Binding of Protein S to Factor Va Associated with Inhibition of Prothrombinase That Is Independent of Activated Protein C*

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Since plasma protein S serves an anticoagulant function by mechanisms which are not completely understood, its possible interaction with Factor Va was investigated. Human protein S bound to immobilized human Factor Va in a calcium-dependent, saturable, and reversible manner and Factor Va bound similarly to immobilized protein S. Binding of protein S to immobilized Factor V was greatly enhanced by pretreatment of the surface-bound Factor V with increasing doses of thrombin up to 1 unit/ml. Binding of protein S to Factor Va was also demonstrated in fluid phase with a KD of 33 ± 9 nM. Biotin-labeled heavy chain of Factor Va bound to immobilized protein S, and this binding was reversed by an 17-fold molar excess of intact unlabeled Factor Va. Protein S competed efficiently with thrombin for binding to immobilized Factor Va. The prothrombinase activity in a reaction mixture of purified clotting factors was inhibited by protein S and exhibited a pattern of mixed inhibition. The concentration of protein S needed for 50% inhibition of the prothrombinase activity of a mixture containing 1 nM Factor Xa, 20 pM Factor Va, and 50 μM phospholipids was about 16 nM. Since not all protein S preparations exhibited this degree of prothrombinase inhibitory activity, extensive control experiments were performed to verify that the inhibitory activity was associated with protein S during immunoaffinity chromatography and was not caused by traces of activated protein C in the protein S preparations. These data show that protein S has an anticoagulant function which is independent of activated protein C and, at least in part, that this is because of its competition with prothrombin for direct binding to Factor Va.

Protein S is a vitamin K-dependent anticoagulant protein (1, 2) which can serve as a nonenzymatic cofactor for activated protein C (APC) in its inactivation of Factors Va and VIIIa (FVAs and FVIIAs) (3, 4). Its biological importance is inferred from the association of recurrent venous thrombosis with hereditary deficiency of protein S (5–8). The mechanism of action of protein S is not completely understood. Evidence has been presented that protein S increases the affinity of APC for phospholipid vesicles (9), endothelial cell surfaces (10), platelets (11, 12), and platelet microparticles (13). It has been proposed that protein S localizes APC to a cell surface on which APC can inactivate FVAs in the prothrombinase complex or FVIIAs in the “tenase” complex. One report described an APC-independent anticoagulant activity of a protein S preparation made using a unique monoclonal antibody coupled to Sepharose (14). Using an independent approach, we were unable to demonstrate that protein S increased the binding of APC to immobilized vesicles composed of various mixtures of phosphatidylinerine and phosphatidylcholine. Moreover, protein S deficiency but not protein C deficiency is associated with arterial as well as venous thrombosis in some patients (15), suggesting that the modes of anticoagulant activity of protein S may be more complex than those of APC. Therefore, we investigated whether protein S might directly interact with FVAs and thereby exhibit anticoagulant activity independent of APC.

MATERIALS AND METHODS

Proteins—Protein S was purified by slight variations of published methods (16). Briefly, the steps for protein S purification were: 1) barium adsorption of 1-day-old citrated human plasma containing 10 mm benzamidine, 1 mm diisopropylfluorophosphate, 1 mm PMSF, 100 units/ml aprotinin, and 0.02% NaCl; 2) elution of the barium citrate pellet with EDTA; 3) chromatography on DEAE-Sephalac (Pharmacia LKB Biotechnology Inc.) in 5 mM MESS, 0.15 M NaCl, 1 mm benzamidine, 5 mM EDTA, 0.02% NaCl, pH 6.0, with a linear gradient of 0–0.4 M NaCl in the same buffer; 4) chromatography on Blue Sepharose (Pharmacia) in 0.05 M Tris-HCl, 0.05 M NaCl, 1 mm benzamidine, 2 mM EDTA, pH 7.4, with a linear gradient of 0–0.4 M NaCl in the same buffer; 5) chromatography on heparin-Sepharose (Pharmacia) in 0.05 M MESS, 2 mM CaCl2, 1 mm benzamidine, 0.01% Tween 20, 0.02% NaCl, pH 6.0, with a linear gradient of 0–0.4 M NaCl in the same buffer. The yield for the two preparations used in most of the experiments was about 46% of the free protein S or 19% of the total protein S in the starting plasma. Following purification, protein S was dialyzed against TBS and stored in aliquots at −70 °C.

