Binding of Calcium to Anticoagulant Protein S: Role of the Fourth EGF Module†

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ABSTRACT: Protein S is an anticoagulant protein containing a Gla (enclosing γ-carboxyglutamic acids) module, a TSR (thrombin sensitive region) module, four EGF (epidermal growth factor)-like modules, and a SHBG (sex hormone binding globulin)-like region. Protein S is a cofactor to activated protein C (APC) in the degradation of coagulation factors Va and VIIIa but also has APC-independent activities. The function of the fourth EGF module (EGF4) in protein S has so far not been clear. We have now investigated this module through studies of recombinant wild-type protein S and a naturally occurring mutant (Asn217Ser). The mutant has essentially normal APC anticoagulant activity and a previously reported secretion defect. In the wild-type protein, Asn217 is normally β-hydroxylated. The binding of calcium to wild-type protein S is characterized by four high-affinity binding sites with K_D values ranging from 10^{-7} to 10^{-9} M. Three of these binding sites are located in EGF modules. Using surface plasmon resonance, competition with a calcium chelator, and antibody-based methods, we found that one high-affinity binding site for calcium was lost in protein S Asn217Ser but that the mutation also affected the calcium-dependent conformation of EGF1. We conclude that binding of calcium to EGF4 of protein S, involving Asn217, is important for the maintenance of the structure of protein S. Also, the abolition of binding of calcium to EGF4, related to Asn217, impairs both the structure and function of EGF1.

Protein S is a vitamin K-dependent anticoagulant protein with a molecular mass of approximately 70 kDa. Its physiological importance is demonstrated by an increased risk of thrombosis in individuals who are heterozygous for protein S deficiency, a risk which is further enhanced if the deficiency occurs in combination with other prothrombotic genetic defects (*1*). Homozygosity for protein S deficiency is a serious condition associated with neonatal purpura fulminans. Protein S consists of an N-terminal vitamin K-dependent γ-carboxyglutamic acid (Gla)-containing module, a module sensitive to cleavage by thrombin and factor Xa (TSR), four epidermal growth factor (EGF)-like modules, and a sex hormone binding globulin (SHBG)-like region, which contains two laminin G-type repeats in the C-terminal part of the molecule.

One of the major functions of protein S is to enhance active protein C (APC)-dependent proteolytic inactivation of coagulation factors Va and VIIIa, which are cofactors in the prothrombinase and tenase complexes of the coagulation cascade. However, protein S also has APC-independent anticoagulant functions, probably through direct inhibition of both the prothrombinase and the tenase complexes, and it is also involved in apoptosis (*2*). Protein S reverses the protective effect that factor Xa exhibits in factor Va inactivation and competes for protein and/or phospholipid binding sites (*3*−*8*). Other proposed functions of protein S are the inhibition of the activation of thrombin-activatable fibrinolysis inhibitor and the acceleration of APC-mediated neutralization of PAI-1 (*9*, *10*).

The domains of protein S that have been implicated in the interaction with APC include the TSR, EGF1, and EGF2 modules (*2*). The Gla module contributes to this interaction by having a high affinity for phospholipid membranes. The SHBG region accommodates the binding site for the C4b-binding protein (C4BP), a regulatory protein in the complement system to which 70% of protein S in plasma is bound. When protein S is bound to C4BP, the function of protein S as a cofactor to APC in the degradation of factor Va is abolished (*11*). Measurements of free protein S levels, as opposed to total levels, have been shown to be superior for prediction of protein S deficiency (*12*). In patients with thrombosis, several mutations in protein S have been found that cause low levels of protein S in plasma, but only a few patients with qualitative defects have been described (*2*, *13*).

EGF modules have been found in many different proteins, such as blood clotting factors, complement proteins, and membrane proteins. In many of the blood clotting factors, a

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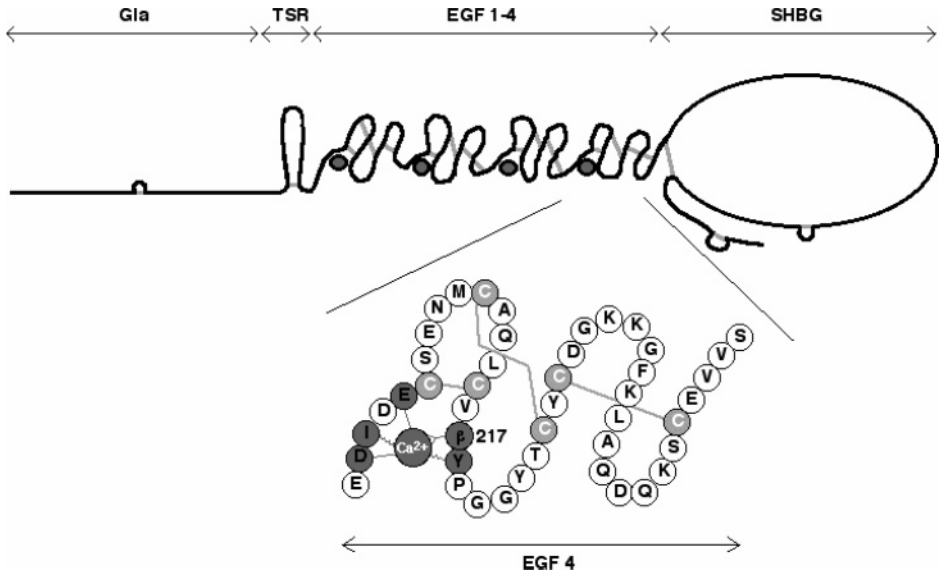


