Purified Protein S Contains Multimeric Forms with Increased APC-Independent Anticoagulant Activity†

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ABSTRACT: Protein S, the cofactor of activated protein C (APC), also expresses anticoagulant activity independent of APC by directly inhibiting prothrombin activation via interactions with factor Xa, factor Va, and phospholipids. In different studies, however, large variations in APC-independent anticoagulant activities have been reported for protein S. The investigation presented here shows that within purified protein S preparations different forms of protein S are present, of which a hitherto unrecognized form (<5% of total protein S) binds with high affinity to phospholipid bilayers (K_d < 1 nM). The remaining protein S (>95%) has a low affinity (K_d = 250 nM) for phospholipids. Using their different affinities for phospholipids, separation of the forms of protein S was achieved. Native polyacrylamide gel electrophoresis demonstrated that the form of protein S that binds to phospholipids with low affinity migrated as a single band, whereas the high-affinity protein S exhibited several bands that migrated with reduced mobility. Size-exclusion chromatography revealed that the slower-migrating bands represented multimeric forms of protein S. Multimeric protein S (<5% of total protein S) appeared to have a 100-fold higher APC-independent anticoagulant activity than the abundant form of protein S. Comparison of purified protein S preparations that exhibited a 4-fold difference in APC-independent anticoagulant activity showed that the ability to inhibit prothrombin activation correlated with the content of multimeric protein S. Multimeric protein S could not be identified in normal human plasma, and it is therefore unlikely that this form of protein S contributes to the APC-independent anticoagulant activity of protein S that is observed in plasma.

Human protein S is a vitamin K-dependent glycoprotein that functions as a nonenzymatic cofactor for activated protein C (APC)[1] (*1*) and which accelerates factor Va inactivation by selectively enhancing APC-mediated cleavage of factor Va at Arg^{306} (*2*). The importance of this anticoagulant protein C pathway is emphasized by the increased risk for thrombotic complications associated with protein S and protein C deficiencies (*3*, *4*).

In 1988, it was reported that protein S also exhibits anticoagulant activity in the absence of APC (*5*) by inhibiting prothrombin activation via direct interactions with the components of the prothrombinase complex: factor Va, factor Xa, and phospholipids (*6*−*8*). In addition, it was shown that protein S reduced the level of F1+2 generation in plasma during perfusion over tissue factor-rich endothelial cell matrices in the absence of APC (*9*). However, up to 100-fold differences in APC-independent anticoagulant activities have been reported for different protein S preparations (*5*−*11*).

The ability of protein S to bind to phospholipids appears to be crucially important for the expression of anticoagulant activity in the absence of APC (*6*, *10*−*12*). This was demonstrated by the observation that a monoclonal antibody directed against the γ-carboxyglutamic acid (Gla) module of protein S inhibited its APC-independent anticoagulant activity (*10*, *13*). More than 10-fold differences have been reported for the dissociation constant of protein S binding to phospholipids (*6*, *10*−*13*), and the APC-independent anticoagulant activity of a protein S preparation was shown to be related to its affinity for phospholipids (*10*). Furthermore, the APC-independent activity appeared to be the consequence of occupation of the lipid membrane by protein S, since increasing the amount of phospholipids abrogated the inhibitory activity of protein S (*11*).

In this study, we investigated the relationship between the APC-independent activity of protein S and its phospholipid binding properties to find an explanation for the variation in APC-independent anticoagulant activity between different protein S preparations.

EXPERIMENTAL PROCEDURES

Materials. Hepes, Tris, EDTA, bovine serum albumin (BSA), and ovalbumin were purchased from Sigma. 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-

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[1] Abbreviations: APC, activated protein C; BSA, bovine serum albumin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DAB, diaminobenzidine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPS, 1,2-dioleoyl-*sn*-glycero-3-phosphoserine; Gla, γ-carboxyglutamic acid module; HBS, Hepes-buffered saline; PVDF, polyvinylidene fluoride; S2238, D-Phe-(pipecolyl)-Arg-pNA; SDS, sodium dodecyl sulfate; TBS, Tris-buffered saline; TMB, 3,5,3′,5′-tetramethylbenzidine.

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dioleoyl-*sn*-glycero-3-phosphoserine (DOPS) were obtained from Avanti Polar Lipids (Alabaster, AL). The chromogenic substrate D-Phe-(pipecolyl)-Arg-pNA (S2238) was supplied by Chromogenix (Mölndal, Sweden). Column materials were obtained from Pharmacia (Uppsala, Sweden). Gradient polyacrylamide gels (4 to 15%) were from Bio-Rad (Hercules, CA). Immobilon polyvinylidene fluoride (PVDF) membranes were from Millipore (Bedford, MA). Diaminobenzidine (DAB) was obtained from Sigma (St. Louis, MO). 3,5,3′,5′-Tetramethylbenzidine (TMB) was obtained from Roche (Mannheim, Germany). Protein S was obtained from Enzyme Research Laboratories (ERL, South Bend, IN). Antibodies against protein S were obtained from DAKO (Glostrup, Denmark). Human activated protein C (APC) and factor Xa were obtained from Enzyme Research Laboratories. Prothrombin (*14*) and factor Va (*15*) were purified as described previously.

Protein S Purification. Human protein S preparations were purchased or purified from 20 units of freshly frozen human plasma (5 L) as described previously (*14*). Both preparations were purified according to the same procedure, i.e., barium citrate adsorption of human plasma followed by ammonium sulfate precipitation, anion exchange chromatography (QAE Sephadex), affinity chromatography (Blue-Sepharose), and anion exchange chromatography (Q Sepharose fast flow). The protein S preparations were analyzed for purity and for the presence of thrombin-cleaved protein S by SDS−PAGE under nonreducing and reducing conditions, respectively. Protein S concentrations were determined by measuring A_{280} using an $\epsilon^{1\%}_{1cm,280\ nm}$ of 9.6 (*1*).

SDS−PAGE, Native Polyacrylamide Gel Electrophoresis, and Western Blotting. Protein S preparations were analyzed using PAGE in the presence of SDS or under native conditions. After electrophoresis, the separated proteins were transferred to PVDF membranes for Western blotting. Blocking of the PVDF membranes was performed with Tris-buffered saline (TBS) [50 mM Tris and 100 mM NaCl (pH 7.4)] with 5% Protifar (blocking buffer), and protein S was detected using peroxidase-conjugated polyclonal antibodies against human protein S diluted 1/1000 in blocking buffer. DAB or TMB was used as a peroxidase substrate.