The two protein S preparations used had APC cofactor activities varying by no more than 20% when compared with each other or with commercially available protein S (Enzyme Research Laboratories) as judged by their prolongation of a FXa one-stage clotting time in protein S-depleted plasma with APC (17). Immediately preceding binding assays, protein S was pretreated for 20 min with 400 μM p-APMSF (Chemicon, Temecula, CA). Prothrombin (1) and FV (18) were purified as described previously, and protein C (19) was purified and activated as described previously. FV (0.2 mg/ml) was activated with 20 μM thrombin in 50 mM Hepes, 175 mM NaCl, pH 7.5, for 30 min at 37 °C. The thrombin was subsequently neutralized by adding 5.0 mg/ml aprotinin. The abbreviations used are: APC, activated protein C; F, Factor; MESS, 4-morpholineethanesulfonic acid; p-APMSF, para-amidinophenylmethylsulfonyl fluoride; TBS, Tris-buffered saline.

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equimolar amounts of Phe-Pro-Arg-chloromethyl ketone (Calbiochem). Human thrombin and FXa were obtained from Enzyme Research Laboratories, South Bend, IN. All of these proteins were judged >95% homogeneous by SDS-polyacrylamide gel electrophoresis, and aliquots were stored at -70 °C. Antibodies to protein S and to prothrombin were prepared as described previously (5). Anti-protein S antibodies were affinity purified using a column of protein S coupled to cyanogen bromide-activated Sepharose (Pharmacia) according to the manufacturer's instructions. Anti-prothrombin antibodies were adsorbed using the same column to remove any anti-protein S antibodies. The Ag fraction of rabbit polyclonal antibodies to FVa was obtained from Dako, Carpinteria, CA. Non-calcium-dependent murine monoclonal antibodies (S7 to protein S, and C3 to protein C) were prepared as described for protein C monoclonal antibodies (20) and purified as described for protein C monoclonal antibodies (20). Monoclonal antibody S7 had at least 1,000-fold higher affinity for protein S than for protein C, FIX, FX, or prothrombin. Ascites fluid containing monoclonal antibody to the heavy chain of FVa was kindly donated by Drs. Dario Altiere and Tom Edgington, Scripps Clinic, La Jolla, CA, and this antibody was purified as described (21). A sample of rabbit anti-tissue factor pathway inhibitor antiserum and a positive control were generously provided by Drs. Ronnie Warn-Cramer and Samuel Rapaport of University of California at San Diego. Antibodies to α1-antitrypsin and antithrombin III were obtained from Behring, Marburg, Germany; antibody to heparin cofactor II was obtained from Diagnostica Stago, Asnieres, France; and antibodies to human serum amyloid P component was from Atlantic Antibodies, Stillwater, MN.

