Self-Association of Human Protein S†

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ABSTRACT: Protein S functions as a cofactor with activated protein C in the down-regulation of the blood coagulation cascade. In vitro studies have historically produced conflicting data with regard to the extent of various protein S activity in clotting assays which typically involve adding $CaCl_2$ to initiate reactions. We report here that protein S reversibly self-associates in the absence of Ca^{2+}. Sedimentation experiments showed a transition in sedimentation velocity from 7.2 to 4.2 S with a transition midpoint (T_m) of 0.42 mM Ca^{2+} for intact protein S. Studies of thrombin cleaved (Arg^{70}) protein S revealed similar results with a transition in sedimentation velocity from 7.9 to 4.4 S with a T_m of 0.42 mM Ca^{2+}. This transition is reversible with the addition of 10 mM EDTA. Sedimentation equilibrium data suggest at a minimum, a monomer−dimer−trimer association. Sedimentation velocity experiments were also performed on mixtures of protein S and prothrombin which showed no heterodimer formation in either Ca^{2+} or EDTA solutions. These data suggest that previous interpretations of protein S structure and function may have been confounded by the self-associative behavior of protein S in non-Ca^{2+} solutions.

Protein S is a vitamin-K-dependent glycoprotein, M_r = 78 000 (*1*), present in human plasma (*2*). The physiologic concentration is approximately 330 nM, with nearly 60% bound to C4b-binding protein (C4BP), the putative plasma protein S carrier (*3*). Protein S is homologous to the vitamin-K-dependent family of zymogens but is not a serine protease precursor. It contains an amino terminal Gla domain, a thrombin-sensitive disulfide loop (Cys^{47} to Cys^{72}), four epidermal growth factor (EGF)-like domains, and a COOH-terminal sex hormone binding globulin-like domain (*4*). Protein S has three cleavage sites within the "thrombin-sensitive loop" at Arg^{49}, Arg^{60}, and Arg^{70}. Thrombin cleaves protein S at Arg^{49} and Arg^{70} while factor Xa cleaves at Arg^{60} (*1*, *5*) (Figure 1).

Vitamin-K-dependent proteins are characterized by their amino-terminal Gla domains. These γ-carboxyglutamic acid-rich domains contain 10−12 Gla residues which give these regions unique Ca^{2+} binding properties (*6*). Ca^{2+} binding plays an important role in stabilizing the three-dimensional structures of these proteins, and this structural rigidity is essential for membrane surface binding (*7*−*10*). Like protein S, many of these vitamin-K-dependent proteins contain EGF-like domains with one or more high affinity Ca^{2+} binding sites (*11*). Ca^{2+} binding has been reported to stabilize conformation in these regions, as well. The Ca^{2+}-dependent stability within the EGF-like region is essential for protein−protein interactions (*12*).

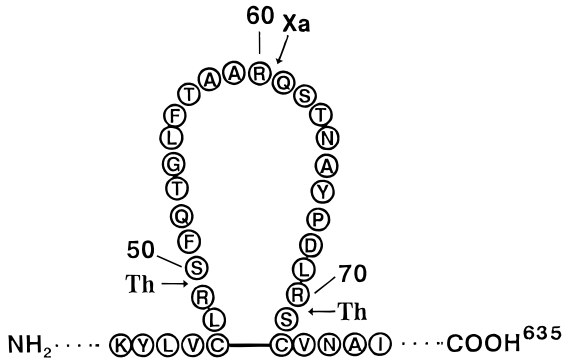

FIGURE 1: Human protein S cleavage sites within the thrombin sensitive disulfide loop. Cleavage sites are numbered on the basis of the mature human protein S (*4*). Thrombin cleavage sites (Th) and the factor Xa (Xa) cleave site are labeled with arrows.

