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Biochemistry

Protein S binds to and inhibits factor Xa

(blood coagulation/prothrombinase/anticoagulant)

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ABSTRACT **Although human protein S binds to human factor Va and inhibits prothrombinase activity, this inhibition is not totally dependent on factor Va. Hence, we investigated possible interaction of protein S with human factor Xa. Factor Xa, diisopropylphospho-factor Xa and their biotin derivatives ligand blotted specifically to protein S and protein S ligand blotted specifically to factor X and factor Xa. Biotinylated factors X and Xa bound to immobilized protein S and, reciprocally, protein S bound to immobilized factor Xa with a K_d of ≈19 nM. In fluid phase, protein S bound to factor Xa with a K_d of ≈18 nM. Protein S at 33 nM reversibly inhibited 50% of factor Xa amidolytic activity. Protein S inhibition of prothrombin conversion to thrombin by factor Xa was phospholipid-independent and was 1.6 times stimulated by Ca^{2+} ions. Inhibition of prothrombinase activity by protein S was 2.3-fold more potent in the presence of factor Va, with 50% inhibition at ≈8 nM protein S. Protein S prolonged the factor Xa one-stage clotting time of protein S-depleted plasma in a dose-dependent manner. These data demonstrate mechanisms of anticoagulant action for protein S that are independent of activated protein C and that involve direct binding to factors Xa and Va and direct inhibition of factor Xa.**

Protein S is a vitamin K-dependent plasma protein that can act as a cofactor for the anticoagulant functions of activated protein C (1–3). Deficiency of protein S is associated with venous thrombosis (4–6) or arterial thrombosis (7, 8). Among young adult patients with venous thrombosis, 4–12% have hereditary protein S deficiency (9). Mechanisms of action of protein S as an antithrombotic factor are not fully understood, although it exerts negative feedback on blood coagulation pathways (10). Protein S increases the affinity of activated protein C for phospholipid vesicles (11), platelets (12), and endothelial cells (13). In purified systems using platelets, the cofactor activity of protein S is modest, increasing the effect of activated protein C by a factor of 2 (14, 15). Yet protein S is antithrombotic in a rabbit thrombosis model (16). We therefore searched for anticoagulant roles of protein S independent of activated protein C. Protein S binds to activated factor V [factor Va (FVa)] with a K_d of 33 nM and inhibits prothrombinase activity (17). However, protein S inhibition of prothrombinase is not totally dependent on the presence of FVa. Studies here describe binding and inhibition of factor Xa (FXa) by protein S in the absence of activated protein C. A preliminary report of this work has been presented (18).

MATERIALS AND METHODS

Materials. FX (19), prothrombin (1), FV (17), protein C (20), and FIX (19) were purified and activated as described. FXa, reference protein S, and thrombin were obtained from Enzyme Research Laboratories (South Bend, IN) and enzymes were active-site-titrated (21). FVII was from Celsus Laboratories (Cincinnati). Proteins were >95% pure by SDS/PAGE and were stored in aliquots at −70°C. Proteins were biotinylated as described (22). Diisopropylphospho (DIP)-FXa (99% inactivated) was prepared by incubation of FXa at 1 mg/ml with 2 mM diisopropyl fluorophosphate (Sigma) on ice for 2 hr and dialysis against Tris-buffered saline (TBS: 50 mM NaCl/100 mM Tris·HCl, pH 7.4). DEGR-FXa was prepared by incubation of FXa with a 1.5-molar excess of 1,5-dansyl-Glu-Gly-Arg (DEGR) chloromethyl ketone (Calbiochem) until >99% inactivated and dialysis against TBS. Goat antibody to protein S was immunoaffinity-purified (17). Monoclonal antibody (mAb) S7 to protein S was prepared (23) and purified (24) as described. *p*-Amidinophenylmethylsulfonyl fluoride and biotin *N*-hydroxysuccinimide were from Clontech. Chromatography gels were from Pharmacia.