Binding Assays—FV or FVa (2.5 μg/ml) was coated to the wells of Nunc (Naperville, IL) Maxisorp microtiter plates in 50 μl/well in 0.1 M sodium carbonate, pH 9.0, for 1 h at 37 °C in a humid chamber. The plates were washed once with Tris-buffered saline, 0.02% NaN3, pH 7.4 (TBST), and blocked for 1 h at room temperature with 0.5% porcine skin gelatin (Sigma) in TBST. Control wells with no FVa were also blocked. In some cases, FV on the plate was treated for 20 min at room temperature with thrombin in blocking solution containing 5 mM CaCl2 followed by four washes with the same buffer. The plates were incubated for 50 min at 37 °C with 50 μl of various concentrations of protein S in binding buffer consisting of 0.5% gelatin, 0.05 M NaCl, 5 mM CaCl2, 0.1 mM MnCl2, 0.02% NaN3, pH 7.4. The plates were washed three times with wash buffer, which was identical to binding buffer except that the gelatin content was 0.1%. The plates were incubated sequentially at room temperature with 50 μl of each of the following: 10 μg/ml antibody in wash buffer, 50 min; biotin-secondary antibody (Pierce Chemical Co.) diluted 1:1,000 in wash buffer, 50 min; and avidin-biotinylated alkaline phosphatase conjugate (Pierce) diluted 1:500 in wash buffer, 30 min. Each step was followed by three washes, which was followed by two washes. The plates were developed with a p-nitrophenyl phosphate substrate kit (Bio-Rad). The initial change in absorbance at 405 nm was recorded over a 5-10-min interval on a Bio-Tek (Winooski, VT) microtiter plate reader using a Kinetic-calc program. The assay was performed in a similar manner except that protein S was coated to the plate instead of FVa at 5 μg/ml in the binding buffer. For some experiments, FVa heavy chain (22) was biotin labeled (23), and bound biotin-FVa heavy chain was detected, omitting the antibody steps. Binding of prothrombin to FVa was monitored in a similar way. For determination of the dissociation constant (Kd) for binding of protein S to FVa in the fluid phase, a method recently applied to determine Kd and stoichiometry. For determination of the dissociation constant (Kd) for binding of protein S to FVa in the fluid phase, a method recently applied to determine Kd and stoichiometry.
sponding wells coated with FVa. Similar results were obtained when bound protein S was detected with polyclonal or monoclonal antibody.

Binding of FVa to immobilized protein S was also demonstrated as seen in Fig. 2A. The FVa was detected with either polyclonal antibodies or monoclonal antibody to FVa heavy chain, although the color intensity observed using a monoclonal antibody was approximately 1/5 of that observed using the polyclonal antibodies. EDTA at 10 mM reduced the binding of FVa by 50–75%, showing that this binding required calcium ions (data not shown). Fig. 2B demonstrates that biotinylated FVa heavy chain also bound to immobilized protein S and that this binding was time-dependent, saturable, and reversed by the addition of a 17-fold molar excess of unlabelled FVa.

In ligand blotting experiments, biotinylated FVa heavy chain blotted to nonreduced protein S which had been electrophoresed on a denaturing SDS-polyacrylamide gel and transferred to an Immobilon membrane (Millipore, Bedford, MA) (data not shown). No bands with an apparent molecular weight other than that of protein S (75,000) was detected by this ligand blotting, suggesting that the FVa binding observed using microtiter plates was to intact protein S and not to any contaminant or to a proteolytic fragment of protein S.

To show that the data for the binding of protein S to FVa observed using immobilized protein S or immobilized FVa were not artifacts of the microtiter plate methods, the binding of protein S to FVa in the fluid phase (24, 25) was determined as described under “Materials and Methods” (Fig. 3). The binding of protein S to FVa was saturable, and the $K_d$ was 33 ± 9 (mean ± standard deviation for five experiments), using a fixed concentration of 3–12 nM FVa in various experiments. The intercept on the abscissa of the Scatchard plot as shown in the insets was consistent with a stoichiometry of 1.0–1.3 mol of protein S/mol of FVa in various experiments. The data from Fig. 3, A and B, were also fit to hyperbolas using the Enzfitter program (not shown). By this method, the respective calculated $K_d$ values were 20 and 50 nM, and the ratios of protein S to FVa were 1.1 and 1.4. Similar $K_d$ values were observed whether the 2-h incubation mixtures were incubated in the protein S-coated detection plate for 10, 15, or 20 min, suggesting that equilibrium was not significantly disturbed during incubation on the detection plate.