Phospholipid Liposome and Vesicle Preparation. Liposomes were prepared essentially as described previously (*16*). Dried phospholipids were suspended in TBS and vortexed for 3 min in the presence of glass beads. The liposomes were centrifuged at 8000*g* and washed once with TBS to remove small lipid structures. Small unilamellar vesicles were prepared as described previously (*15*). Phospholipid concentrations were determined by microphosphate analysis (*17*).

APC-Independent Anticoagulant Activity of Protein S. The APC-independent anticoagulant activity of protein S was measured in a prothrombinase assay. Factor Va, factor Xa, phospholipid vesicles (20/80 DOPS/DOPC molar ratio), and protein S were preincubated at 37 °C in Hepes-buffered saline (HBS) [25 mM Hepes and 175 mM NaCl (pH 7.7)] containing 3 mM $CaCl_2$ and 5 mg/mL BSA. Prothrombin was added after 15 min, resulting in final concentrations of 10 pM factor Va, 10 pM factor Xa, 100 nM phospholipid vesicles, 500 nM prothrombin, and various concentrations of protein S in a volume of 300 μL. Rates of prothrombin activation were determined with the chromogenic substrate S2238.

Ellipsometric Determination of the Extent of Protein Adsorption to Lipid Bilayers. Protein adsorption to planar bilayers was assessed by ellipsometry (*18*). Briefly, planar bilayers deposited on silicon slides were prepared as described previously (*19*) by immersion of the hydrophilic silicon slide for 5 min in a stirred suspension of small unilamellar lipid vesicles (30 μM) in binding buffer [50 mM Tris, 100 mM NaCl, and 3 mM $CaCl_2$ (pH 7.5)]. The instrument and data analysis have been described previously (*20*). Measurements were performed using silicon slides at an angle of incidence of the light beam (HeNe laser) of 68° (*21*). Experiments were performed in binding buffer containing 0.5 mg/mL BSA at room temperature (20−22 °C) with continuous stirring in a trapezoidal cuvette.

Separation of Protein S Forms by Liposome Centrifugation. Liposomes (200 μM) were incubated with 900 nM protein S (protein S-*total*) in 200 μL of TBS containing 3 mM $CaCl_2$ and 5 mg/mL BSA for 45 min with continuous mixing. The mixture was centrifuged for 10 min at 8000*g* in a Hettich EBA 12 Eppendorf centrifuge, and the supernatant was collected (protein S-*supernatant*). The liposome pellet was washed with TBS containing 3 mM $CaCl_2$ and 5 mg/mL BSA and again centrifuged. Then, phospholipid-bound protein S was eluted by resuspending the pellet in 200 μL of TBS containing 3 mM EDTA, and the liposomes were quantitatively removed by centrifugation in a Beckman TLA-100 ultracentrifuge (25 min at 110000*g*). The eluted protein S thus obtained was designated protein S-*pellet*. All steps during the separation were performed at room temperature in siliconized tubes. Protein S concentrations were determined using an ELISA.

Protein S ELISA. An ELISA was used to determine protein S concentrations of the separated protein S forms. Microtiter plate wells were coated overnight with a polyclonal antibody against human protein S diluted 1/1000 in 0.1 M Na_2CO_3 at pH 9.0 (50 μL/well) and 4 °C. Further steps were performed at room temperature. The wells were subsequently blocked with 200 μL of HBS containing 30 mg/mL BSA for 2 h. Protein S samples were diluted in HBS containing 5 mg/mL BSA and 3 mM EDTA, and 50 μL of each dilution was incubated in the wells for 1 h. After washing was performed three times with 200 μL of wash buffer (HBS containing 0.02% Tween-20), 50 μL of a peroxidase-conjugated polyclonal anti-human protein S antibody in HBS containing 5 mg/mL BSA was added to the wells and incubated for 1 h. After washing was performed five times with 200 μL of wash buffer, peroxidase activity was determined with the chromogenic substrate (TMB enzymatic kit, Roche) according to the manufacturer's instructions. ERL protein S (1540L) was used as a standard.

Size-Exclusion Chromatography. Protein S-*supernatant* and protein S-*pellet* (10 nM, 100 μL) were injected separately on a Superose 6 size-exclusion chromatography column (Pharmacia) at a flow rate of 0.25 mL/min in 25 mM Hepes, 150 mM NaCl, and 1 mM EDTA (pH 7.4). Fractions (0.25 mL) were collected and immediately diluted 1:1 in HBS containing 30 mg/mL BSA. The amount of protein S in the column fractions was determined with an ELISA.

Isolation of Protein S Multimers Using Phospholipid-Coated Capillaries. Glass capillaries (0.58 mm internal diameter and 127 mm length, volume of 35 μL, Brand AG, Wertheim, Germany) were thoroughly cleaned before phos-

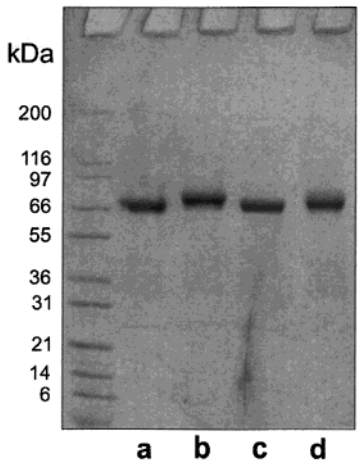

FIGURE 1: Characterization of protein S by SDS−PAGE. Protein S (4 μg) was applied to a gradient SDS gel (from 4 to 15% polyacrylamide). Lanes a and c contained nonreduced protein S_A and protein S_B, respectively. Lanes b and d contained reduced and alkylated protein S_A and protein S_B, respectively. Reduction was performed with 10 mM dithiothreitol for 15 min at 37 °C followed by alkylation of the reduced sulfhydryl side chains with 20 mM iodoacetamide. Coomassie Brilliant Blue was used for staining.

pholipid coating was carried out as described previously (*22*). The phospholipid bilayer was deposited by incubating the capillaries for 1 h with a solution of 2 mM 20/80 DOPS/DOPC vesicles. To remove unbound phospholipid, the capillaries were washed with TBS containing 5 mg/mL BSA and 3 mM $CaCl_2$ for 30 min at a flow rate of 200 μL/min. Defibrinated, recalcified normal pooled plasma (3 mL), with or without addition of 50 ng of protein S-*pellet*, was recirculated over three capillaries in series for 1 h at a flow rate of 200 μL/min. Then, a 30 min wash was performed with TBS containing 5 mg/mL BSA and 3 mM $CaCl_2$ at the same flow rate, and finally, protein S elution was performed with TBS (130 μL) containing 5 mg/mL BSA and 4 mM EDTA. Samples of eluted fractions (30 μL) and purified protein S-*pellet* (10 ng) were subjected to native PAGE (4 to 15%) and Western blotting. Protein S was detected with peroxidase-conjugated polyclonal antibodies directed against protein S, using TMB as a substrate.