FIGURE 1: Schematic drawing of protein S. Gla, module with γ-carboxyglutamic acids; TSR, thrombin sensitive region; EGF, epidermal growth factor-like module; SHBG, sex hormone-binding globulin-like region. In the inset, EGF4 is shown enlarged. Residue 217, which is normally a β-hydroxylated Asn, was mutated to a Ser. The suggested location of the calcium ion is colored dark gray, and the disulfide bridges are colored light gray. The dark gray residues are those assumed to be responsible for calcium binding; straight lines represent side chain ligands, and zigzag lines represent backbone ligands.

set of EGF modules functions as a spacer between the Gla module and the serine protease region to position the active site at a distance from the phospholipid membrane that is commensurate with biological activity (*14*). EGF modules are also directly involved in protein−protein interactions, for example, between tissue factor and coagulation factor VIIa (*15*). In fibrillin, binding of calcium to multiple EGF modules has been shown to stabilize the structure of the protein. Without calcium, fibrillin is highly flexible, but with calcium bound, the protein adopts a rodlike conformation (*16*).

The functions of the third and fourth EGF modules in human protein S remain to be clarified, but very high affinity calcium binding sites have been identified (*17*, *18*). In this study, we have determined the calcium affinity for intact protein S, both the wild-type protein and a variant protein S, harboring a mutation in EGF4 (Asn217Ser; the Asn residue is normally β-hydroxylated) that has been found in patients with venous thrombosis (Figure 1) (*19*, *20*). We show that this mutation results in the loss of one calcium binding site and that it also affects the calcium-dependent conformation of EGF1.

MATERIALS AND METHODS

Proteins. Recombinant wild-type protein S and protein S with the mutation Asn217Ser were produced in HEK 293 cells as described previously (*20*). Site-directed mutagenesis was performed using the Transformer Site-Directed Mutagenesis Kit (Clontech). Vitamin K_1 (Konakion, Roche, U.K.) was added to the medium to allow γ-carboxylation of the proteins. Protein S purified from human plasma was purchased from Enzyme Research Laboratories (South Bend, IN).

SDS−PAGE and Immunoblotting. Protein samples were analyzed by SDS−PAGE, using 12% (w/v) gels stained with Coomassie Brilliant Blue R-250 (*21*). For immunoblotting, the proteins were transferred to an Immobillon membrane (Millipore, Bedford, MA), which was blocked with 5% dry milk dissolved in quenching buffer [0.05% Tween 20, 150 mM NaCl, and 10 mM Tris (pH 8.0)]. The membrane was subsequently incubated with 10 μg/mL polyclonal anti-human protein S (in house), then washed and incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG (Dako, Glostrup, Denmark), and thereafter washed again and developed with 0.15 mg/mL 5-bromo-4-chloro-3-indolyl phosphate and 0.3 mg/mL nitroblue tetrazolium.

Preparation of Calcium-Free Buffers. Water (18 Ω resistance) and buffers were rendered calcium-free by being passed over a column packed with Chelex 100 (Bio-Rad, Hercules, CA), and they were stored in plastic containers with dialysis bags containing Chelex 100. The pH meter was left for 30 min in 0.1 M EDTA (pH 8.0) and then washed with calcium-free water before use. The concentrations of calcium in both buffers and protein solutions were measured by atomic absorption. The cuvettes were made calcium-free by leaving them in 0.1 mM EDTA (pH 8.0) for 30 min and then washed with calcium-free water, after which they were kept in a 1:1:3 $HF/HNO_3/H_2O$ mixture for 3 min. Finally, they were washed again with calcium-free water.

Protein Purification. Recombinant protein S was purified by affinity chromatography on a HiTrap column (5 mL, Amersham Pharmacia Biotech, Buckinghamshire, U.K.) with immobilized HPS 21 (a calcium-dependent monoclonal antibody directed against the Gla module of human protein S) (*22*). Benzamidine (final concentration of 2 mM), $CaCl_2$ (2 mM), and Triton X-100 (0.1%) were added to the medium, after which the column was washed with 50 mM Tris, 150 mM NaCl, and 2 mM $CaCl_2$ (pH 7.5). To eliminate nonspecific binding, a solution containing 1 M NaCl, 50 mM Tris, and 2 mM $CaCl_2$ (pH 7.5) was applied to the column. The protein was eluted with 0.1 M glycine (pH 2.7), and pH was immediately adjusted with Tris and NaCl to give final concentrations of 50 mM Tris and 150 mM NaCl (pH 7.5). Thereafter, the protein was concentrated (Macrosep 10

K Omega, Pall Life Sciences) to approximately 1 g/L and dialyzed in calcium-free 2 mM Tris-HCl (pH 7.5). To further lower the concentration of calcium, 1 volume of protein was mixed with 1 volume of Chelex 100 and the mixture was gently rocked for 6 days at 8 °C and then centrifuged at 2000 rpm for 5 min to dispose of the Chelex 100.