Purification of Protein S. For most experiments below, protein S was purified (17) by barium adsorption of fresh citrate-treated plasma and chromatography with DEAE-Sephacel, Blue Sepharose, and heparin-Sepharose. For one study, using a modified protocol, fractions were screened throughout purification for ability to inhibit prothrombinase and to bind to FXa. Citrate-treated plasma (4 liters) was treated within 2 hr of collection with inhibitors and kept 1 day at 4°C, and $BaCl_2$ was added. The barium citrate pellet was washed and was then eluted with EDTA. The eluate was dialyzed against 5 mM Mes/120 mM NaCl/20 mM EDTA/1 mM benzamidine/0.02% NaN_3, pH 6.0, then against the same buffer containing 1 mM EDTA, which was also used in the first chromatography step on a column (5 cm × 12.5 cm) of DEAE-Sephacel. After the column was washed, a 2-liter gradient of 0–0.5 M NaCl in starting buffer was applied at 2.0 ml/min and 10-ml fractions were collected. Protein S and complement component C4b-binding protein (C4BP) were analyzed by ELISA (25), and serum amyloid P component was analyzed by quantitative immunoblotting (23). Two peaks of protein S antigen were eluted. The first peak also contained C4BP and serum amyloid P component and was presumed to contain protein S complexed with C4BP. The second peak of protein S, containing no C4BP or serum amyloid P component, was pooled and one-third was dialyzed against 10 mM Mes/50 mM NaCl/1 mM benzamidine/1 mM EDTA/0.02% NaN_3, pH 7.4, and applied to a Blue Sepharose column (2.5 cm × 17 cm) at 1 ml/min. After unadsorbed protein was eluted, a 1 liter gradient of 0–0.35 M NaCl in the starting buffer was applied, and 4-ml fractions were collected and analyzed for protein S antigen, protein S binding to FXa (described below) or to FVa (17), and ability to inhibit prothrombinase (described below). Fractions were also screened for protein C activity, FX clotting activity, and SDS/PAGE profile.

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Abbreviations: C4BP, C4b-binding protein; DIP, diisopropylphospho; FVa, factor Va; FXa, factor Xa; mAb, monoclonal antibody.

The ascending and descending halves of the protein S peak were separately pooled. Each pool was dialyzed against the same buffer used for DEAE-Sephacel chromatography above. A column (1.6 cm × 5.8 cm) of DEAE-Sephacel was equilibrated with the same buffer except that 2 mM $CaCl_2$ was used in place of EDTA. After the column was washed, an 80-ml gradient of 0–0.35 M NaCl in the column buffer was applied at 0.5 ml/min, 2-ml fractions were collected, and 3 mM EDTA was added to each. Fractions were analyzed as above.

The peak of protein S antigen from each DEAE-Sephacel column run was pooled and dialyzed against TBS. A portion of protein S (200 μg) from the ascending run was further analyzed on a column (1 cm × 3.7 cm) containing mAb S7 coupled to CNBr-activated Sepharose. After application of protein S this column was washed and bound protein was eluted with 0.1 M glycine/0.05 M NaCl, pH 2.5. Fractions were neutralized with Tris base and tested as above.

Ligand Blotting. Various protein S preparations and other proteins (100 ng each) were subjected to SDS/7.5% PAGE (Phast system, Pharmacia) and transferred to Immobilon membranes (Millipore) according to Pharmacia instructions. Membranes were blocked for 1 hr in TBS/1% casein (Sigma)/2 mM $CaCl_2$/0.02% NaN_3, pH 7.4, and then incubated sequentially at 23°C in the same buffer with either biotin-DIP-FXa at 4 μg/ml or [protein S at 4 μg/ml followed by anti-protein S mAb (10 μg/ml) and biotin-anti-mouse IgG (1:1000; Pierce) for 1 hr, streptavidin-alkaline phosphatase (1:500; Pierce)] for 0.5 hr, and phosphatase substrate (*p*-nitrophenyl phosphate; Bio-Rad) until color development was achieved. Three washes were included after each step, except the substrate step, which included two additional washes with TBS before and after.

