Identification of a New Protein Involved in the Regulation of the Anticoagulant Activity of Activated Protein C

PROTEIN S-BINDING PROTEIN*

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The apparent molecular weight of functional protein S in citrated plasma was observed to be between 115,000 and 130,000 as measured by sedimentation equilibrium in the air-driven ultracentrifuge. The molecular weight of the functional protein decreased to approximately 62,000 when copper ions were added to the plasma. This suggested the presence of a protein S-binding protein in plasma, which was confirmed by gel filtration experiments. Frontal analysis of plasma indicated that functional protein S could exist in as many as three forms. Addition of copper ions to plasma reduced the number of forms to one. In order to isolate the binding protein, plasma was fractionated first on a column of immobilized iminodiacetic acid that had been equilibrated with copper ions. The proteins that eluted in a 0.6 M NaCl wash were passed over a column of protein S immobilized on agarose beads. A protein, eluted in the 0.8 M NaCl wash, was observed to bind to protein S in gel filtration experiments. When added to plasma depleted of both protein S and the binding protein, the binding protein was observed to enhance the anticoagulant activity of activated protein C only in the presence of protein S. Protein S-binding protein was also observed to enhance the rate of factor Va inactivation by activated protein C and protein S.

Protein S is a vitamin K-dependent protein that has been found in the blood plasma of several species (1). It has been observed to be a cofactor required for the maximum expression of the anticoagulant activity of activated protein C (2). Activated protein C is a serine protease that can inhibit blood coagulation through the inactivation of either coagulation factors Va (3-6) or factor VIIIa (6-8). The importance of protein S in the regulation of activated protein C is derived from several experiments. First, plasma from which protein S has been extracted is no longer susceptible to the anticoagulant activity of activated protein C (2). Second, plasma derived from patients with hereditary protein S deficiency cannot be anticoagulated with activated protein C (9). Studies with purified proteins have shown that activated protein C and protein S can form a stoichiometric complex on the surface of membranes that contain negatively charged phospholipids (10), making activated protein C a more effective inhibitor than free activated protein C. Protein S can be inactivated by thrombin (11, 12) which catalyzes the cleavage of a single peptide bond. The resulting two-chain protein has no cofactor activity and has lost its lipid binding properties (12).

Protein S has been observed to bind to another plasma protein, complement component C4-binding protein (12). The functional significance of this interaction is unclear. However, it has been reported that when protein S is complexed with C4-binding protein it is unable to participate as a cofactor for the anticoagulant activity of activated protein C (9). In this paper evidence is presented that active forms of protein S exist in plasma as a mixture of free protein S and protein S in complex with one or more plasma proteins. One of these proteins, protein S-binding protein, has been identified and some of its properties are reported. This protein appears to differ from C4-binding protein as it is able to enhance the anticoagulant activity of activated protein C.

EXPERIMENTAL PROCEDURES

MATERIALS—Iminodiacetic acid immobilized onto agarose (IAA-agarose) was prepared essentially by the method of Moroux and co-workers (14). 100 ml of aminoethyl-agarose (Sigma) was suspended in 50 ml of 0.5 M sodium carbonate (pH 9.5). 50 ml of epichlorhydrin (Aldrich) was added and mixed for 24 h at room temperature. The gel was then washed with 0.5 M sodium carbonate (pH 5.5) and suspended in 40 ml of 0.1 M sodium bicarbonate. 10 mg of iminodiacetic acid was added and the gel was stirred for 3 h at 70 °C. The gel was extensively washed and equilibrated in 0.1 M NaCl, 0.02 M Tris-HCl (pH 7.5), and 5 mM CuCl2. Protein S immobilized-agarose was prepared by the method of Cuáreas (15). Phospholipids were prepared from bovine brain as described previously (2). All other reagents used in this study were of the highest quality commercially available.

Preparation of Proteins—All of the proteins used in this study were of bovine origin. Protein S and protein C were purified by barium citrate absorption of plasma and ion-exchange chromatography as described previously (2). The protein S used in these studies appeared to be greater than 90% composed of a single-chain species as observed by electrophoresis of reduced samples. Protein C was activated with the factor X activator from Russell's viper venom as previously described (3). Factor V was purified by the method described by Esmon (16). Activated factor V was prepared by thrombin treatment of factor V followed by ion exchange chromatography (16). The purity of all proteins was ascertained by SDS-polyacrylamide gel electrophoresis. The concentrations of the proteins were calculated from previously reported extinction coefficients (2). For protein S-binding protein, an arbitrary extinction coefficient at 280 nm of 10.0×10−3 cm−1 was used.