There are multiple calcium binding sites in the Gla domain of protein S, and four additional high-affinity calcium binding sites are contained in the EGF repeats (*13*, *14*). An apparent Ca^{2+} effect on protein S conformation has been reported which was detected by alterations in the rate of reduction of disulfide bonds by thioredoxin in the presence of ethylenediaminetetraacetic acid (EDTA). In contrast, these bonds are relatively inaccessible in the presence of Ca^{2+} (*13*). The EGF-mediated Ca^{2+} binding of protein S plays an important role, presumably through enhanced structural rigidity of the EGF segments, in mediating the protein S/C4BP interaction. In the presence of saturating Ca^{2+} concentrations the two proteins have been reported to be tightly bound (K_d < 0.5 nM). Removal of the Ca^{2+} has been reported to result in a 100-fold increase in K_d for this complex (*15*). Evidence for a structural transformation after thrombin cleavage is displayed in reports of a requirement for higher Ca^{2+} concentrations to sustain normal levels of protein S phospholipid

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binding by the thrombin-cleaved protein S molecule (*16*). This phenomenon has been hypothesized to be due to reduced Ca^{2+} binding by the cleaved protein S (*13*). Cleavage by thrombin does not appear to significantly alter the binding of protein S and C4BP (*3, 17*).

Protein S has been reported to play multiple roles in the anticoagulant system, functioning both as a cofactor to activated protein C and a direct inhibitor of *prothrombinase* activity. The cleavage of factor Va by activated protein C is accelerated by the addition of protein S, but different groups have reported variable levels of "cofactor" effect with regard to the extent of activity protein S induces on the inactivation of Va. The magnitude of the cofactor role has been reported to range from 2- to 25-fold (*18*−*21*). A similar function by protein S in activated protein C inactivation of factor VIIIa has been reported to increase the rate of inactivation of this protein by as much as 9-fold (*22*−*23*). Thrombin-cleaved protein S has been reported to display a significant loss in activated protein C cofactor activity (*16*).

Protein S has also been shown to directly play a part in the anticoagulation system through inhibition of *prothrombinase* independent of activated protein C. It presumably accomplishes this by binding to and inhibiting the activities of both factors Va and Xa (*24*, *25*). Protein S also has been reported to directly inhibit *prothrombinase* complex assembly by competing for *prothrombinase* binding sites on membrane surfaces (*26*, *27*).

Patients with protein S deficiencies are subjected to perinatal life-threatening thrombotic episodes (*28*−*31*) suggesting that protein S plays a far greater role than marginally accelerating the activated protein C cleavage of factor Va, limited factor Va and Xa inhibition properties, and competition for *prothrombinase* sites. These anticoagulant activities, while reasonably well documented, do not appear to fully explain the severe pathology associated with protein S deficiency. The lack of any one of these activities by itself may account for a predisposition toward thrombosis, but the absence of limited contributions do not reconcile the observed pathology.

Many of the enzymatic reactions described above involve multiprotein complexes bound to a membrane surface. The kinetics of these reactions and the ensuing rearrangements are multifaceted, and accurate measurements are elusive as evidenced by the varied results in the literature. The complex nature of the measurements surrounding protein S and the role Ca^{2+} plays in conformation, C4BP binding, and function led us to investigate the influence of Ca^{2+} on the hydrodynamic properties, the solution molecular weight, and, ultimately, the association status of protein S.

Prothrombin self-association studies have produced variable results in Ca^{2+} and non-Ca^{2+} environments. The most complete report of self-associative behavior of prothrombin showed that there was no significant association in the presence or absence of Ca^{2+} (*32*). Protein S commonly contaminates prothrombin isolates. Further, the two proteins comigrate on SDS−polyacrylamide gels and exhibit similar sedimentation and light-scattering behavior. The potential contribution of protein S contamination to the prothrombin observation led us to analyze the potential of a prothrombin−protein S interaction.