Enzyme-Linked Immunosorbent Assay (ELISA). HPS 56 [10 μg/mL in 0.05 M sodium carbonate (pH 9.6)], a non-calcium-dependent monoclonal antibody directed against EGF3 in human protein S (*22*, *23*), was coated on 96-well microtiter plates (Costar, Corning, NY) overnight at 4 °C. The plates were washed three times with 50 mM Tris, 100 mM NaCl, 1 mM EDTA, and 0.1% Tween (pH 7.5) and then blocked with 1% ovalbumin (Sigma), 50 mM Tris, and 1 mM EDTA (pH 7.5) for 1 h at room temperature. After this, the plates were washed three times with wash buffer containing different concentrations of calcium (from 0 to 0.9 mM), and from this time forward in the assay, the same concentrations of calcium were used for each well. Calcium-free protein S (wild type or with the Asn217Ser mutant, 0.5 g/mL in the same buffers) was added to the plates, and they were incubated for 1 h at room temperature. Thereafter, the plates were washed four times with the same buffers, followed by the addition of horseradish peroxidase-conjugated HPS 54 (0.2 μg/mL), a calcium-dependent monoclonal antibody directed against EGF1 in human protein S (*22*). After incubation for 30 min at room temperature, the plates were washed four times with the different buffers. For development, 100 μL of TMB peroxidase substrate (Moss, Inc., Pasadena, MA) per well was added. The reaction was stopped by adding 150 μL of H_2SO_4/well, and the absorbance was measured at 450 nM. As a control experiment, HPS 61 (a monoclonal, non-calcium-dependent antibody directed against the SHBG region of human protein S; *22*) was used following biotinylation according to the manufacturer's instructions (Pierce, Rockford, IL) with detection using StreptABComplex HRP (Dako).

Factor Va Inactivation Assay. The assay for the concentration dependence of protein S was performed as described previously (*24*). To assess factor Va degradation by APC as a function of time (*25*), 0.16 nM APC was incubated with 75 μM phospholipid vesicles (60:20:20 PC/PE/PS mixture, Avanti Polar Lipids Inc.), 4 nM factor Va, and 600 nM protein S in 40 mM Tris-HCl, 140 mM NaCl, 3 mM $CaCl_2$, and 0.3% (w/v) BSA (0.04 nM APC, 19 μM phospholipids, 150 nM protein S, and 1 nM FVa, final concentrations). The mixture was incubated at 37 °C, and 2 μL aliquots were removed and added to a prothrombinase mixture, consisting of 75 μM phospholipids (60:20:20 PC/PE/PS mixture), 3 nM factor Xa, and 1.5 μM prothrombin (25 μM phospholipids, 1 nM factor Xa, and 0.5 μM prothrombin, final concentrations) at defined time points between 0 and 20 min. Each reaction was stopped after 3 min using 5 μL of ice-cold 0.5 M EDTA. The reaction mixture (100 μL) was then removed and incubated with 50 μL of chromogenic substrate S-2238 (Chromogenix) to assess thrombin generation. The coagulation factors used in these assays were purchased from Haematologic Technologies Inc. All experiments were performed in triplicate, and kinetic rate constants for cleavage of factor Va were determined by curve fitting (*25*).

Macroscopic Ca^{2+} Binding Constants and Number of Binding Sites. Calcium-free protein S was titrated with $CaCl_2$ (0.5 mM) in portions of 0.5 μL in the presence of Quin2 (tetrapotassium salt, Fluka Chemie, Buchs, Switzerland). The concentration of calcium was determined by atomic absorption spectroscopy. Titrations were performed at 25 °C in 2 mM Tris (pH 7.5), with or without 150 mM NaCl using a total volume of 70−90 μL, and all experiments were carried out in duplicate. The protein concentration (3−11 μM) was determined by amino acid analysis after acid hydrolysis. The concentration of chelator (15−30 μM) was quantitated in a calcium-saturated solution in the absence of protein by measuring the absorbance at 239 nM [$\epsilon_{239} = 4.2 \times 10^4$ L mol^{-1} cm^{-1} (*26*)]. The titrations were performed at 263 nM, and the absorbance was recorded on a Cary 400E spectrophotometer (Varian). The number of binding sites and macroscopic binding constants were obtained from a least-squares fit to the measured absorbance as a function of the total calcium concentration, using CaLigator (*27*). The K_D used for Quin2 was 5.2×10^{-9} M (low salt) and 1.2×10^{-7} M (150 mM NaCl) (*26*). The K_D of the chelator−calcium complex was a fixed parameter, as were the initial protein, chelator, and calcium concentrations. The program corrected for the effect of dilution when calcium was added.