**Competition for Prothrombin Binding to FVa by Protein S**

![Fig. 2. Binding of FVa or biotin-FVa heavy chain to immobilized protein S. In panel A, various concentrations of protein S were coated to the wells of a microtiter plate. Protein S coated to the plate was detected directly in some wells using monoclonal antibody to protein S (triangles). In other wells, 2 μg/ml FVa was incubated, and bound FVa was detected as described under “Materials and Methods,” using a polyclonal antibody (closed circles) or a monoclonal antibody (squares). In panel B, 5 μg/ml (66 nM) protein S was coated to the wells of a microtiter plate. Biotin-FVa heavy chain (1 μg/ml, 9 nM) was incubated in the wells for various times as indicated on the abscissa (closed circles). For some wells, unlabelled FVa (50 μg/ml, 150 nM) was added at 15, 30, or 45 min of incubation and the incubation continued until 60 min (open symbols). Biotin-FVa heavy chain was detected as described under “Materials and Methods.”](image)

**Fig. 3. Binding of protein S to FVa in fluid phase.** A constant amount of FVa (panel A, 9 nM; panel B, 6 nM) was incubated in fluid phase with various amounts of protein S as described under “Materials and Methods,” and then free FVa was determined by 20-min (panel A) or 10-min (panel B) exposure to a protein S-coated plate and comparison of FVa bound to a standard curve for free FVa performed on the same plate. Bound and free FVa and protein S in the fluid phase mixtures were then calculated. The insets show Scatchard analysis of the data, the calculated $K_d$, and the correlation to a straight-line function (r). The probability (p) that the slope of the line is zero is 0.002 in panel A and 0.008 in panel B.

![Fig. 4. Competition by protein S with prothrombin for binding to FVa. In panel A, 15 μg/ml prothrombin was incubated in FVa-coated wells at 37°C. At 15, 30, or 45 min of incubation, protein S was added to indicated incubation mixtures (dashed lines and open circles), and the incubation was continued until 60 min. Prothrombin binding was detected as described under “Materials and Methods.” Closed circles indicate incubations of prothrombin alone for the indicated times. In panel B, protein S in binding buffer at several concentrations as indicated or binding buffer alone was preincubated in FVa-coated wells for 1 h at room temperature. One set of these wells (open circles) was washed, and protein S was allowed to remain in another set of wells (closed circles) prior to the addition of prothrombin to a final concentration of 15 μg/ml and further incubation at 37°C for 60 min. Bound prothrombin was detected as described under “Materials and Methods.”](image)
Protein S binds Factor Va and inhibits prothrombinase.

Protein S was washed from the wells (Fig. 4B, open circles) or left in the wells (Fig. 4B, closed circles) when prothrombin was added.

Control experiments in reverse of the above showed that prothrombin competed weakly with protein S for binding to FVa. Preincubation of immobilized FVa with 20 μg/ml prothrombin resulted in a 17–25% decrease in the protein S bound to FVa when protein S was subsequently incubated at 2.5–5.0 μg/ml in FVa-coated wells (data not shown). Disopyrrol-FXa and FXa competed modestly with protein S for binding to FVa, since preincubation of immobilized FVa with 20 μg/ml disopyrrol-FXa resulted in a 16–38% decrease, and 20 μg/ml FXa resulted in a 29–48% decrease, in the protein S bound to FVa when protein S was subsequently incubated at 5–10 μg/ml in FVa-coated wells (data not shown).

Inhibition of prothrombinase by protein S—Experiments were performed to test whether protein S could inhibit prothrombin activation under conditions at which the rate of prothrombin activation was linearly dependent on the amount of FVa present (i.e. limiting FVa). Preincubation of protein S with the purified components of the prothrombinase complex (FXa, FVa, calcium ions, and phospholipid) before the addition of prothrombin resulted in a gradual decrease of the rate of prothrombin activation. Incubation times of 15 min were required for maximal inhibition of prothrombinase activity by protein S. Inhibition of prothrombin activation depended on the prothrombin and protein S concentrations present in the reaction mixture. To obtain information on the mechanism of prothrombinase inhibition by protein S, Lineweaver-Burk plots were determined in the absence and presence of 36 nm protein S (Fig. 5). The apparent K₅₀ for prothrombin shifted from 0.30 μM in the absence of protein S to 0.55 μM in the presence of 36 nm protein S, and the V₅₀ decreased by a factor of 2.2. These kinetic data are characteristic of a mixed type of inhibition. Experiments performed at different phospholipid concentrations (data not shown) indicated that the inhibition by 39 nm protein S was independent of phospholipid concentration between 10 and 150 μM.