EXPERIMENTAL PROCEDURES

Materials. Calcium chloride, sodium chloride, and ethylenediaminetetraacetic acid (EDTA) were purchased from J. T. Baker (Phillipsburg, NJ). (*N*-[2-Hydroxymethyl]piperazine-*N*′-[2-ethanesulfonic acid]) (HEPES) was purchased from Sigma (St. Louis, MO). FC-43 was purchased from Beckman Instruments (Fullerton, CA). Human plasma derived protein S was obtained as a gift from Dr. Richard J. Jenny at Haematologic Technologies (Essex Junction, VT). Routine preparations of protein S are ∼85% single chain as determined by densitometric analysis of a Coomassie stained SDS−polyacryimide gel. Thrombin-cleaved protein S was prepared by digestion of 200 μM protein S with 2 nM thrombin for 30 min at 37 °C.

Sedimentation Velocity Experiments. Sedimentation velocity experiments were performed using a Beckman Optima XL-I analytical ultracentrifuge. All velocity experiments were performed at 50 000 rpm in an An50-Ti, 8-hole rotor at 20 °C. Protein S was dialyzed twice for 4 h versus 4 L of 20 mM HEPES, 150 mM NaCl, pH 7.4 (HBS), in the presence of either 5 mM EDTA or 5 mM calcium chloride. The 400 μL samples (17 μM for the 85% single-chain protein S experiments; 13 μM for the fully cleaved protein S experiments) were analyzed in 12 mm double-sector cells with sapphire windows against a reference of dialysis buffer. Data were obtained by UV−visible absorbance scanning at 280 nm and Rayleigh interference optics at 90 s intervals. The data were analyzed with DC_DT (*33*) and plotted on Microsoft Excel.

Calcium Titration of Protein S. Protein S was dialyzed for 4 h in 2 L of HBS with 5 mM EDTA and then dialyzed two more times for 4 h in 4 L of HBS. The titration was started with 300 μL protein S at a concentration of 13 μM in HBS with no Ca^{2+}. Concentrations were evaluated by checking protein S absorbance at 280 nm using the reported extinction coefficient (280 nm, 1%) of 9.5 (*34*). An initial sedimentation velocity experiment was performed on the protein S in the absence of $CaCl_2$. After the run, the cell contents were mixed by gentle inversion and the housing plugs and plug gaskets were carefully removed. Additions of appropriate volumes of the $CaCl_2$ stock (10 mM) were supplemented with HBS to a final addition volume of 5 μL. This particular volume was chosen to ease sample handling and to ensure complete Ca^{2+} addition. The $CaCl_2$ solutions were added via microsyringe directly to both the sample and reference sectors of the centrifuge cell, and the housing plug gaskets were replaced and the plugs reset. In this fashion the Ca^{2+} concentrations were incrementally raised by 100 μM. The cells were gently inverted several times before each experiment. Titrations were concluded when a $CaCl_2$ concentration of 1.0 mM was reached. To ensure saturation was achieved, the Ca^{2+} concentration was raised to 1.5 mM and subsequently to 2.0 mM. Sedimentation velocity experiments were performed as previously described after each successive addition of $CaCl_2$. Upon completion of a titration, EDTA (0.5 M) was added to the protein S sample and reference to yield a final concentration of 10 mM. The sample was again analyzed at 50 000 rpm and a $g(s^*)$ plot was made to check for reversibility.

Sedimentation Equilibrium Experiments. Sedimentation equilibrium experiments were performed using the high-