RESULTS

APC-Independent Anticoagulant Activity of Protein S. To investigate the nature of differences in APC-independent anticoagulant activities between protein S preparations, we studied protein S obtained from two different sources: a protein S isolated from freshly frozen plasma (protein S_A) and a commercially available protein S (protein S_B). Both protein S preparations were characterized by SDS−PAGE. No differences between these protein S preparations were apparent under nonreducing and reducing conditions. SDS−PAGE analysis under reducing conditions indicated that in both preparations no thrombin-cleaved protein S was detectable (Figure 1).

Protein S_A and protein S_B were tested for their ability to inhibit prothrombin activation. Factor Va, factor Xa, and phospholipid vesicles were preincubated in the presence of various concentrations of protein S. After 15 min, prothrombin was added and its activation was followed over time (Figure 2A,B). The extent of prothrombin activation was linear with time, and both protein S_A (panel A) and protein S_B (panel B) inhibited prothrombin activation in a dose-dependent manner, although to different extents. Panel C shows the rate of prothrombin activation as a function of the protein S concentration. Half-maximal inhibition was reached at concentrations of 7 nM protein S_A and 28 nM protein S_B, indicating that protein S_A expressed a 4-fold higher inhibitory activity than protein S_B (Figure 2C).

Phospholipid Binding Properties of Protein S. Since binding of protein S to phospholipid is essential to expression of its anticoagulant activity, we compared the phospholipid binding properties of protein S_A and protein S_B using ellipsometry. The level of concentration-dependent adsorption of protein S to DOPS/DOPC bilayers containing 20 mol % DOPS was measured as a function of time (Figure 3). The adsorption of both protein S_A and protein S_B was biphasic. A fast initial adsorption phase which was complete within 50 s after addition of protein S was followed by a secondary slow adsorption during which additional protein accumulated on the lipid layer until equilibrium was reached.

The extent of protein adsorption during the first phase of binding was concentration-dependent and comparable for both preparations. This became apparent when the amount of protein bound to the lipid surface 50 s after addition of protein S was plotted as a function of the total protein S concentration in the cuvette (Figure 3C). The Langmuir model was used to analyze the adsorption at this time point. No difference was found between the two preparations in this first adsorption phase, and therefore, the data were combined for further analysis. The solid line in Figure 3C represents the best fit to the combined data. The maximal level of binding (Γ_{max}) to the phospholipid bilayer was estimated to be 0.34 μg/cm^2, and the dissociation constant (K_d) was 246 nM.

The fast initial phase of binding was followed by a second phase of slow, continuing adsorption. For protein S_A (Figure 3A) and protein S_B (Figure 3B), the adsorption rate during the second phase was concentration-dependent, as shown by the faster saturation of the lipid layer at higher protein S concentrations (Figure 3A,B). However, protein adsorption during the second phase was much slower for protein S_B than for protein S_A. At 25 nM protein S, the extra amounts of protein S bound during the second phase of adsorption (between 50 and 3800 s after addition of protein S) were 0.20 μg/cm^2 for protein S_A and 0.09 μg/cm^2 for protein S_B.

After 3800 s, the cuvette was flushed with a calcium-containing buffer. Surprisingly, only very limited desorption was observed (see below), implying that at the end of the adsorption phase protein S was bound with a high affinity to the phospholipid surface. Addition of EDTA, however, caused an instantaneous and complete dissociation of protein S from the lipid bilayer (see below), indicating that the high-affinity binding was Ca^{2+}-dependent.

The biphasic adsorption behavior can tentatively be explained by assuming that protein S preparations contain two forms of protein S with different phospholipid binding properties. The initial fast adsorption phase likely originates from a fraction of protein S that is present abundantly and therefore binds rapidly but with a relatively low affinity, while the fraction that causes the additional slower binding is present in a small amount, resulting in a slow but high-

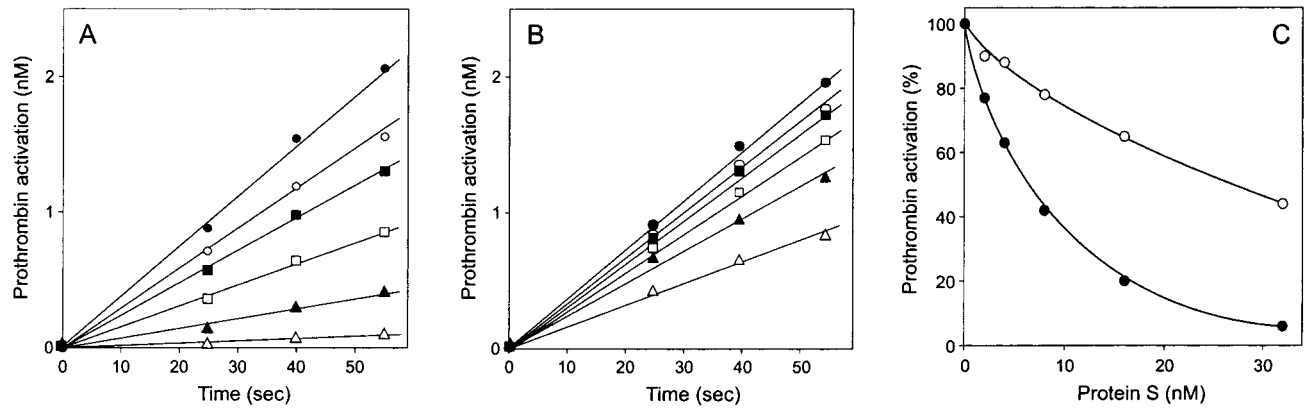

FIGURE 2: Effect of protein S on prothrombin activation in the absence of APC. Factor Va, factor Xa, phospholipid vesicles (20/80 DOPS/DOPC), and protein S_A (A) or protein S_B (B) were preincubated at 37 °C in Hepes-buffered saline [25 mM Hepes and 175 mM NaCl (pH 7.7)] containing 3 mM $CaCl_2$ and 5 mg/mL BSA. After 15 min, prothrombin was added, resulting in final concentrations of 10 pM factor Va, 10 pM factor Xa, 100 nM phospholipid vesicles, 500 nM prothrombin, and 0 (●), 2 (○), 4 (■), 8 (□), 16 (▲), or 32 nM protein S (△). At the indicated time points, the amounts of thrombin formed were determined with the chromogenic substrate S2238. Panel C shows inhibition of prothrombin activation by protein S_A (●) and protein S_B (○) plotted as a function of the final protein S concentration.