Surface Plasmon Resonance Studies. The interaction between protein S and the HPS 54 antibody was studied by surface plasmon resonance technology, using the Biacore-3000 apparatus (Biacore AB, Neuchâtel, Switzerland) at 25 °C. The flow buffer was 10 mM Tris-HCl (pH 7.4) with 0.15 M NaCl, 0.005% Tween 20, and 0.02% NaN_3, with different free Ca^{2+} concentrations ranging from 32 nM to 10 mM. Ca^{2+} concentrations in the range of 32−430 nM were achieved by mixing calcium-free buffer with 1 mM EDTA and 0.1−0.95 mM $CaCl_2$ (total concentration), and the free Ca^{2+} concentration was quantified using Quin2 fluorescence. All buffers were filtered through sterile 0.22 μm filters before use and degassed for at least 30 min.

Immobilization of HPS 54 to the sensor chip was performed through amine coupling. HEPES-NaOH (10 mM) at pH 7.4, 0.15 M NaCl, 0.005% Tween 20, 0.02% NaN_3, and 3.4 mM EDTA were used as the flow buffer during coupling. Amine coupling was performed as described previously at a constant flow rate of 5 μL/min (*28*). Equal volumes of 0.1 M NHS and 0.4 M EDC were mixed, and 25 μL of the mixture was allowed to flow over the sensor chip surface to activate the carboxymethylated dextran (5 min). Three solutions (70 μL of 50, 60, or 75 μg/mL HPS 54 in sodium acetate buffer at pH 5.0) were then injected over three different lanes of the sensor chip. Typically, three levels of immobilization ranging from 4000 to 11 000 RU were achieved on each chip. For a second set of experiments, coupling levels between 800 and 4000 RU were achieved using lower concentrations of HPS 54 in the coupling step (70 μL of 20−50 μg/mL HPS 54 in sodium acetate buffer at pH 5.0). Injection of ethanolamine for 4 min deactivated excess reactive groups on the chip. One of the four flow cells on each sensor chip was reserved for a blank immobilization with no protein in the coupling step and was used as reference. The signal from the reference cell was subtracted from the signal in cells with HPS 54. Each chip with immobilized HPS 54 was used for 1 week.

The kinetics of the reconstitution reactions was studied at constant flow rates of 10−30 μL/min with no significant change in the rate constants that were obtained. Therefore,

a flow rate of 10 μL/min was used in subsequent experiments. The association of protein S (wild-type or mutant) with immobilized HPS 54 and the following dissociation were studied at several different Ca^{2+} concentrations ranging from 25 nM to 10 mM. Protein stock solutions were diluted using the flow buffer, and 300 μL was injected during the association phase, which was followed for 30 min. The dissociation process was followed for 30−45 h. After each experiment, the surfaces were regenerated by injecting 10 mM glycine-HCl (pH 2.9) for 5 min.

The data were evaluated using the Levenberg−Marqardt nonlinear least-square method. Data analysis was conducted using Kaleidagraph (Synergy Software, Reading, PA). The dissociation of the complex was modeled as a single-exponential decay, and attempts were made to fit the signal using a single-exponential decay plus a constant baseline

$$R(t) = Ce^{-k_{off}t} + R_0 \quad (1)$$

or a sum of two exponential decays plus a constant baseline

$$R(t) = C_1e^{-k_{off1}t} + C_2e^{-k_{off2}t} + R_0 \quad (2)$$

where k_{off}, k_{off1}, and k_{off2} are dissociation rate constants and C, C_1, and C_2 are the amplitudes of the corresponding processes.

Other Methods. N-Terminal sequences were determined on an Applied Biosystems (Foster City, CA) 494 Procise Sequencer. Protein concentrations were determined by amino acid analysis of acid hydrolysates as previously described (*11*). The amount of Gla was measured following alkaline hydrolysis as described previously (*29*).

RESULTS

Characterization of Recombinant Proteins. Wild-type protein S and protein S with the mutation Asn217Ser were produced in HEK293 cells. The affinity-purified proteins were more than 95% homogeneous as judged by N-terminal sequencing and SDS−PAGE, and there were no signs of internal cleavage. Amino acid analysis of alkaline hydrolysates established that the protein S preparations were almost fully carboxylated (10.1 mol of Gla/mol of protein S for the wild-type protein and 9.8 mol of Gla/mol of protein S for the Asn217Ser protein). Western blotting of the proteins was performed after extensive decalcification (Figure 2).

Factor Va Inactivation Assay. Preliminary results demonstrated high dilutions of wild-type protein S dialyzed into assay buffer from culture medium, and purified plasma-derived protein S exhibited the same APC cofactor activities in a factor Va inactivation assay. Different concentrations of recombinant protein S (1.25, 2.5, 5, 10, and 20 nM) were therefore employed to asses the ability of the mutated protein to function as a cofactor to APC. The Asn217Ser mutant exhibited a minimal reduction in cofactor function compared to wild-type protein S, but only at low protein S concentrations (Figure 3A). Dialyzed conditioned medium from nontransfected cells, diluted to the same extent as wild-type protein S, had no activity in the assay. Neither the wild type nor the mutated protein S (Asn217Ser) had any APC-independent effects in this assay. To confirm that the mutant had essentially normal activity, a higher (approximately plasma) concentration (150 nM) of protein S was used and