speed meniscus depletion method (*35*) using the Beckman Optima XL-I analytical ultracentrifuge. Protein S samples were treated with 1 mM diisopropylfluorophosphate and prepared by two dialyses versus 4 L of either HBS with 5 mM EDTA or HBS with 5 mM $CaCl_2$. The dialyzed protein S samples were diluted to 1.3 μM, and 150 μL was loaded into each cell. The protein S samples were run in 12 mm double-sector cells with sapphire windows against a reference of the dialysate. To elevate the protein solution off the cell bottom, 20 μL of the immiscible fluorocarbon FC-43 was added to each protein S sample. All samples were run at 12 000, 17 000, and 22 000 rpm at 20 °C in an An50-Ti, 8-hole rotor. The cells were thoroughly mixed between rotor speed changes to evenly distribute the protein throughout the cell. Data were obtained by Rayleigh interference optics and absorbance scans taken at 280 nm. The equilibrium was checked by subtracting interference scans taken at 2 h intervals. When the subtraction of consecutive scans produced a flat residual, the protein S solution was determined to be at equilibrium. The raw concentration (c) versus radial position (r) equilibrium data were then transformed to plot of ln(c) versus radius (r) squared. From this plot the point average molecular weights were determined as a function of protein concentration within the cell using eq 1, where $\bar{\nu}$ is the calculated partial specific volume from amino acid sequence, ρ is the solution density, and ω is the rotor angle velocity (*36*).

$$M_r = [2RT/\omega^2(1 - \bar{\nu}\rho)](\mathrm{d}\ln C/\mathrm{d}r^2) \quad (1)$$

Dissociation constants were estimated from the data obtained from the protein S in EDTA spun at 22 000 rpm. Use of conservation of mass for protein S was applied given $[M_o] = [M] + 2[D] + 3[T]$, where $[M_o]$, [M], [D], and [T] are the concentrations within the cell of the monomer, the dimer, and the trimer, respectively (*37*). Combining this with the equilibrium expressions $K_{d1} = [M]^2/[D]$ and $K_{d2} = [M][D]/[T]$ gives a cubic polynomial through substitution. Use of a FORTRAN 77 code employing Newton's method ($\epsilon = 0.1$ pM) allowed for convergence.

Sedimentation of Protein S and Prothrombin. Protein S and prothrombin were dialyzed twice into 4 L of HBS with 5 mM EDTA or HBS with 5 mM $CaCl_2$. Sedimentation velocity experiments were performed on a 400 μL solution of protein S (15 μM) and prothrombin (15 μM) against a reference of the dialysis buffer as described above. The data were analyzed for solution oligomeric or monomeric status using the time-dependent $g(s^*)$ analysis algorithm contained in the software DC_DT.

Transfer for Amino Acid Sequencing. A poly(vinylidene difluoride) (PVDF) membrane was rinsed with methanol and immersed in a transfer buffer (25 mM tris, 0.192 M glycine, 0.1% SDS). The gel was placed on the PVDF membrane and covered with four sheets of 3 mm Whatman chromatography paper. The proteins were electroeluted from the gel using a Bio-Rad trans blot semidry transfer cell. The transfer took 150 min at a 250 mA constant current using an E-C Apparatus Corp. power supply. The PVDF membrane was subsequently stained with a 0.25% Coomassie blue, 45% methanol, and 9% acetic acid solution for 5 min. It was destained for 24 h with a 18% methanol and 9% acetic acid solution. The membrane was finally rinsed with distilled water and air-dried in a fume hood. The amino acid sequencing was performed by Dr. Alex Kurosky's laboratory (University of Texas, Medical Branch, Galveston, TX).

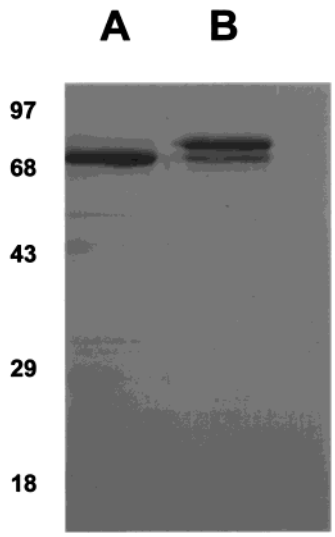

FIGURE 2: SDS−PAGE gel of purified human protein S and protein S cleaved at Arg^{70}. Lane A is human protein S that has been cleaved at Arg^{70}. Lane B is protein S isolated from human plasma. Roughly 85% of the sample is single chain.