Microtiter Plate Assays. Binding assays were as described for binding of protein S to FVa (17). Protein S, FX, DIP-FXa, or FXa (5 μg/ml) was coated on the wells of microtiter plates. After washing and blocking with 0.5% porcine skin gelatin (Sigma) in TBS, biotin-FXa or protein S was incubated in the wells in binding buffer consisting of 0.5% gelatin in 0.05 M Tris/0.2 M NaCl/5 mM $CaCl_2$/0.1 mM $MnCl_2$/0.02% NaN_3, pH 7.4. Wells were washed in the same buffer, except that gelatin concentration was 0.1%. Bound protein S was detected with mAb S7 (10 μg/ml), followed by biotin-anti-mouse IgG, streptavidin-alkaline phosphatase, and phosphatase substrate. Bound biotin-FX or biotin-FXa was detected in the same manner, with antibodies omitted. For each data point in duplicate, a paired set of wells that were not coated with protein served as nonspecific controls whose values were subtracted from the specific data points. Nonspecific binding was 10–30%. The apparent equilibrium dissociation constant (K_d) for binding of protein S to FXa in fluid phase was determined as described for binding of protein S to FVa (17, 26).

Prothrombinase and Clotting Assays. Prothrombinase assays were performed in binding buffer containing bovine serum albumin (Calbiochem) in place of gelatin (17). Phospholipid vesicles (50 μM), 1 nM FXa, and 20 pM FVa were preincubated alone or with protein S for 15 min at 23°C. After addition of 0.3 μM prothrombin, aliquots were taken over time and quenched with TBS/10 mM EDTA/0.1% albumin. Thrombin formed was assessed with 0.2 mM S2238 substrate (Kabi Pharmacia Hesper, Franklin, OH).

For FXa one-stage clotting assays, 10 μl of 3 nM FXa was preincubated with 50 μl of normal or immune-depleted plasma with or without 15 μl of exogenous protein S for 4 min at 37°C. Clotting was initiated by addition of 100 μl of a 150-μg/ml cephalin solution and 125 μl of 20 mM $CaCl_2$ at 37°C. Clotting time measured on a Stago (Asnieres, France) ST4 coagulometer was 100 ± 15 sec in the absence of exogenous protein S.

RESULTS

Binding of Protein S to FX Derivatives. When protein S was electrophoresed and transferred to a membrane, direct ligand binding by biotin-DIP-FXa (Fig. 1*A*), FXa, or biotin-FXa was demonstrated. There was no observed binding of any FX species at any M_r other than 76,000, and there was no binding to other vitamin K-dependent proteins. The protein S preparation used for most of the binding and inhibition experiments is shown in Fig. 1*A*, lane 7. The other preparations were purified by somewhat different methods, bound FXa species less avidly, and inhibited prothrombinase in the absence of activated protein C less efficiently, although they had normal activated protein C cofactor activity. Blotted protein S bound FX and FXa and weakly bound activated protein C but did not bind other vitamin K-dependent proteins (Fig. 1*B*).

Biotin-FXa and biotin-FX bound in a similar dose-dependent manner to protein S immobilized in the wells of microtiter plates (Fig. 2*A*). Binding in the presence of EDTA was similar to binding in the presence of divalent metal ions (data not shown). Binding of 54 nM biotin-FX or biotin-FXa was reversed by ≥72% in the presence of a 50-fold excess of unlabeled ligand. Protein S bound in a dose-dependent manner to immobilized FXa, DIP-FXa, or FX (Fig. 2*B*). The apparent K_d for FXa was ≈19 nM and for FX was ≈25 nM.