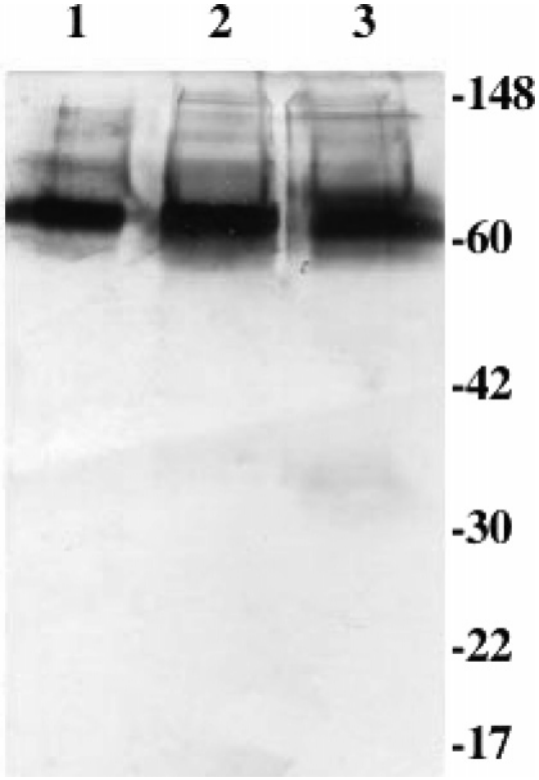
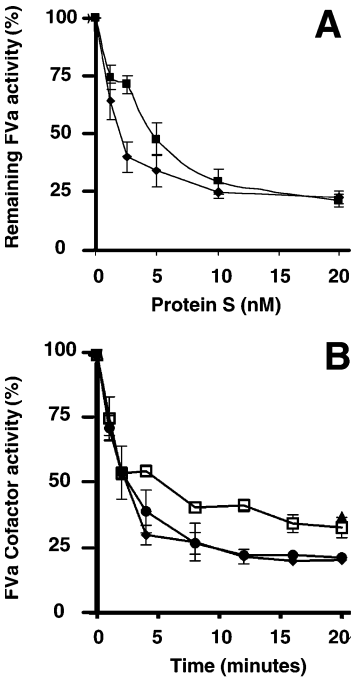


FIGURE 2: Immunoblotting of protein S. The recombinant proteins had been rocked for 6 days with Chelex 100 and thereafter centrifuged to dispose of the Chelex. Lane 1 contained purified protein S from human plasma, lane 2 wild-type recombinant protein S, and lane 3 recombinant protein S with the Asn217Ser mutation. Polyclonal anti-human protein S was used as the primary antibody and goat anti-rabbit protein IgG as the secondary antibody. The gel was performed under nonreducing conditions; there is no evidence of increased slow mobility bands, suggesting that decalcification does not cause appreciable aggregation of the protein S preparations.

the extent of factor Va degradation measured as a function of time. As can be seen in Figure 3B, the Asn217Ser mutant was as effective as wild-type protein S in enhancing factor Va inactivation, compared to the absence of protein S. The time course curves were fitted using an equation describing biphasic factor Va proteolysis. Identical kinetic constants were obtained for mutant and wild-type protein S (see the inset table in Figure 3B).

Affinity of Ca^{2+} Binding and Number of Binding Sites. To determine the number of calcium binding sites and their binding constants, the Chelex-treated protein was used for calcium titrations in the presence of the chelator Quin2. All protein concentrations were determined by acid hydrolysis. The buffers and solutions with Quin2 were found to contain between 0.25 and 0.4 μM calcium before addition of the protein. As a control, titrations were made with only Quin2 and the different buffers, and the data could easily be fitted to a straight line with the correct concentration of Quin2 in accordance with one strong binding site (not shown). After the protein had been introduced into the cuvette, approximately 1 mol of calcium was bound per mole of protein. The binding constants and stoichiometry were obtained from least-squares fitting to the data points using CaLigator (Figure 4). The macroscopic binding constants that were obtained are listed in Table 1, and they reveal four high-affinity calcium binding sites in wild-type protein S and three sites



	APC	APC + wtPS	APC + N217S
k_{506} (M^{-1} s^{-1})	2.9×10^8	2.8×10^8	2.8×10^8
k_{306} (M^{-1} s^{-1})	1.9×10^6	4.6×10^6	4.6×10^6

FIGURE 3: (A) Concentration dependence of protein S on the enhancement of APC-dependent factor Va inactivation. Protein S at different concentrations was incubated with 0.1 nM APC, 3 nM factor Va, and phospholipid vesicles (40:40:20 PC/PE/PS mixture, 25 μM) at 37 °C. The remaining factor Va activity was quantified in a prothrombinase assay using a chromogenic substrate. Results are expressed relative to the remaining activity, with no protein S present (100%), as a mean of three experiments ± the standard deviation: (◆) wild-type protein S and (■) protein S with the Asn217Ser mutation. (B) Time course of inactivation of factor Va by APC in the presence of protein S. Factor Va (1 nM) was incubated with APC (0.04 nM) and phospholipids (60:20:20 PC/PE/PS mixture, 25 μM) in the presence or absence of recombinant protein S (150 nM). At specified time points, an aliquot was removed and the factor Va cofactor activity determined using a prothrombinase assay [phospholipids (25 μM), FXa (1 nM), and prothrombin (1.5 μM)], as described in Materials and Methods. APC in the absence of protein S (□), APC and wild-type protein S (◆), and APC with protein S variant N217S (●) were assessed, and the kinetic rate constants for factor Va proteolysis were determined (see the inset table).