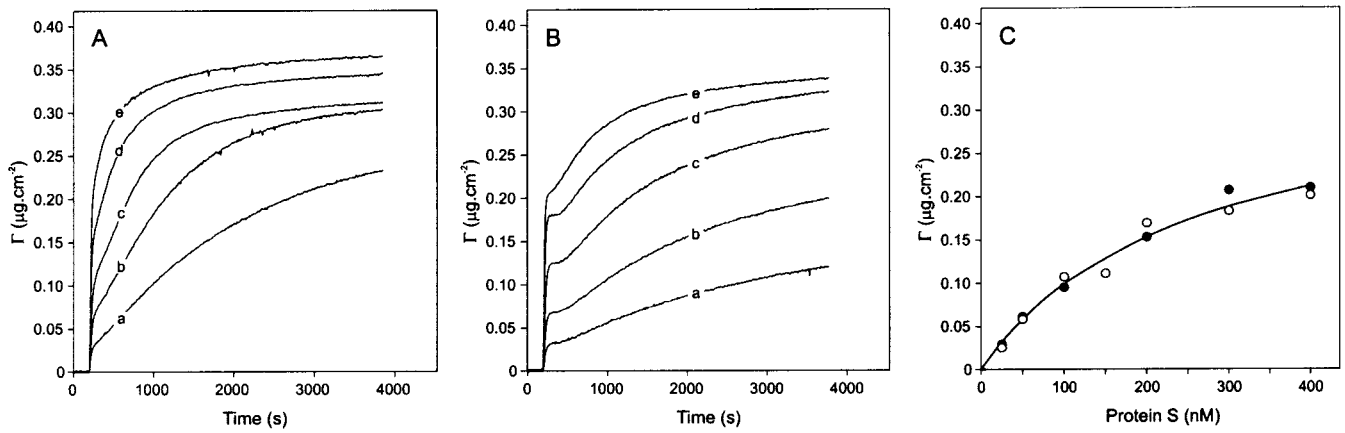

FIGURE 3: Concentration-dependent binding of protein S to a phospholipid bilayer. The mass Γ (in micrograms per square centimeter) of protein adsorbed to DOPS/DOPC bilayers containing 20 mol % DOPS was measured by ellipsometry as described in Experimental Procedures. (A) At 200 s, protein S_A (25, 50, 100, 200, and 400 nM in curves a−e, respectively) was added to the cuvette, and adsorption was followed over time for 1 h. (B) The same experiment was performed with protein S_B (25, 50, 100, 200, and 400 nM in curves a−e, respectively). (C) The amount of protein bound to the bilayer during the first, fast phase (50 s after addition of protein S to the cuvette) is plotted as a function of the final concentration of protein S_A (●) and protein S_B (○). The solid line represents the best fit to these data according to the Langmuir model. Experiments were performed at room temperature (20−22 °C) in binding buffer [50 mM Tris, 100 mM NaCl, and 3 mM $CaCl_2$ (pH 7.5)] containing 0.5 mg/mL BSA.

affinity accumulation of protein S on the phospholipid bilayer.

Adsorption and Readsorption of Protein S on a DOPS/DOPC Bilayer. To test the hypothesis that the slow adsorption process is associated with a high-affinity binding fraction of protein S, we characterized the readsorption of the bound protein to the planar lipid layer at 50 nM protein S (Figure 4A) and at 300 nM protein S (Figure 4B).

After the characteristic biphasic adsorption of protein S, nonbound protein S was removed by rapidly flushing the cuvette with 50 mL of calcium-containing buffer at 4700 s (Figure 4, black arrowheads). This depletion of protein S from the solution caused only a slow and limited desorption, and most of the protein remained bound to the phospholipid bilayer. After addition of EDTA at 5000 s (Figure 4, white arrowheads), all protein S dissociated from the phospholipid bilayer. The amounts of protein S present in the EDTA-containing buffer (Figure 4A), as determined by an ELISA, were 0.23 μg of protein S_A (0.74 nM) and 0.13 μg of protein S_B (0.43 nM), which are close to the amounts of protein S that were bound to the phospholipid surface (0.6 cm^2) before the addition of EDTA (0.18 μg of protein S_A and 0.11 μg of protein S_B). This indicates that the phospholipid-bound protein was protein S.

Subsequently, the free calcium concentration in the cuvette was restored to 3 mM, and readsorption of protein S was observed (Figure 4). However, the initial fast phase of adsorption was no longer present. This observation indicates that (1) the high-affinity protein S was responsible for the secondary, slow binding to the phospholipid bilayer and (2) the high-affinity protein S, which was adsorbed during the slow binding phase, quantitatively replaced the low-affinity protein S that was bound during the first rapid phase. In agreement with this, washing the cuvette before the initially bound protein S was replaced by the high-affinity form (300 s after protein S addition) resulted in desorption of more than 75% of the bound protein S (data not shown). As mentioned earlier, the concentration of protein S in the incubation mixture after the release of protein S from the phospholipid bilayer by EDTA was <1 nM. The fact that,