Binding of protein S to FXa in fluid phase was determined by using a detection plate with immobilized protein S to determine the amount of free FXa in equilibrium mixtures with a fixed total FXa concentration and various protein S concentrations. The short incubation on the detection plate did not disturb the equilibrium between protein S and FXa in solution, since similar results were obtained when the incubation time on the detection plate was 10, 15, or 20 min. Binding was saturable, K_d was 18 ± 12 nM, and stoichiometry was 1.1 molecules of protein S bound per FXa by Scatchard analysis (n = 4). Fitting representative data to the equation for a single-site binding isotherm (ENZFITTER computer program, Elsevier Biosoft, Cambridge, U.K.) yielded a K_d of 15 ± 3 nM and a protein S/FXa stoichiometry of 1.3 ± 0.6.

Inhibition of FXa by Protein S. Protein S inhibited the amidolytic activity and the prothrombin-converting activity of FXa in a dose-dependent manner, with 15 min required for

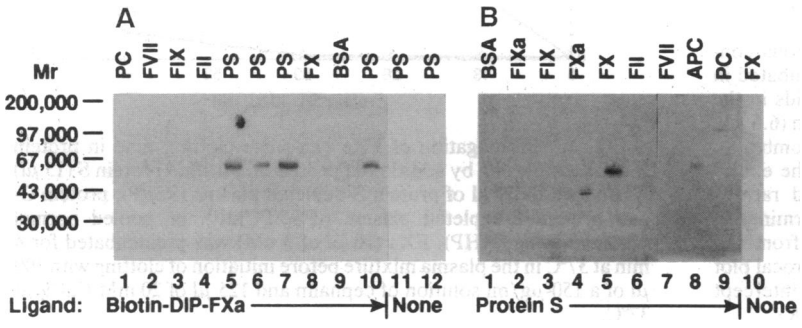

FIG. 1. Ligand blots of vitamin K-dependent factors with biotin-DIP-FXa (*A*) or protein S (*B*). Purified protein S preparations and other proteins were electrophoresed and ligand blotting was performed. Biotin-DIP-FXa ligand was omitted from lanes 11 and 12 in *A*, and protein S ligand was omitted from lane 10 in *B*. PC, protein C; APC, activated protein C; PS, protein S; BSA, bovine serum albumin.

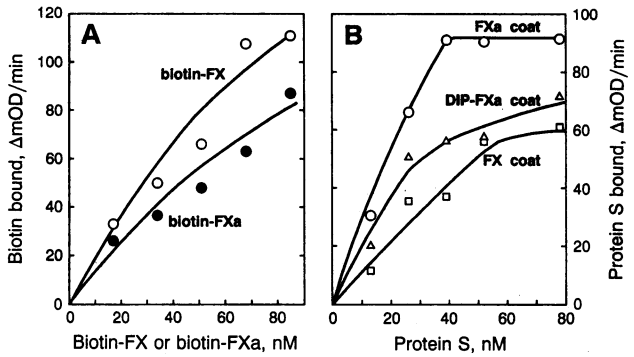

FIG. 2. (A) Binding of biotin-FX (○) or biotin-FXa (●) to immobilized protein S. (B) Binding of protein S to FX (□), FXa (○), or DIP-FXa (△) coated on the wells of microtiter plates. Incubations were at 37°C for 50 min, followed by detection of bound biotin. mOD, OD milliunits.

maximum inhibition and half-maximal inhibition achieved after 1–2 min of incubation (data not shown). Thirty-three nanomolar protein S was required for 50% inhibition of FXa amidolytic activity, but only 5.5 nM protein S was required for 50% inhibition of FXa prothrombinase activity (data not shown). Kinetic analysis suggested that inhibition was not due to traces of activated protein C or other protease, since inhibition reached a maximum after 15 min. Also, protein S treated with *p*-amidinophenylmethyl sulfonyl fluoride, which inhibits activated protein C and other trypsin-like proteases, behaved identically to untreated protein S (data not shown). Protein S did not inhibit the amidolytic activity of thrombin, activated protein C, or FXIa.