in protein S Asn217Ser. The values represent the average of two determinations. To evaluate the accuracy of the stoichiometry, the program was forced to use one fewer or more high-affinity sites, compared to the best fit. This resulted in an increase in the error square sum of a factor of ≥5−10. Hence, it was not possible to fit the data to a model with more or fewer than four sites for the wild type or three sites for the mutant. In the evaluation of the accuracy of the fits, a change in log K_A for an individual site of 0.5 resulted in an increase in the error square sum of a factor of at least 5−10. When the individual macroscopic binding constants K_1−K_3 for the wild-type protein were compared with the values for the mutated protein, the changes were not significant, while for K_4, representing the fourth calcium ion bound, the change was clearly significant. Also, when the product $K_1K_2K_3$ was considered, the change was significant. Therefore, it can be concluded that the Asn217Ser mutation results in loss of one binding site for calcium and also that

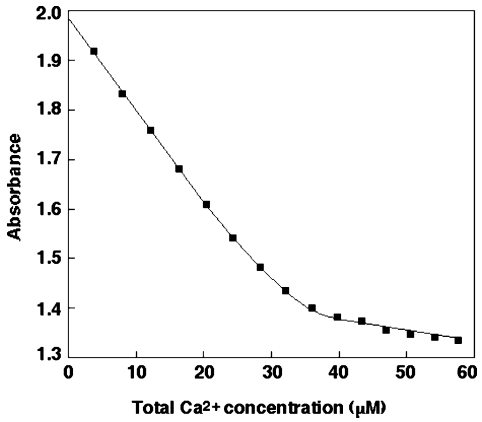


FIGURE 4: Ca^{2+} titration of Quin2 in the presence of protein S. The experiment that is shown was performed with the mutated protein S Asn217Ser (3.2 μM) in 2 mM Tris (pH 7.5). The absorbance at 263 nM is plotted vs the total concentration of calcium. Squares are experimental points, and the line represents the curve obtained by least-squares fitting to the data.

it results in a slightly reduced affinity of calcium in one or more of the other three sites. We cannot conclude, however, from these measurements whether the effect is located to one site or if it is due to smaller changes in all the other three sites.

Enzyme-Linked Immunosorbent Assay (ELISA). To further investigate the effect of the Asn217Ser mutation on the other EGF modules, especially EGF1, an ELISA was performed (Figure 5). Protein S was bound to the microtiter plate through the calcium-independent antibody HPS 56, directed against EGF3. A calcium titration was accomplished using the strongly calcium dependent antibody HPS 54, directed against EGF1, as a reporter. There was a clear difference between the wild-type and mutated proteins, indicating that the Asn217Ser mutation in EGF4 affects binding of calcium to EGF1. As a control experiment, calcium-independent antibody HPS 61, directed against the SHBG region, was used instead of HPS 54. With this antibody, there was no difference in absorbance for the wild-type protein when different calcium concentrations were used (not shown), indicating that the studied effect of calcium is dependent on HPS 54.

Surface Plasmon Resonance Studies of Binding of Protein S to Monoclonal Antibody HPS 54. The interaction between wild-type protein S and HPS 54 occurs with extremely high affinity in the presence of Ca^{2+}, and the dissociation is so slow that a large fraction of protein S remains bound to HPS 54 even after dissociation for 30−60 h (Figure 6). Both the association and dissociation processes are clearly Ca^{2+}-dependent, although the affinity is still very high at 32 nM Ca^{2+}. The dissociation data can in most cases be fitted only if two exponential decays are included (eq 2), representing one very slow process and one extremely slow process. The rate of the extremely slow process is so slow that we can only say that k_{off2} is less than 10^{-6} s^{-1}. The rate of the slow process decreases with an increase in Ca^{2+} concentration. The extremely slow process represents ∼50% of the bound wild-type protein at low nanomolar concentrations of free Ca^{2+}, and its fraction increases as the Ca^{2+} concentration is increased. In contrast, the Asn217Ser mutant displays Ca^{2+}-independent kinetics for dissociation from HPS 54. At all

Table 1: K_D (Molar) for Binding of Ca^{2+} to Protein S[a]