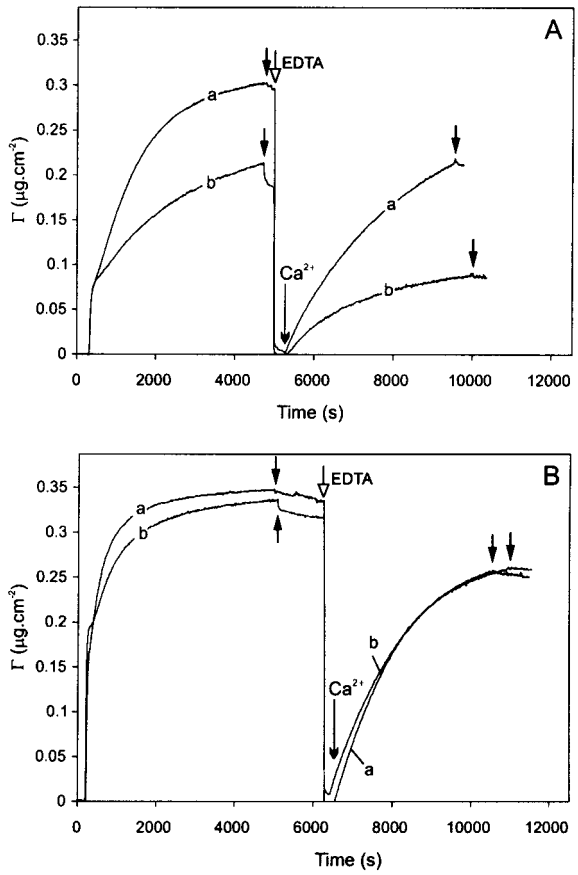

FIGURE 4: Adsorption and readsorption of protein S to phospholipid bilayers. Adsorption and readsorption of protein S to a DOPS/DOPC bilayer (20 mol % DOPS) was analyzed by ellipsometry at two protein S concentrations [50 (A) and 300 nM (B)]. In both panels A and B, curves a and b represent protein S_A and protein S_B, respectively. Protein adsorption was initiated by addition of protein S to the cuvette at 200 s and was followed until maximal adsorption was reached. At ~5000 s (black arrowheads), the cuvette was rapidly flushed with 50 mL of calcium-containing buffer. The reversibly bound protein was allowed to desorb, and when the desorption leveled off, EDTA was added (3.2 mM, white arrowheads) so that the remaining protein could be desorbed from the lipid layer. When desorption was complete, the calcium level was restored to 3 mM to allow the readsorption of the dissociated protein. About 4000 s later (at ~10000 s), the cuvette was rapidly flushed with 50 mL of calcium-containing buffer (black arrowheads).

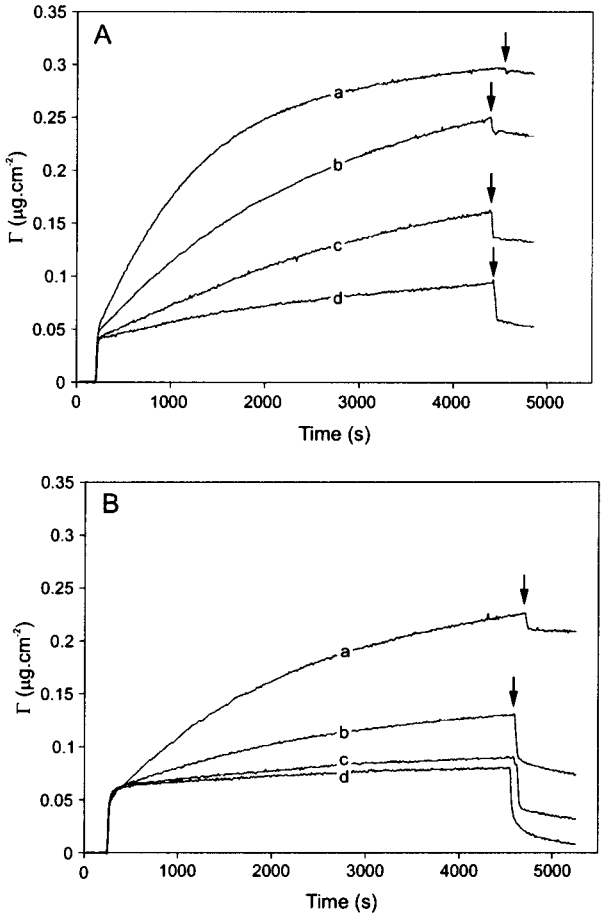

FIGURE 5: Binding of protein S to a lipid bilayer after preadsorption to small unilamellar phospholipid vesicles. Protein S (500 nM) was preincubated for 45 min at room temperature with DOPS/DOPC vesicles at various concentrations (20 mol % DOPS) in binding buffer [50 mM Tris, 100 mM NaCl, and 3 mM $CaCl_2$ (pH 7.5)] containing 0.5 mg/mL BSA with incidental shaking. The preincubation mixture (0.5 mL) was added to 4.5 mL of binding buffer in the ellipsometer cuvette, yielding final concentrations of 50 nM protein S (protein S_A in panel A and protein S_B in panel B) and 0, 62.5, 125, or 250 nM phospholipid vesicles (curves a−d, respectively, in both panels). The adsorption to the planar DOPS/DOPC (20 mol % DOPS) layer was quantified using ellipsometry. At ~4500 s (marked with arrows), the cuvette was flushed with 50 mL of calcium-containing buffer.

after recalcification of the medium, approximately 50% of maximal protein S readsorbed indicates that the high-affinity form of protein S did bind to the phospholipid bilayer with a K_d of <1 nM.

At 300 nM protein S, the adsorption and readsorption characteristics were similar for both preparations (Figure 4B), indicating that in this case both forms of protein S were present in excess of the number of binding sites on the phospholipid bilayer. This observation suggests a quantitative, but not qualitative, difference between the two protein S forms present in the protein S_A and protein S_B preparations.

Preincubation of Protein S Preparations with Small Unilamellar Vesicles. Since a small fraction of total protein S binds with such a high affinity to the phospholipid bilayers that it can only be released by addition of EDTA, it was reasoned that a protein S preparation could be depleted from this high-affinity fraction upon preincubation with phospholipid vesicles. To verify this, we preincubated protein S_A and protein S_B with different concentrations of small unilamellar DOPS/DOPC (20/80) vesicles in the presence of 3 mM $CaCl_2$. The protein S/vesicle mixtures were subsequently transferred to the ellipsometer cuvette, yielding final concentrations of 50 nM protein S and 0, 62.5, 125, or 250 nM phospholipid vesicles (Figure 5). For both preparations, the fast initial phase remained constant, but with increasing amounts of phospholipid vesicles present in the preincubation mixture, the amount of protein S that adsorbed to the phospholipid bilayer during the second, slow phase was reduced for more than 90% after preincubation of protein S with 250 nM phospholipid vesicles. This suggests that the protein S form that is responsible for the secondary, slow binding was bound to the phospholipid vesicles during the preincubation and was not available anymore for binding to the planar phospholipid layer in the ellipsometer.

At saturation of protein S adsorption to the phospholipid bilayer (t = 4500 s), bound protein was allowed to desorb by rapidly washing the cuvette with 50 mL of $CaCl_2$-

containing buffer (Figure 5A,B). Almost no protein S desorbed from the planar bilayer in the control experiment in which the preincubation mixture did not contain phospholipids (Figure 5, curves a). However, with increasing amounts of phospholipid vesicles present in the preincubation mixture, increasing amounts of protein S desorbed after flushing the cuvette (Figure 5). This indicates that incubation of the protein S preparation with DOPS/DOPC vesicles resulted in depletion of the protein S preparations from high-binding affinity protein S.