The inhibition of prethrombinase activity by protein S was not affected when protein S was preincubated with p-APMSF and with antibody to protein C under conditions shown to inhibit APC completely at concentrations of 2 ng/ml to 4 μg/ml. Thus, the inhibitory activity of protein S was not because of contamination with APC or other protease sensitive to p-APMSF. Furthermore, immunoblotting of the inhibited prothrombinase mixtures revealed no apparent proteolytic cleavage of FVa.

Studies of the Protein S Preparations—The protein S preparations were examined for the presence of known protease inhibitors of FXa and thrombin. By immunoblotting analysis (20, 26), no evidence for the presence of α₂-antitrypsin, antithrombin III, tissue factor pathway inhibitor, or heparin cofactor II could be found (data not shown). Since the last three of these inhibitors are heparin-dependent, the effect of 1 unit/ml heparin in combination with protein S was assessed, but no stimulation of inhibition of prothrombinase activity was observed (data not shown). Thus, no obvious protease inhibitor contamination or heparin dependence was found for protein S preparations that inhibited prothrombinase activity.

To show directly that the prothrombinase inhibitor was protein S, a preparation of purified protein S was adsorbed using an immobilized monoclonal anti-protein S antibody. A 100-μg aliquot of protein S in TBS was chromatographed on a 2-ml column of monoclonal antibody S7-Sepharose. The eluted fractions were analyzed for their ability to inhibit prothrombinase and for the distinctive protein S doublet near Mᵣ = 75,000 on SDS-polyacrylamide gels (data not shown). Both the prothrombinase inhibitory activity and the protein S antigen were retained on the column after washing with 10 ml of TBS, followed by 10 ml of TBS containing 10 mM EDTA. Inhibitory activity and protein S could be recovered when the column was subsequently eluted with 0.1 M glycine, pH 2.5, followed by neutralization of the fractions with 1 M Tris base. A control showed that a test aliquot of neutralized glycine did not inhibit prothrombinase. This experiment supports the hypothesis that protein S itself is responsible for direct inhibition of prothrombinase activity.

Fig. 6 shows a silver stain of a number of different protein S preparations following SDS-polyacrylamide gel electrophoresis under nonreduced or reduced conditions. The relative potencies in APC-independent inhibition of prothrombinase and in APC cofactor activity are indicated below the figure. Preparations H89–4C and H89–5 were used for all of the experiments described above, but preparation H89–4A was equally potent in inhibition of prothrombinase. Two different commercial preparations of protein S (C410 and C450) and one of our own preparations (H89–3A, B, and C) only weakly or moderately inhibited prothrombinase activity and bound.

![Fig. 6. SDS-polyacrylamide gel electrophoresis of different protein S preparations.](image-url)
weakly to FVα, yet they were equally active as the protein S preparations described here when they were tested for APC cofactor activity in a FXα one-stage clotting assay. Preparations H89-5, H90, and C450 had the same NH₂-terminal sequence of 5 amino acids. Preparations H89-4A and H89-5 contained a trace contaminant of mobility $M_r = 125,000-130,000$. This contaminant was separated on a small scale trial chromatography from the protein S without loss of the protein S ability to inhibit prothrombinase or to bind to FVα (data not shown).