Protein S was preincubated with various concentrations of FXa. Prothrombin was added and the rate of thrombin formation was determined (Fig. 3*A*). When the inhibited FXa was assumed to be bound to protein S, a calculation of bound and free FXa was made and a double reciprocal plot was constructed (Fig. 3*B*). The intercept on the *y* axis yielded a concentration of "inhibitory" protein S of about 9.6 nM. Since the FXa used was 48% active, and protein S binds to both active and inactive FXa (Fig. 2), this corresponds to 20 nM protein S bound to total FXa. Since 73 nM protein S neutralized ≈20 nM FXa, we estimate that 27% of protein S bound FXa. An alternative explanation is that more than one

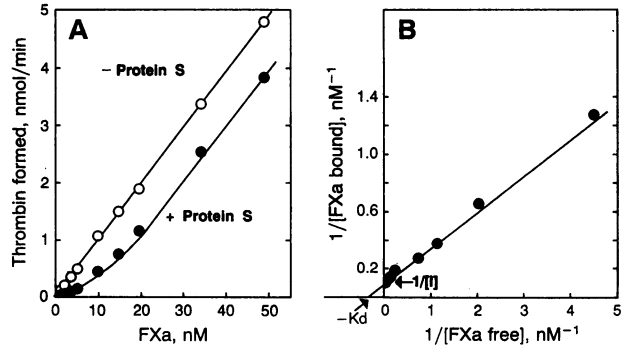

FIG. 3. Protein S inhibition of prothrombinase at various concentrations of FXa. Mixtures containing FXa were preincubated in binding buffer for 15 min at 23°C with 50 μM phospholipids in the absence (○) or presence (●) of 73 nM protein S. Prothrombin (6.6 μM final) was added and the rate of conversion of prothrombin to thrombin was measured (*A*). Free FXa concentration in the experiment with protein S was calculated from the observed rate of prothrombin activation by using the calibration curve determined in the absence of protein S. Bound FXa was calculated from the difference between free and added FXa and a double reciprocal plot of FXa free versus FXa bound was constructed (*B*). The *y* intercept was taken as 1/[inhibitor] (1/[I]) and the *x* intercept as $-K_d$.

Table 1. Inhibition of FXa-catalyzed prothrombin activation by protein S

Components	Protein S for 50% inhibition, nM
FXa, EDTA	30
FXa, $CaCl_2$	19
FXa, $CaCl_2$, PL	19
FXa, $CaCl_2$, PL, FVa	8.1

Protein S at various concentrations was added to a preformed mixture of the components listed and preincubated for 15 min at 23°C prior to the addition of prothrombin (final concentration, 1 μM) and measurement of rate of thrombin formed with S2238 as substrate. FXa was used at 1 nM, FVa at 20 pM, EDTA or $CaCl_2$ at 5 mM, and phospholipid (PL) at 50 μM.

molecule of protein S bound per molecule of FXa. The intercept on the *x* axis of Fig. 3*B* was taken as K_d = 2.5–5.2 nM (presumably the K_d for complex formation between FXa and a subpopulation of protein S).

Protein S inhibition of FXa conversion of prothrombin to thrombin was examined in the presence of various components (Table 1). The concentration of protein S required for 50% inhibition of FXa in the presence of EDTA was 1.6 times higher (30 nM) than that required in the presence of divalent metal ions (19 nM). No increase in potency of protein S was observed when phospholipid vesicles were included, suggesting that protein S inhibition was not due to its competition for phospholipid binding sites. The concentration of protein S required for 50% inhibition in the presence of FVa was 2.3-fold less (8.1 nM) than in the absence of FVa (19 nM).

Inhibition of FXa amidolytic activity by protein S was reversible, since a mixture of 72 nM protein S and 3 nM FXa in which the remaining FXa activity was 23% regained 95% of its original FXa activity when the mixture was diluted 10-fold and allowed to stand for 10 min at 23°C. No covalent complex of FXa was detected when an inhibited mixture of FXa and protein S was subjected to SDS/PAGE and immunoblotting (data not shown).