Figure 5 also shows that, compared to protein S_B, more phospholipid vesicles were needed to deplete protein S_A from the high-affinity protein S fraction, which confirms the presence of larger amounts of high-affinity protein S in protein S_A.

Separation of Protein S Forms by Liposome Sedimentation. The experiments presented above suggest that phospholipid vesicles can be used to separate and subsequently isolate the low- and high-affinity binding forms of protein S. Since protein S_A contains more high-affinity protein S than protein S_B, an attempt to separate the two forms of protein S was only performed with protein S_A.

To separate the protein S forms, 200 μM centrifugable phospholipid liposomes were incubated with 900 nM protein S_A in a $CaCl_2$-containing buffer. After centrifugation of the suspension, the majority of the protein S (840 nM) remained in the supernatant as measured by an ELISA, and this fraction was designated protein S-*supernatant*. The phospholipid-bound protein S was isolated by resuspending the liposome pellet in an EDTA-containing buffer. The liposomes were removed by centrifugation, and the protein S present in the supernatant was designated protein S-*pellet.* In addition, an experiment was performed in which protein S was subjected to the same sample handling in the absence of liposomes. This preparation was called protein S-*total*. ELISA measurements showed that the protein S-*pellet* concentration was 26 nM, which constituted ~3% of protein S-*total.*

Figure 6A shows the time courses of protein S-*total*, protein S-*supernatant*, and protein S-*pellet* binding to phospholipid. Protein S-*total* showed the characteristic biphasic adsorption to the lipid bilayer (Figure 6A, curve a). At ~4500 s, the cuvette was flushed with 50 mL of calcium-containing buffer, resulting in a minor desorption of protein S. The protein S-*supernatant*, however, only exhibited the first, rapid phase of protein S adsorption (Figure 6A, curve b). No secondary slow adsorption was observed, indicating that the high-binding affinity form was absent. This was confirmed by the fact that during washing at ~4500 s, all the bound protein desorbed, which indicates reversible and low-affinity binding of protein S. The lipid binding pattern of protein S-*pellet* exhibited only the second, slow phase of protein S adsorption (Figure 6A, curve c), and no protein S desorbed when the cuvette was flushed with calcium-containing buffer at ~4500 s.

When the protein S fractions were tested for their ability to inhibit prothrombin activation, the major part of the APC-independent activity of protein S was shown to be associated with the protein S-*pellet* fraction (Figure 6B). It was observed that protein S-*pellet* is 100-fold more effective in inhibiting prothrombin activation than protein S-*supernatant* under the experimental conditions that were used (Figure 6B).

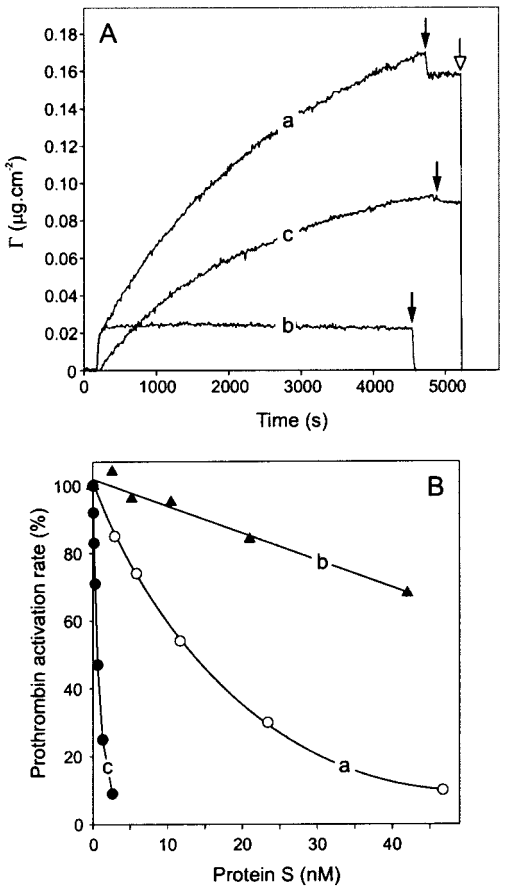

FIGURE 6: Separation of protein S forms by liposome centrifugation. Liposomes were used to separate different protein S forms present in the protein S_A preparation as described in Experimental Procedures. At the end of the procedure, protein S-*total*, protein S-*supernatant*, and protein S-*pellet* fractions were obtained. (A) Kinetics of binding of protein S fractions to DOPS/DOPC planar bilayers. At 200 s, 100 μL of protein S-*total* (a), protein S-*supernatant* (b), or protein S-*pellet* (c) was added to the ellipsometry cuvette, and adsorption to the DOPS/DOPC bilayer (20 mol % DOPS) was followed over time. The unbound protein was depleted from the cuvette at ~4500 s by washing with 50 mL of $CaCl_2$-containing buffer (black arrowheads). Adsorbed protein was released from the phospholipid bilayer by addition of 3.2 mM EDTA (white arrowhead). (B) APC-independent inhibition of prothrombin activation by protein S-*total* (○), protein S-*supernatant* (▲), and protein S-*pellet* (●) assessed as described in Experimental Procedures.

Difference in the Mobility of Protein S Preparations on PAGE Analysis. Although protein S_A and protein S_B preparations exhibited different APC-independent anticoagulant activities and phospholipid binding properties, they could not be distinguished by SDS−PAGE analysis (Figure 1). However, using native PAGE in the absence of SDS, a difference between the preparations was observed. Both protein S preparations contained more than one form of protein S, which migrated with different rates during native PAGE. To confirm that all protein bands represented protein S, the separated proteins were transferred to PVDF membranes for Western blotting with polyclonal antibodies against protein S. As can be observed, different bands migrating with different rates on native PAGE stained positive for protein S (Figure 7A). The form of protein S with high mobility appears to be the most abundant, whereas the form of protein S with low mobility is present at a low concentration. Interestingly, the amount of low-mobility

