca}^{2+})^n \]

\[ K_2 = \frac{[S^*(\text{Ca}^{2+})^n]}{[S^*][\text{Ca}^{2+}]^n} \]

If 2a and 4a react preferentially with S*, they will show a mixed Ca²⁺-dependence since they will react with both S* and with S*(Ca²⁺)n. The Ca²⁺-specific antibody 1b would only react with S*(Ca²⁺)n and its concentration would be expressed by:

\[ [S^*(\text{Ca}^{2+})^n] = K_1K_2[S][\text{Ca}^{2+}]^n \]

Antibodies that are Ca²⁺-dependent are particularly useful for isolating protein S since recovery of antibody-bound protein can be achieved, at least in part, by elution with EDTA. Inhomogeneous, conventionally isolated protein S was purified on antiprotein S-sepharose (Figs 3 through 5). Essentially homogeneous protein S was isolated with immobilized antibody from the 35% ammonium sulfate supernatant of barium citrate-adsorbed, vitamin K-dependent proteins (Fig 6).
PROTEIN S MONOCLONAL ANTIBODIES

Protein S—complement C4b-binding protein complex has been isolated directly from plasma by adsorption of the vitamin K-dependent proteins on barium citrate followed by chromatography on DEAE-Sephaclone and heparin-Sepharose. It is likely that the material of highest mol wt that was isolated directly from plasma with immobilized antibody (Fig 7, lanes 1 and 5) is this complex. The electrophoretic mobility found is consistent with the nonreduced mol wt of 570,000 and a reduced mol wt of 70,000 of complement C4b-binding protein.

The identities of the materials of intermediate nonreduced mol wt (Fig 7, lanes 1, 3, and 4) and the materials that are eluted with EDTA and have an apparent mol wt that is less than that of protein S (Fig 7, lane 3) are not known; some are potentially heterophile human immunoglobins which bind to the mouse immunoglobulin on the affinity resin, however.

The use of denaturants such as sodium thiocyanate for the elution of antigen from immunoaffinity chromatography columns has proved to be effective for isolating biologically active protein. Homogenous protein S that had been eluted from immobilized antibody 2a with 3 mol/L of sodium thiocyanate was dialyzed to remove the denaturant and was then assayed for cofactor activity. The fact that this protein S was shown to be biologically active clearly demonstrates the usefulness of this procedure for isolating protein S. In addition, the results show directly for the first time that human protein S provides the same cofactor activity toward the factor Va inactivation by APC as does its bovine counterpart.

The reagents established in this article should be useful in the further investigation of protein S binding substances and in the continuing study of protein S and its role in the coagulation and complement cascades.

ACKNOWLEDGMENT

We wish to thank Dr Paul Bajaj for generously providing protein S. We also thank Sandra Schumann, Dorothy Suddendorf, and Jeanette Mann for their patient assistance in preparing the manuscript.

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