Sedimentation Equilibrium—For the determination of the functional apparent molecular weight of protein S, sedimentation equilibrium was carried out in an air-driven ultracentrifuge (Beckman...
Airfuge) by the method described by Bock and Halvorson (17). Solutions (0.150 ml) were sedimented at 36,000 rpm for 24 h at room temperature. Tubes were then fractionated with a micropipette and assayed for protein S activity. All samples were sedimented in duplicate tubes. Calculations of molecular weights were performed using the computer program described by Bock and Halvorson (17). For an internal standard, factor X was determined in the same samples as was protein S. The molecular weight of factor X was within 5% of the reported value (55,000 (18)).

Assay of Protein S—Protein S was assayed by a modification of previously reported methods (2). Samples to be assayed for protein S were added to protein S-deficient plasma. Clotting was initiated by the addition of phospholipid (0.11 mg/ml), calcium (6 mM), activated protein C (0.01 mg/ml), and factor Xa (the factor Xa concentration was adjusted to give a blank time of 18 s). The total volume of the assay was 0.4 ml. Standard curves were prepared by diluting citrated plasma into the deficient plasma. Protein S-deficient plasma was prepared as described previously (3). Plasma was absorbed with barium citrate. 1 M BaCl₂ (80 ml/liter) was added slowly to the plasma which was kept on ice. After a 66-min incubation the precipitate was removed and the plasma was dialyzed against 0.1 M NaCl, 0.02 M Tris-HCl (pH 7.5). Prothrombin was then added to the plasma so that the factor Xa clotting time was the same as a control, untreated plasma. Plasma that was deficient in both protein S and protein S-binding protein (factor X in plasma has a molecular weight of 64,000 as determined by frontal analysis. In this technique plasma is continuously added to the column until the eluate has the same concentration of the components being measured as the starting material. When plasma was filtered on Ultrogel 44 (exclusion limits 10,000–130,000) two fronts of protein S activity were observed (Fig. 2). The leading front contained approximately 40% of the activity found in plasma. The rest of the protein S activity emerged one fraction before prothrombin and well ahead of factor X. The fractions in the leading front were pooled and a molecular weight of 140,000 was determined for protein S activity in the air-driven ultracentrifuge. In the same run the average molecular weight of protein S from a plasma sample was 128,000. Since the leading front emerged with the void volume it was unclear whether it might be heterogeneous with respect to size. Plasma was then filtered on Bio-Rad A-15M (exclusion limit 15,000,000). In this case the protein S emerged in three fronts (Fig. 2). The first front emerged two fractions after factor V. The third front emerged with prothrombin. When copper ions (2 mM) were included in the plasma, the two early peaks were lost and the protein S emerged as a single peak. These results suggest that active protein S exists in plasma as a complex mixture that consists of free protein as well as protein S in complex with other plasma proteins.

To test this hypothesis, purification of a protein S-binding protein (PSBP) was attempted. The first step was to pass citrated plasma through a column of IAA-agarose (2.5 x 15 cm) that had been equilibrated with CuCl₂ (4 mM) and washed with 10 volumes of 0.1 M NaCl (0.02 M Tris-HCl, pH 7.5). The plasma was followed with an extensive wash with 0.1 M NaCl. This continued until the absorbance at 280 nm was less than 0.05. The column was then washed with 0.6 M NaCl (0.02 M Tris-HCl, pH 7.5). The protein that eluted with the 0.6 M wash (this peak was always characterized by a high degree of turbidity) was dialyzed into 0.1 M NaCl (0.02 M Tris-HCl, pH 7.5) and applied to a column of agarose-immobilized protein S (1.5 x 15 cm). This column was washed with the starting buffer until the absorbance (280 nm) was less than 0.05 and then washed with 0.6 M NaCl (0.02 M Tris-HCl, pH 7.5). The protein that eluted was dialyzed into 0.1 M

FIG. 1. Sedimentation equilibrium of protein S in plasma. 0.150 ml of plasma either in the presence (circles) or absence of 2 mM CuCl₂ (squares) was sedimented at 36,000 rpm at room temperature in an air-driven ultracentrifuge. Samples were fractionated and protein S was determined as described under "Experimental Procedures." The data are representative of the results of three separate runs. Factor X was also assayed as an internal standard in all fractions. Its molecular weight was found to be approximately 55,000.