RESULTS

Sedimentation Velocity Studies of Ca^{2+} Effects on Protein S. Sedimentation velocity experiments were initially performed on protein S (17 μM) (~85% single chain) (Figure 2) in HBS and 5 mM Ca^{2+} and protein S in HBS and 5 mM EDTA. A significant difference in sedimentation velocity was seen in the initial experiment with protein S in EDTA compared to the initial experiment with protein S in $CaCl_2$. The $S_{20,w}$ for protein S in the presence of Ca^{2+} was determined to be 4.2. In the presence of EDTA, $S_{20,w}$ was found to be 7.0 (Figure 3). At this concentration (17 μM) a 2:1 molecular weight ratio was estimated between the protein S/EDTA and protein S/Ca^{2+} using the relationship between sedimentation coefficient and molecular weight for nearly globular proteins expressed in eq 2.

$$(S_1/S_2)^{3/2} = M_1/M_2 \quad (2)$$

Experiments were performed with protein S (13 μM) that was cleaved in the thrombin-sensitive loop, since this cleavage has been reported to be significant for Ca^{2+}-dependent processes. The cleavage site (Figure 2) was confirmed to be the thrombin cleavage site at Arg^{70} by amino acid sequence analysis (data not shown). The cleaved species had a sedimentation coefficient of 7.9 in the presence of 5 mM EDTA and 4.4 in the presence of 5 mM $CaCl_2$.

Ca^{2+} Titration of Protein S. A titration was performed with protein S (13 μM), and the change in sedimentation behavior was observed with the incremental additions of Ca^{2+}. The resulting $g(s)_{max}$ versus Ca^{2+} concentration plot is presented in Figure 4 (closed circles). The transformation from the higher molecular weight species to lower molecular weight is observed through a shift of $S_{20,w}$ from the initial 7.2 to the final 4.2. The curve shows a transition midpoint (T_m) of 0.42 mM Ca^{2+}. EDTA (10 mM) was added after the transformation was complete, and a shift in $S_{20,w}$ to 6.8 was observed.

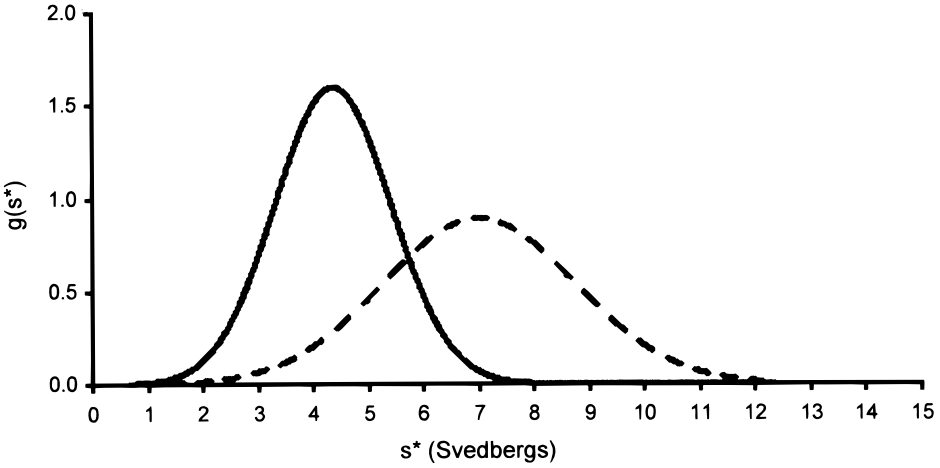

FIGURE 3: $g(s^*)$ plots of protein S (17 μM) in the presence of 5 mM Ca^{2+} (—) and 5 mM EDTA (- - -) in HBS. The 400 μL samples were centrifuged against a reference buffer at 50 000 rpm. Rayleigh interference data were collected. These $g(s^*)$ plots show the distribution of sedimentation velocity for the given sample.