Protein S prolonged the clotting time of protein S-depleted plasma in FXa one-stage assays in a dose-dependent manner in the absence of activated protein C (Fig. 4). This anticoagulant effect was not due to traces of activated protein C,

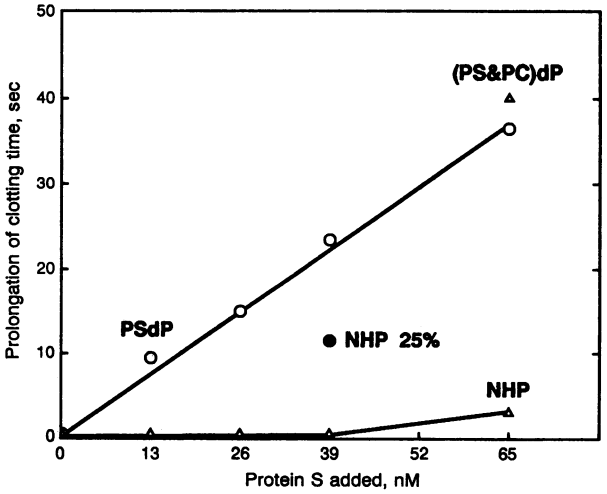

FIG. 4. Prolongation of FXa one-stage clotting time in protein S-depleted plasma by addition of protein S. Purified protein S (15 μl) was added to 50 μl of protein S-depleted plasma (PSdP), protein C- and protein S-depleted plasma [(PS&PC)dP], or pooled normal human plasma (NHP). FXa (10 μl of 3 nM) was preincubated for 4 min at 37°C in the plasma mixture before initiation of clotting with 100 μl of a 150-μg/ml solution of cephalin and 125 μl of 20 mM $CaCl_2$ at 37°C.

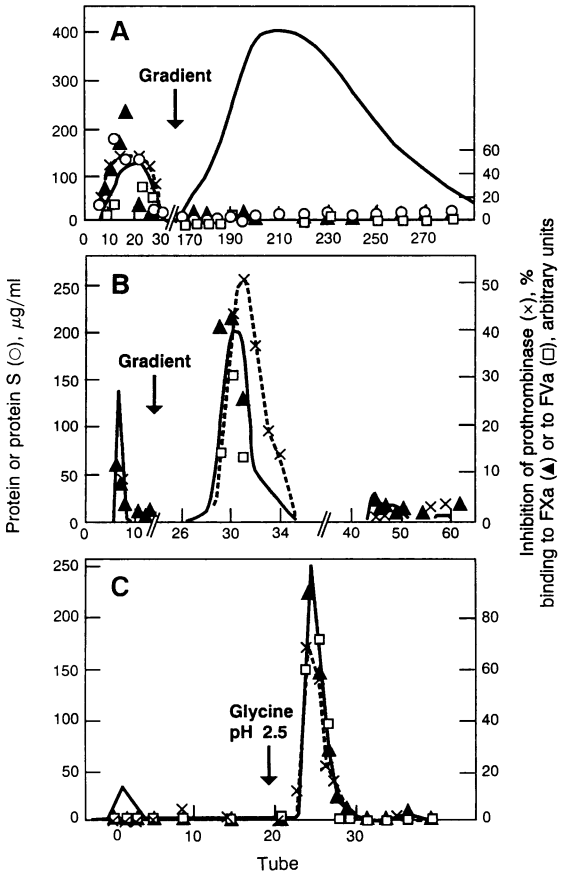

FIG. 5. Analysis of protein S-binding properties and inhibition of FXa during purification of protein S. (*A*) Blue Sepharose chromatography of the free protein S pool from DEAE-Sephacel chromatography in the presence of 1 mM EDTA. (*B*) DEAE-Sephacel chromatography of the protein S pool from *A* in the presence of 2 mM $CaCl_2$. (*C*) Immunoaffinity chromatography of the protein S pool from *B*. Protein (A_{280}) (solid lines without symbols), protein S binding to immobilized FXa (△) or immobilized FVa (□), and inhibition of prothrombinase activity (×) are indicated.

since response to protein S was the same in plasma depleted of both protein S and protein C as in plasma depleted of only protein S. A mixture of 25% normal and 75% protein S-depleted plasma gave an anticoagulant response to added protein S that was intermediate to the response of the two plasmas. There was <5 sec prolongation of clotting time by addition of protein S to normal plasma, suggesting that endogenous protein S in normal plasma exerts an anticoagulant effect that is not greatly enhanced by exogenous protein S.