We examined differences in purification procedures for the different protein S preparations. H89-5, H89-4A, and H89-4C were purified as described under "Materials and Methods," except that heparin-Sepharose chromatography was omitted for H89-4A and H89-4C and replaced with chromatography on monoclonal anti-protein C-Sepharose to remove traces of protein C. Preparation H89-3 began with frozen cryosupernatant of plasma, included an ammonium sulfate fractionation following barium elution, and omitted the heparin-Sepharose chromatography. Different pools A, B, and C of preparation H89-3 were collected based on the proportion of upper and lower band of the protein S doublet at $M_r = 75,000-85,000$ on reduced SDS-polyacrylamide gel electrophoresis (Fig. 6), but none of the pools was very inhibitory toward prothrombinase activity. Preparation H90 was partially purified in another laboratory and was not well studied because it had low APC cofactor activity and modest ability to inhibit prothrombinase. The commercial preparations were purified using ammonium sulfate fractionation following barium adsorption and included hydroxylapatite chromatography instead of heparin-Sepharose chromatography. No differences were noted in the procedures for preparation C410, which inhibited prothrombinase moderately, as compared with preparation C450, which inhibited weakly or not at all. A third commercial preparation (C570) purified with heparin-Sepharose in place of hydroxylapatite inhibited prothrombinase by 30-75% at 25 µg/ml (data not shown). Several other preparations made in our laboratory by the procedure given under "Materials and Methods" and by slight variations (such as using 3-day-old plasma) inhibited prothrombinase by 50-75% at approximately 20 µg/ml (267 nM) and bound FVα, but less well than preparations H89-4A, H89-4C, and H89-5. Several treatments were tested in an attempt to convert a less active protein S preparation to a more active one, or vice versa. These included treatment with thrombin, neuraminidase, 0.01% Tween 20, 0.1 M glycine, pH 2.5, EDTA, and repeated freeze-thaws. None of these treatments was effective. Treatment with N-glycanase in SDS did not change the ability of protein S to bind biotin-FVα heavy chain on a ligand blot.

In summary, the less active protein S preparations were derived from older plasma or cryosupernatant or were prepared using hydroxylapatite chromatography, but we were unable to determine with certainty whether or how the various protein S preparations differed in molecular form or conformation or in the presence of a trace protein S-binding component. Nonetheless, the data suggest that some form of protein S binds directly to FVα and has anticoagulant functional activity that is independent of APC.

**DISCUSSION**

Evidence from clinical studies strongly demonstrates that protein S is an important natural antithrombotic factor. One study of 141 unrelated patients with venous thrombotic dis-

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4 M. Morris, Enzyme Research Laboratories, personal communication.
vated" in some way that increases its ability to inhibit prothrombinase; or 3) a subpopulation of protein S possesses a subtle difference in structure or conformation that enables it to bind FXa and inhibit prothrombinase.

FXa has the property of protecting FXa from inactivation by APC (35), and protein S negates this protective effect (30, 31). Both FX and FXa apparently block an important binding site for FXa (36). The finding here that protein S binds to FXa is consistent with those findings and suggests that protein S may negate the protective effect of FXa by displacing FXa or FX from its binding site on FXa, thereby making or keeping available a FXa site for APC binding. Our findings may be also related to another recent study, in which protein S purified on an immunoaffinity column that contained a unique monoclonal antibody had anticoagulant properties independent of APC. A novel conformational form of protein S, designated PSM, appeared to act as a competitive inhibitor of prothrombin in clotting assays (14). In a related study, Mitchell and Salem (36) showed that prothrombin could inhibit the APC cofactor activity of protein S. Our protein S preparations used in the studies reported here were not prepared using an immunoaffinity column yet displayed anticoagulant activity as great or greater than that described by Mitchell et al. (14). Thus, there exist molecular forms of protein S, as demonstrated here and previously by Salem's group (14), that directly bind to FXa and that have an anticoagulant activity which is independent of APC. Presumably this activity, at least in part, is caused by competition with prothrombin, but future studies will investigate possible interactions of protein S with FXa or thrombin. Since platelets contain protein S that is secreted upon platelet activation (37), the anticoagulant activity of protein S may be important in regulating coagulation reactions in the locus of activated platelets. If this is an important activity of protein S, its deficiency could contribute to risks of arterial thrombosis as recently described (15).

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