	K_1	K_2	K_3	K_4
wild type, low salt	$(2.5 \pm 0.5) \times 10^{-9}$	$(3.9 \pm 2.1) \times 10^{-8}$	$(3.1 \pm 0.4) \times 10^{-8}$	$(2.0 \pm 1.2) \times 10^{-7}$
wild type, 150 mM NaCl	6.3×10^{-9}	$(4.0 \pm 0.7) \times 10^{-7}$	$(5.0 \pm 0.6) \times 10^{-7}$	$(8.0 \pm 0.5) \times 10^{-7}$
Asn217Ser, low salt	$(1.2 \pm 0.2) \times 10^{-8}$	$(6.3 \pm 0.5) \times 10^{-8}$	$(3.2 \pm 2.2) \times 10^{-7}$	ND[b]
Asn217Ser, 150 mM NaCl	1.0×10^{-8}	$(5.0 \pm 1.2) \times 10^{-7}$	$(8.0 \pm 0.9) \times 10^{-7}$	ND[b]

[a] K_D values were obtained from titration of Ca^{2+} with the chelator Quin2 in the presence or absence of 150 mM NaCl in 2 mM Tris (pH 7.5) ± one standard deviation. [b] Values were too low to be determined with the chelator Quin2.

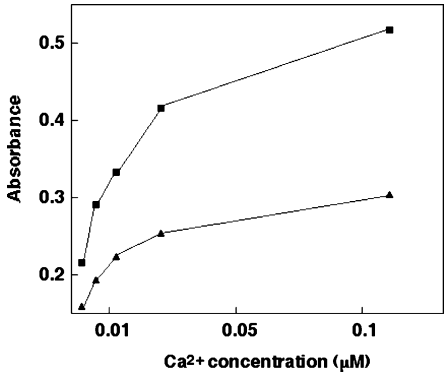


FIGURE 5: Enzyme-linked immunosorbent assay. Protein S was bound to the microtiter plate through non-calcium-dependent monoclonal antibody HPS 56. Peroxidase-conjugated HPS 54, a calcium-dependent monoclonal antibody directed against EGF1, was used as a marker. Squares depict data for wild-type protein S and triangles data for the Asn217Ser protein S mutant. All experiments were performed with 1 mM EDTA in the assay, and the shown concentrations of calcium are the concentrations calculated not to be bound to EDTA.

Ca^{2+} concentrations that were examined, the dissociation phase is dominated by an extremely slow process ($k_{off} < 10^{-6}$ s^{-1}). Dissociation data obtained using different levels of immobilized HPS 54 ranging from 800 to 11 000 RU display the same behavior with Ca^{2+}-dependent kinetics for the wild type and Ca^{2+}-independent kinetics for the mutant. The association phase has a considerably lower amplitude for the mutant than for the wild type for all Ca^{2+} concentrations that were examined.

DISCUSSION

In protein S, there are four EGF modules. So far, no structure of protein S has been determined. However, there is a recent structure of a fragment comprising the SHBG domain of growth arrest specific protein 6 (Gas6) that is homologous to protein S (*30*). The structures of other coagulation proteins with EGF modules, such as factors VIIa and IXa, have been determined as well as the structure of single or double EGF modules. The structure of a pair of EGF modules may be extended as in fibrillin or bent with the calcium binding area acting as a hinge, as in many of the coagulation proteins. It can also be U-shaped as in *Plasmodium falciparum* (*31*). A recent NMR and SAXS study of the four EGF modules from protein S found that there is exchange between two conformations corresponding to one straight and one bent arrangement (*32*). The structures of the individual EGF modules are similar with a characteristic 1−3, 2−4, and 5−6 pairing of the six cysteines into three disulfide bonds. More than 25% of the EGF modules described in the literature bind one calcium ion. The calcium

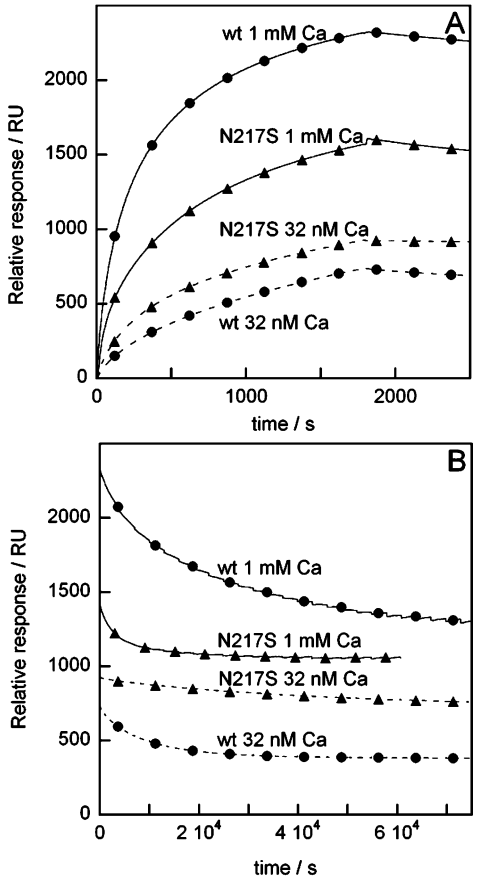


FIGURE 6: Binding of protein S to HPS 54. Surface plasmon resonance performed with monoclonal antibody HPS 54 coupled to the chip and human recombinant protein S (wild type or with the Asn217Ser mutation) in the flow buffer. Several different Ca^{2+} concentrations were used; the data shown here were recorded at 32 nM and 1 mM: (A) Association phase data and (B) dissociation phase data.