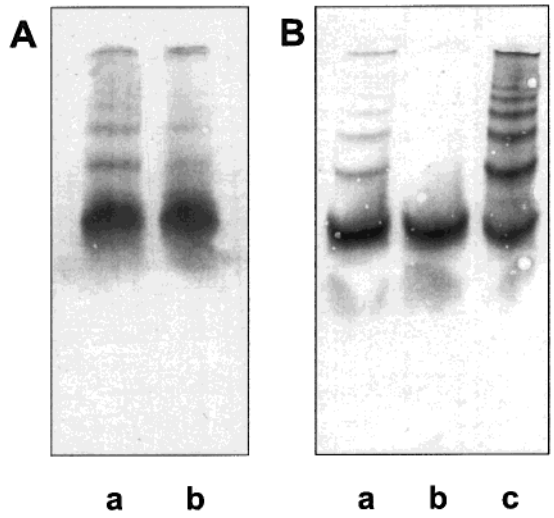

FIGURE 7: Analysis of protein S by native PAGE and Western blotting. (A) Native PAGE immunoblot analysis of protein S_A and protein S_B. Seventy-five nanograms of protein S_A (lane a) or protein S_B (lane b) was subjected to native PAGE (4 to 15%). After being transferred to a PVDF membrane, protein S was detected with peroxidase-conjugated polyclonal antibodies against protein S. (B) Native PAGE immunoblot analysis of protein S-*total*, protein S-*supernatant*, and protein S-*pellet*. Seventy-five nanograms of protein S-*total* (a), protein S-*supernatant* (b), or protein S-*pellet* (c) was subjected to native PAGE (4 to 15%), transferred to a PVDF membrane, and detected with peroxidase-labeled polyclonal antibodies against protein S.

protein S is greater in protein S_A than in protein S_B (Figure 7A).

Protein S-*total*, protein S-*supernatant*, and protein S-*pellet* were also subjected to native PAGE and Western blotting (Figure 7B). Protein S-*total* showed the characteristic pattern with the different migrating forms. Protein S-*supernatant*, however, contained only the form with the highest mobility, which was abundantly present in protein S-*total*. Strikingly, protein S-*pellet* predominantly contained the slower-migrating bands.

To rule out the possibility of cross-staining of the polyclonal anti-protein S antibodies with other proteins, we used a monoclonal antibody against the Ca^{2+}-bound conformation of the Gla module of protein S (CLB-13) to detect purified protein S on Western blotting. The same multimeric patterns of protein S were observed on Western blotting when CLB13 was used in the presence of $CaCl_2$ (data not shown). Furthermore, in an ELISA in which monoclonal anti-protein S antibody CLB-13 was used as the capture antibody and peroxidase-labeled polyclonal antibodies against protein S were used for detection, purified multimers of protein S (protein S-*pellet*) were detected in the presence but not in the absence of $CaCl_2$ (data not shown).

It is also possible that other protein components are incorporated in the protein S aggregates. However, on SDS−PAGE, only one band was visible on gel (Figure 1). This rules out a major second component to the multimeric structures unless the second component has the same mass as protein S. Specifically, prothrombin had to be excluded as a possible component of the multimers because (1) it is frequently observed as a contaminant of protein S preparations and (2) prothrombin has a molecular weight comparable to that of protein S on SDS−PAGE. The protein S preparations were tested for the presence of prothrombin with the

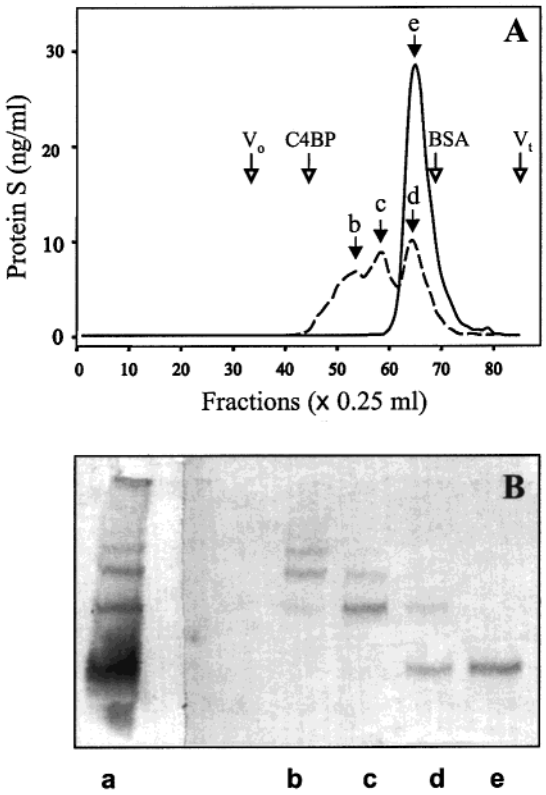

FIGURE 8: Size-exclusion chromatography of protein S. Seventy-five nanograms of protein S-*supernatant* and protein S-*pellet* were injected separately on a Superose 6 column. (A) Elution profiles of protein S-*supernatant* (—) and protein S-*pellet* (− − −) in the column effluent as detected by an ELISA. White arrowheads indicate the void volume of the Superose column (V_o), the elution positions of C4b-binding protein (C4BP) and BSA, and the total volume of the Superose column (V_t). (B) Native PAGE immunoblot analysis of the column fractions (lanes b−e) indicated by black arrowheads in panel A. Samples were subjected to native PAGE (4 to 15%), transferred to PVDF membranes, and detected with peroxidase-conjugated polyclonal antibodies against protein S using DAB (lane a) or TMB (lane b−e) as a substrate.

prothrombin activator from *Echis carinatus* venom. No measurable levels of thrombin in the preparations could be detected which indicates that prothrombin is absent. In addition, protein S-*pellet* was subjected to native PAGE and Western blotting, and developed using peroxidase-labeled anti-prothrombin antibodies and DAB. No signal was obtained for the purified protein S sample, indicating that prothrombin is not part of the multimeric structures of protein S. Furthermore, in a control sample of purified prothrombin, no multimeric forms of prothrombin were present, indicating that multimerization is specific for protein S (data not shown).