Analysis of Protein S Activity During Purification of Protein S. Data presented in Fig. 3 suggested that a subpopulation of protein S possesses the ability to inhibit FXa. Also, different preparations of protein S inhibited prothrombinase to different extents. Therefore, a protein S purification procedure was monitored to determine whether different fractions of protein S have different abilities to bind to and inhibit FXa or whether a trace contaminant might be associated with protein S activity. During barium adsorption and DEAE-Sephacel chromatography of plasma, it was not possible to screen for these activities due to the presence of other vitamin K-dependent factors. During the remaining steps, fractions were screened for SDS/PAGE profile, protein S antigen, ability to bind FXa and FVa, and ability to inhibit prothrombinase activity (Fig. 5). Protein S adsorbed weakly to Blue Sepharose and was eluted as the first peak (Fig. 5*A*), >90% pure by SDS/PAGE. Prothrombinase inhibitory activity was present throughout the protein S peak. The peak contained no detectable C4BP, serum amyloid P component, protein C, or Factor X.

The ascending and descending halves of the protein S peak from the Blue Sepharose column were pooled separately for DEAE-Sephacel chromatography in the presence of Ca^{2+}. Results for the two pools were very similar and the results for only the ascending half are presented (Fig. 5*B*). A small amount of protein S did not adsorb to the column (fractions 7–9). The largest peak contained >95% pure protein S by SDS/PAGE, with trace contaminants at M_r 30,000 and 50,000, most visible by SDS/PAGE in fraction 31 (data not shown). The peaks for binding FXa and FVa were at fraction 30, and the peak for prothrombinase inhibitory activity was at fraction 31. The minor peak at fraction 45 formed a band of M_r 50,000 on SDS/PAGE and had no ability to bind FXa or inhibit prothrombinase.

The major peak of protein S shown in Fig. 5*B* was pooled and a portion was subjected to chromatography on mAb S7-Sepharose (Fig. 5*C*). Protein S antigen, FXa-binding properties, and prothrombinase-inhibitory activity bound to this column and were eluted with glycine at pH 2.5. Silver staining revealed no contaminants in the protein S peak. Overall, prothrombinase-inhibitory activity was coeluted with protein S from Blue Sepharose, DEAE-Sephacel, and immunoaffinity chromatography and was not apparently due to any contaminant. Chromatography on DEAE-Sephacel was done in the presence of EDTA and later in the presence of Ca^{2+} to decrease the chance that a contaminant responsible for the observed inhibition was coeluted with protein S on both columns. No subpopulation of protein S was isolated that was more active than another in inhibition of prothrombinase, although prothrombinase inhibitory activity displayed a slight asymmetry with respect to protein S antigen peaks on some chromatographic profiles.

Protein S isolated in the experiment described in Fig. 5 had an apparent K_i of 130 nM—i.e. it was less active than two other protein S preparations that did not include immunoaffinity chromatography but did include heparin-Sepharose chromatography. Therefore, protein S from Blue Sepharose chromatography above (Fig. 5*B*) was subjected to heparin-Sepharose chromatography in the same manner as was used for the previous two more-active preparations. No change in prothrombinase-inhibitory activity was observed and no distinct subpopulation of active protein S was isolated. Various treatments were employed to attempt to make either our or commercial preparations of protein S more inhibitory toward FXa, but none was successful. These included freeze–thaw, thrombin, neuraminidase, *N*-Glycanase, glycine (pH 2.5), and 0.01% Tween 20.