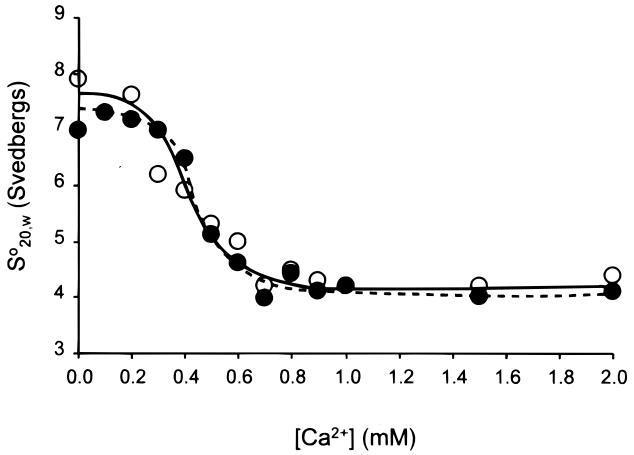

FIGURE 4: Calcium ion dependence of sedimentation coefficient for human protein S. Sedimentation coefficient is plotted against absolute calcium ion concentration. Shown is the sedimentation behavior of 85% intact protein S with the addition of Ca^{2+} (●- - -●), and the sedimentation behavior of fully cleaved (Arg^{70}) protein S with the addition of Ca^{2+} (○—○).

A similar titration performed with protein S cleaved at Arg^{70} (Figure 4, open circles) resulted in an initial sedimentation coefficient of 7.9 and final $S_{20,w}$ of 4.4. The T_m for the cleaved species was 0.42 mM Ca^{2+}. The reversibility was duplicated with the addition of 10 mM EDTA after the completion of the titration as seen by an upward shift in sedimentation velocity of 6.8.

Sedimentation Equilibrium Studies. Two protein S samples were dialyzed into HBS with 5 mM $CaCl_2$ or 5 mM EDTA, respectively. The samples were diluted to 1.3 μM and spun to equilibrium at 12 000, 17 000, and 22 000 rpm. Point weight average molecular weights were determined for each sample at each speed. The protein S in Ca^{2+} revealed a homogeneous solution with an apparent molecular weight of 75 800 ± 4200. Experiments performed with protein S in EDTA showed a heterogeneous solution of higher molecular weights. The molecular weights calculated from the protein S/EDTA equilibrium data collected revealed species as high as 185 000 (Figure 5).

A fit of the molecular weight versus concentration plot for the protein S in EDTA suggested dissociation constants of 17 and 0.1 μM for the monomer association to dimer and the dimer−monomer association to trimer, respectively. Since the biological relevance of the self-association process is most likely limited (see Discussion), a more extensive analysis was not merited.

Sedimentation Velocity Experiments with Protein S and Prothrombin. Sedimentation velocity experiments were performed on protein S (15 μM) in solution with equimolar