ion is bound in the N-terminal part of the module in a pentagonal bipyramidal geometry, coordinated by oxygen atoms that are supplied either by the backbone or by the side chains of the protein (*14*). The residues that can be assumed to be responsible for calcium binding in protein S EGF4 are Asp202, Ile203, Glu205, Asn217, and Tyr218 (Figure 1). Residue 217 normally carries a post-translationally modified asparagine (erythro-β-hydroxyasparagine). The function of this modification is not known, but in aspartyl β-hydroxylase knockout mice, there are developmental defects that have been assumed to be caused by defects in EGF-containing Notch gene family members (*33*). For coagulation factors IX and X, it has been shown that the β-hydroxylation does not affect binding of calcium, probably because the β-hydroxyl group points away from the calcium binding site (*34*).

In this paper, we wanted to clarify the function and importance of the fourth EGF module in protein S. Patients with the naturally occurring Asn217Ser mutation in EGF4 have thrombosis, indicating that residue 217 is important for maintaining the anticoagulant function of protein S. Since it has been suggested that residue 217 is a calcium-coordinating residue, we wanted to compare the calcium affinity of the mutated protein with that of wild-type protein S. For bovine protein S, four high-affinity sites have been identified (*35*). We have now shown that also human protein S has four high-affinity binding sites for calcium (Table 1). Stenberg et al. have shown (using a variety of fragments) that there are high-affinity sites in human EGF2−4, with the highest-affinity (nanomolar range) site in EGF4 (*17*, *23*). Since the affinity for calcium is so high, direct methods cannot be used, and we therefore employed chelator methods instead. In our studies, it was not possible to see a calcium binding site in EGF1, but the fragments that were used did not include the TSR module which might have affected the binding. Studies of the protein S−C4BP interaction using protein S−factor IX chimeras show that the calcium dependence of this interaction is not due to the EGF domains of protein S (*36*), suggesting that one of the high-affinity sites is present in the SHBG domain. Indeed, a calcium ion is observed in the crystal structure of SHBG (*37*), as well as in the SHBG domain of Gas6 (*30*).

Dahlbäck et al. (*22*) have produced a monoclonal antibody directed against human EGF1 (HPS 54), and this antibody requires <1 μM calcium for maximum binding. Addition of another module (Gla or EGF) N-terminal to an EGF module has been known to increase the affinity of the calcium site (*17*, *38*). The binding of calcium to the Gla module is probably not sufficiently strong to be seen in our calcium binding studies. This is in line with earlier investigations of other coagulation proteins, which showed calcium affinities for the Gla modules in the millimolar range, and it is also in line with studies of monoclonal antibody HPS 21 (directed against the Gla module in human protein S) which requires 1 mM calcium for maximal binding (*22*, *39*). In EGF1 in protein S, there is no complete consensus sequence for binding of calcium, but it has been shown in the EGF module of the LDL receptor that calcium can bind to an EGF module even though it contains only part of the consensus sequence (*40*). Therefore, of the four high-affinity binding sites for calcium in human protein S, three are most likely located in EGF2−4 and one is likely located in the SHBG domain.

We investigated the affinity of calcium for the mutated protein Asn217Ser and found that in the range accessible for the chelator that was used, one high-affinity binding site was lost. This shows that residue 217 is important for forming the calcium binding site. Moreover, the calcium affinity is affected also for the other sites. Whether this effect is located in only one other EGF module, or whether it is a smaller effect on several other sites, cannot be determined from these investigations. However, to confirm the existence of long-range intramolecular effects in protein S, we studied the mutated protein in a sandwich assay, including calcium-dependent monoclonal antibody HPS 54. There was a clear difference between wild-type protein S and the mutated protein, indicating that the mutation in EGF4 affects the calcium-induced response in the conformation of EGF1. More importantly, in the surface plasmon resonance studies, the mutated protein did not exhibit the pronounced calcium dependence in binding to HPS 54 that was found in the case of wild-type protein S. When the calcium binding site in EGF4 is destroyed, the structure might be more flexible, and this affects other parts of the protein, such as EGF1.

One of the major functions of protein S is to be a cofactor to APC in the degradation of factor Va. We have now shown that the calcium binding region in EGF4 is not critical for this interaction, as the Asn217Ser mutation had a minimal, if any, effect upon this function. Our finding of essentially normal function is compatible with prior studies that showed crucial roles for the TSR−EGF1−EGF2 region (*2*) and with a domain deletion/swap study in which complete removal of EGF4 resulted in a mutant that retained ∼30% activity (*41*). It is interesting to note that the level of cellular expression of the Asn217Ser mutant following transient transfection of COS-1 cells was reduced to ∼30% of normal and that pulse chase experiments following metabolic labeling suggested a decreased stability of the mutant within the cell (*20*). It may be that one of the main functions of the high-affinity calcium binding site in EGF4 is to keep the protein in the correct conformation for secretion. The abolition of this binding site will lead to intracellular degradation and a reduced level of secretion of protein S.

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