DISCUSSION

Protein S binds to FVa, inhibits prothrombinase independently of activated protein C, and competes efficiently with prothrombin for binding to FVa (17). However, we reported that Lineweaver–Burk plots of inhibition of prothrombinase at variable prothrombin concentrations indicated a pattern of mixed inhibition rather than simple competitive inhibition (17). Thus, we investigated possible interaction of protein S with thrombin or FXa. Protein S inhibited the amidolytic activity of FXa but not of thrombin. Protein S inhibition of FXa amidolytic activity was similar in efficiency to protein S inhibition of prothrombin-to-thrombin conversion by FXa in the presence of EDTA (Table 1). The efficiency of inhibition of FXa by protein S improved in the presence of divalent metal ions and FVa, but phospholipids had no effect without FVa. Thus, protein S interacts with both FVa and FXa and inhibits prothrombinase in the absence of activated protein C.

FX and FXa derivatives bound to immobilized protein S and not to any contaminant in the protein S preparations. The apparent K_d for protein S binding to FXa in the fluid phase or solid phase was 18 nM. With an alternative method of titrating protein S with FXa and measuring residual FXa activity, a K_d of 5.2 nM was obtained. Since these experiments suggested that a subpopulation of ≈27% of the protein S was inhibitory, the apparent K_d of 5.2 nM would yield an apparent K_d for the total protein S population of ≈19 nM, in good agreement with the K_d calculated from binding data and the apparent K_i for inhibition of FXa. Other evidence of interaction between protein S and FXa in fluid phase was provided by the observation that the fluorescence yield of the dansyl group in DEGR-FXa was changed by 17% when 200 nM protein S was mixed with 66 nM DEGR-FXa in TBS/5 mM $CaCl_2$ (unpublished results). Considerable effort was made to exclude the possibility that the inhibition observed was due to a contaminating protease or enzyme inhibitor in the protein S preparations. Inhibition of FXa was not affected by pretreatment of protein S with protease inhibitors, by extensive dialysis, by addition of heparin, or by treatment with neutralizing antibody to protein C or to tissue factor pathway inhibitor (27).

These findings may be related to observations that protein S was more effective as an activated protein C cofactor in the presence of FXa (15), that both FX and FXa can displace activated protein C from FVa (28), and that protein S can negate the protective effect of either FX or FXa toward FVa (15, 28). Hackeng *et al.* (29) found that protein S directly inhibited prothrombinase on the surface of endothelial cells. Mitchell *et al.* (30) described a form of protein S, purified on an immunoaffinity column, that was anticoagulant in the absence of activated protein C and appeared to act as a competitive inhibitor in prothrombin activation. Exposure to the unique anti-protein S mAb was reported to render nonanticoagulant protein S temporarily anticoagulant (30), suggesting an induced change in conformation. Two different protein S preparations made in our lab appeared at least as active as the protein S described earlier (30), yet they were not prepared by immunoaffinity chromatography. We hypothesize that protein S in blood exists in a form that is anticoagulant in the absence of activated protein C and that some of this activity is lost during purification. Among possibilities, this loss could be due to a change in conformation, loss of a posttranslational modification, proteolytic cleavage, loss of a small cofactor, disulfide bond exchange, or formation of multimers. Evidence for direct anticoagulant activity in plasma came from data showing that protein S prolonged the FXa one-stage clotting time in a dose-dependent manner in protein S-depleted plasma.

We investigated whether a subpopulation of protein S could be isolated that was more active in inhibition of FXa, since some of our protein S preparations inhibited FXa better than others (K_i 8–130 nM) even though they had similar activated protein C cofactor activity (17). No subpopulation of protein S could be isolated that inhibited FXa more efficiently. Immunoaffinity chromatography suggests that protein S *per se* or protein S in close association with an undetectable trace component is responsible for the FXa-inhibitory activity.

Although the molecular basis for the differing activities of different protein S preparations has not been identified, this work and previous studies (17) show that protein S can independently bind FXa and FVa and inhibit prothrombinase. This may help explain the clinical findings of both arterial and venous thrombosis associated with protein S deficiency.

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