FIG. 2. Gel filtration of plasma. Plasma (35 ml) was added to columns (0.9 x 60 cm) of either Ultrogel 44 (squares) or Bio-Rad A-15 M (circles) and 15-dip fractions were collected. Plasma containing 2 mM CuCl₂ was also run on the A-15M column (triangles). Samples (6.010 ml) were removed from each fraction and assayed for protein S activity as described under "Experimental Procedures." For the Ultrogel 44 column the void volume emerged with fraction 18. From this same column the half-heights of factor V, prothrombin, and factor X were, respectively, fractions 18, 30, and 34. For the Bio-Rad A-15 column factor V emerged with fraction 24. Prothrombin emerged in the same fractions as the main protein S peak.
NaCl (0.02 M Tris-HCl, pH 7.5) and reapplied to the protein S-agarose column that had been equilibrated in the same buffer. On the second pass through the protein S-agarose column no protein eluted in the void volume. A protein peak eluted with the 0.6 M wash (Fig. 3). Co-eluting with the protein was an activity that could prolong clotting times in the presence of protein S and activated protein C (Fig. 3) when assayed using protein S-PSBP-deficient plasma. Only a single band of protein was observed when the peak was analyzed by SDS-polyacrylamide gel electrophoresis. When run under non-reducing conditions it had an apparent molecular weight of approximately 138,000. When electrophoresed following reduction two bands with molecular weights of approximately 46,000 and 94,000 were observed (Fig. 4).

If the protein eluted from the protein S-agarose is the PSBP predicted from the frontal analysis of plasma, then it should shift the position of protein S when both proteins are co-chromatographed by gel filtration. When protein S and PSBP were chromatographed together, protein S activity eluted in two peaks (Fig. 5). The first peak eluted in advance of free protein S which co-eluted with the second peak (Fig. 5). This suggests that protein S and PSBP can form a complex. PSBP was not observed to contain any protein S activity (Fig. 6).

The effect of PSBP on protein S was tested in plasma made deficient of protein S and PSBP. PSBP had no effect on the clotting times of plasma alone (Fig. 6). PSBP had no effect on the clotting time when only activated protein C was added (Fig. 6). However, if protein S and activated protein C were added to the assay, PSBP caused a prolongation of the clotting time (Fig. 6). The same change in clotting time could be observed by a 500-fold dilution of the factor Xa used in the reaction mixture. The concentration dependence of PSBP appeared to saturate. To test whether the effect of PSBP could be due to a contamination with protein S, the anticoagulant activity was titrated with protein S. Even with a 10-fold increase in protein S in the assay, it was not possible to equal the effect observed with the combination of PSBP, protein S, and activated protein C (Fig. 7). It therefore seems unlikely that the effect seen with PSBP could be due to a small contamination of protein S. In a few experiments it was observed that high calcium concentrations (25 mM) could inhibit the activity of PSBP (data not shown). This seems to indicate that the role of PSBP in the regulation of the anticoagulant activity of protein C may have an ion involvement.

Fig. 5. Gel filtration of protein S in the presence and absence of protein S-binding protein. A sample (0.8 ml) containing 0.1 mg of PSBP and 0.25 mg of protein S was applied to a column (0.9 x 60 cm) of Superose 6 (preparative grade) equilibrated in 0.1 M NaCl, 0.02 M Tris-HCl (pH 7.5), and 1 mg/ml bovine serum albumin and fractionated at a flow rate of 0.4 ml/min. Fractions (1.0 ml) were assayed for protein S (circles) activity using protein S-deficient plasma as described under “Experimental Procedures” (upper panel) and protein was determined by measuring absorbance at 280 nm (lower panel). In a separate experiment free protein S was found to elute at the position of the second peak (fraction 34).