Characterization of Protein S-Pellet and Protein S-Supernatant. The ladder pattern of protein S that is visible on native PAGE Western blots (Figure 7) might be caused by the existence of either multimers or charge variants of protein S. To discriminate between these two possibilities, both protein S-*supernatant* and protein S-*pellet* were subjected to size-exclusion chromatography on a Superose 6 column (Figure 8A). The protein S-*supernatant* eluted as a symmetrical peak, suggesting a homogeneous protein S fraction. In the case of protein S-*pellet*, however, several smaller peaks eluted from the column: one at the same position of the protein S-*supernatant* peak and the others at

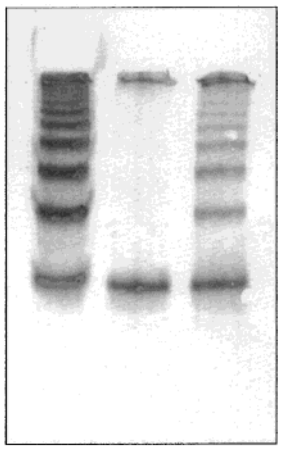

FIGURE 9: Isolation of protein S multimers. Normal human plasma and normal human plasma supplemented with 0.25 nM purified protein S multimers were recirculated through phospholipid-coated glass capillaries (20/80 DOPS/DOPC) in the presence of $CaCl_2$ as described in Experimental Procedures. Phospholipid-bound protein S was eluted with EDTA buffer and subjected to native PAGE and Western blot analysis using peroxidase-conjugated polyclonal anti-protein S antibodies: lane a, purified protein S multimers; lane b, protein S from normal human plasma; and lane c, protein S from normal human plasma supplemented with purified protein S multimers.

earlier elution times. This indicates the presence of multimers of protein S in the protein S-*pellet* fraction. Native PAGE immunoblot analysis of the peak fractions confirmed that the protein S fractions that eluted earlier from the Superose 6 column represented protein S forms with low electrophoretic mobility (Figure 8B).

Prolonged incubation at 4 °C for 2 weeks or repetitive freezing and thawing of protein S-*supernatant* or protein S-*pellet* did not result in generation or disappearance of the protein S multimers (data not shown). Dissociation of purified protein S multimers (protein S-*pellet*) could only be achieved by incubation with the anionic detergent SDS, resulting in a single band on a SDS−PAGE Western blot (data not shown), in accordance with the results of SDS−PAGE of protein S-*total* (Figure 1).

Protein S Multimers in Normal Human Plasma. To investigate whether protein S multimers are present in normal human plasma, we recirculated defibrinated normal plasma (3 mL) through a thin glass capillary coated with DOPS/DOPC vesicles in the presence of 3 mM $CaCl_2$. After 75 min, the capillary was washed with TBS containing 3 mM $CaCl_2$ and 5 mg/mL BSA to remove unbound protein from the capillaries, and the proteins that had bound Ca^{2+}-dependently to the phospholipid bilayer in the capillary were eluted with TBS containing 4 mM EDTA and 5 mg/mL BSA. The feasibility of this technique in detecting multimeric forms of protein S in plasma was verified in a similar experiment with defibrinated normal plasma to which protein S multimers were added to a final concentration of 0.25 nM (<1% of total protein S). Western blotting after native PAGE (Figure 9) shows the characteristic pattern of multimeric protein S in a purified sample of protein S-*pellet* (lane a) and in normal human plasma that was supplemented with protein S-*pellet* (lane c). However, protein S multimers were undetectable in normal plasma (Figure 9, lane b). The protein band on top of the blot (Figure 9, lane b) likely represents the noncovalent complex between protein S and C4b-binding protein that is abundantly present in plasma (*23*). When the purified protein S−C4b-binding protein complex was subjected to native PAGE and Western blotting, an identical band on top of the blot was observed (data not shown).

DISCUSSION

In this study, we have demonstrated that purified protein S preparations contain two forms of protein S that can be separated and that have different functional and biochemical properties. Characterization of the phospholipid binding properties of protein S and its ability to inhibit prothrombin activation revealed the presence of multimeric protein S that binds with high affinity to phospholipid bilayers (K_d < 1 nM). This multimeric form of protein S, which constitutes a small fraction (<5%) of the total protein S, has a 100-fold higher APC-independent anticoagulant activity than the residual protein S form (>95%) that binds with low affinity to phospholipids (K_d = 250 nM).

Comparison of two different protein S preparations, which exhibited a 4-fold difference in APC-independent anticoagulant activities, showed that the preparation with the lower inhibitory activity contained substantially less of the multimeric form of protein S. This indicates that differences in APC-independent anticoagulant activities between protein S preparations reported in the literature (*5*−*11*) are likely caused by the presence of different amounts of multimeric protein S.

The kinetics of protein S binding to phospholipid bilayers show typical biphasic adsorption behavior. During the first rapid phase of adsorption, protein S molecules with a low affinity for lipids that are abundantly (∼95%) present in protein S preparations reversibly bind to the phospholipid surface. During the second slow phase of adsorption, these protein S molecules are subsequently replaced by the high-binding affinity form of protein S. This phenomenon of sequential binding on a phospholipid surface in which a relatively abundant ligand with low affinity is replaced in time by a much less abundant protein with higher affinity has been observed before in protein mixtures (*24*−*26*).

We succeeded in separating the low- and high-binding affinity forms of protein S. Gel electrophoresis under native conditions (i.e., in the absence of SDS) indicated that the form of protein S that binds with a low affinity to phospholipids migrates as a single band, whereas the high-binding affinity form contains multimeric protein S, as revealed by size-exclusion chromatography and native PAGE.

Multimerization of protein S can well explain the high affinity for phospholipids provided that it does not result in shielding of the phospholipid binding site in the Gla module of protein S. The availability of the Gla modules in protein S multimers was confirmed with a monoclonal antibody directed against the Ca^{2+}-bound conformation of the Gla module of protein S (CLB-13). This antibody detected purified protein S multimers in the presence, but not in the absence, of $CaCl_2$, indicating that in protein S multimers the Gla modules are functionally exposed and accessible. If bundling of Gla modules offers a rationale for the observed high phospholipid binding affinity of protein S multimers, it is likely that protein S multimerization proceeds in an

ordered fashion and that Gla modules in protein S multimers are not randomly oriented. We were not able to detect multimeric protein S in normal human plasma. It is therefore unlikely that multimeric protein S contributes to the APC-independent anticoagulant activity of protein S that is observed in plasma (*6*, *9*, *27*). However, it is important to realize that when studying APC-independent activities of purified protein S, the presence of multimeric protein S will significantly enhance the overall anticoagulant activity of the protein S preparation being investigated. Therefore, it is preferable to use the here described protein S-*supernatant* in studies that are aimed at unraveling the mechanisms underlying APC-independent activities of protein S.

Our study indicates that noncovalent association of protein S molecules, which changes its biochemical and functional properties, most likely occurred during the purification procedure. The suggestion that protein S multimerizes in the absence of calcium ions (*28*) might play a role in the observed phenomenon as EDTA is frequently used during purification procedures. However, complete reversal of multimerization in the presence of $CaCl_2$ is not observed in the study presented here.

Ongoing investigations are focused on how protein S multimers can be generated and on how to avoid protein S multimerization during protein purification. Identification of conditions that induce multimerization will benefit the unraveling of mechanisms by which protein S exerts its anticoagulant activities in the absence of activated protein C.

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