Fig. 6. Effect of protein S-binding protein on the anticoagulant activity of activated protein C. The effect of PSBP on the clotting of protein S-PSBP-deficient plasma was determined by adding the indicated amount of PSBP to factor Xa-initiated clotting assays with no additions (triangles), activated protein C (0.5 μg) (squares), and activated protein C (0.5 μg) and protein S (1.5 μg) (circles). Clotting was initiated as described for the protein S assay under “Experimental Procedures.”
Identification of Protein S-binding Protein

The discovery of protein S led to the proposition that the anticoagulant activity of activated protein C was due to the formation of a complex between protein S and activated protein C on the surface of membranes (2, 10). The results of this paper suggest that this explanation was incomplete. In this paper, I propose that there is an additional protein, protein S-binding protein, involved in the expression of the anticoagulant activity of activated protein C.

Experiments carried out either with plasma or purified proteins indicated that plasma might contain a protein S-binding protein that does not inhibit protein S activity. When the size of functional protein S in plasma was determined either by sedimentation equilibrium or gel filtration it was found that the molecular weight associated with protein S activity was greater than that of the purified protein. The active molecules of protein S exist in plasma as a complex mixture of free protein S, and protein S bound to one or more plasma proteins. Both molecular weight measurements as well as chromatography on gel exclusion columns support the hypothesis that the activity is heterogeneous with respect to molecular weight.

Protein S is known to associate with another plasma protein, complement component C4-binding protein (13). This protein has a molecular weight of approximately 600,000. Although it is possible that some of the size heterogeneity of protein S is due to an interaction with this protein, several observations have been made that tend to discount the importance of this interaction. First, it has been observed that the protein S-C4-binding protein complex is not active as a cofactor for activated protein C (9). Therefore, its contribution to the average molecular weight would not significantly alter this analysis since only cofactor activity was measured. Second, if it contributes to the cofactor activity its size falls outside the range observed with the molecular weight gradient formed in the sedimentation equilibrium run. At the speeds used in these experiments it has been calculated that more than 90% of the C4-binding protein would be in the bottom 0.015-ml fraction. This fraction was not measured in any of these experiments. Factor V, which is smaller than C4-binding protein, was found only in the bottom two fractions with the rotor speeds used in these experiments. Third, PSBP appears to be a two-chain protein with an apparent molecular weight of 138,000. Analysis by polyacrylamide gel electrophoresis suggests that it is composed of a 94-kDa heavy chain and a 46-kDa light chain.

In plasma that has been made deficient both in PSBP and protein S, PSBP was observed to have a large effect on the anticoagulant activity of activated protein C. The effect was only observed, however, in the presence of protein S. In the absence of protein S, PSBP had no detectable effect on clotting times. No evidence was observed to suggest that the effect of PSBP could be due to contaminating protein S. It was also observed that PSBP could enhance the rate of factor Va inactivation in the presence of activated protein C and protein S.

These experiments suggest that protein S can exist in plasma as a protein that contains two non-covalently associated subunits. One subunit is the vitamin K-dependent protein S and the second subunit is protein S-binding protein. In plasma, this protein appears to be a cofactor for the anticoagulant activity of activated protein C. If this is indeed the case, then defects in either of these subunits could lead to a lowering of the apparent ability of activated protein C to inhibit blood coagulation.

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FIG. 7. Dependence of clotting time on the concentration of protein S in the presence and absence of PSBP. Clotting was initiated with factor Xa as described for the protein S assay in the presence of activated protein C (6.5 μg) and indicated amounts of protein S either in the presence (circles) or absence (squares) of PSBP (4 μg/ml).

FIG. 8. Effect of PSBP on the inactivation of factor Va by activated protein C and protein S. Factor Va (1.2 units/ml) was inactivated in the presence of calcium chloride (5 mM), phospholipid (25 μg/ml), and activated protein C (2.5 μg/ml) (triangles) with the addition of protein S (5 μg/ml) (squares) and protein S (5 μg/ml) and PSBP (10 μg/ml) (circles). Samples were removed from the reaction mixture at the indicated times and assayed for factor Va which is inactivated in the presence of calcium chloride (0.5 mM), phospholipid (5 mM), and activated protein C and protein S.