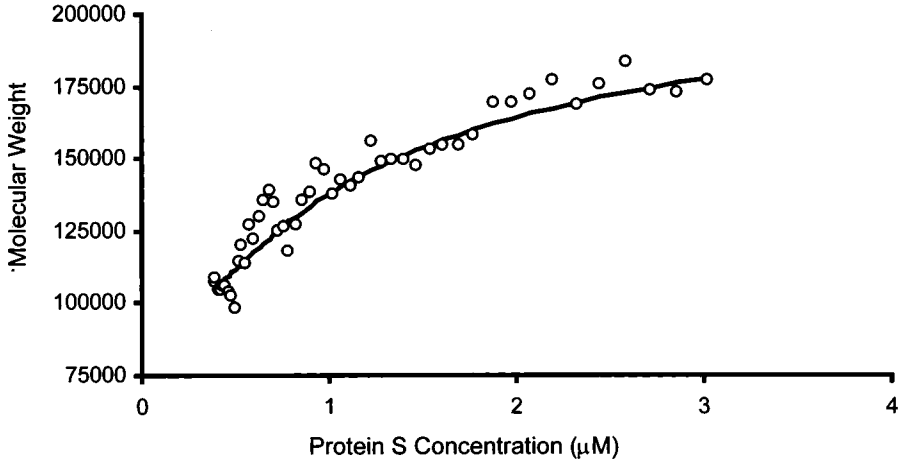

FIGURE 5: Apparent molecular weight of the protein S oligomer as determined by sedimentation equilibrium data in the presence of 5 mM EDTA. The apparent molecular weight was determined by the equation $M_r = [2RT/\omega^2(1 - \bar{\nu}\rho)](\mathrm{dln}\ C/\mathrm{d}r^2)$.

prothrombin in the presence of 5 mM EDTA and in the presence of 5 mM $CaCl_2$. The two species were indistinguishable in the presence of $CaCl_2$ with a wide $g(s^*)$ peak at 4.5. There were two peaks on the $g(s^*)$ plot of the two proteins in EDTA correlating with the protein S oligomer (6.7) and the prothrombin monomer (4.7).

DISCUSSION

These studies show that human protein S reversibly self-associates in Ca^{2+}-depleted solutions. A significant downward shift in sedimentation coefficient with the addition of Ca^{2+} to protein S solutions indicates that a Ca^{2+}-driven dissociation occurs. At 17 μM, the associated product is principally a dimer. The width of the $g(s^*)$ peak in Figure 3 indicates a level of heterogeneity within the protein sample in a Ca^{2+}-depleated solution. The broad Gaussian distribution is centered at 7.0 S, a value consistent with a species about twice the size of protein S. However, sedimentation equilibrium experiments suggest that higher order stoichiometries of association also occur.

Prothrombin fragment 1 has been shown to undergo a Ca^{2+}-dependent conformational change with a transition midpoint of 0.35 mM (*38*). Given the homology of the amino-terminal sequences of protein S and prothrombin (*39*), our transition midpoint of 0.42 mM suggests that the depolymerization of protein S may be driven by a Ca^{2+}-induced conformation change similar to that of prothrombin fragment 1. The biological concentrations of Ca^{2+} in the extracellular space are normally in excess of 1 mM. Therefore, it is unlikely that the self-association of protein S in the absence of Ca^{2+} has any biological significance, at least in extracellular space, where protein S is thought to carry out its functions. However, the observation of the polymerization of protein S in the absence of Ca^{2+} has profound effects on the interpretations of already published work dealing with protein S structure and function. In all of the studies thus far published, most protein S functions have been shown to be Ca^{2+} dependent, and it has been assumed that the only transition involved is the equilibrium among Ca^{2+}, protein S, and its partners. The additional equilibrium and/or kinetic processes associated with depolymerization upon transfer of protein S from a Ca^{2+}-free to a Ca^{2+}-containing environment are explicitly associated with the observed structural, functional, and binding interactions. For example, in many of the kinetic studies of protein S function as a cofactor, the assays conducted represent the time for clot formation following $CaCl_2$ addition to the reaction mixture. Superimposed on any activated protein C function in these assays would be the rate and extent of the Ca^{2+}-dependent depolymerization of protein S present in the assay mixture (*18*, *20*, *22*−*23*). This may contribute to the wide and varied influence protein S has been reported to make on activated protein C inactivation of factors Va and VIIIa (*18*−*23*). Protein S has been reported to speed the inactivation of factor Va by as much as 25-fold, but the most complete studies estimate the change in $\Delta k_{cat}/K_m$ at only 2-fold (*21*). Similarly, studies involving protein S binding to C4BP and the Ca^{2+} influence thereon will require attention to this Ca^{2+}-dependent process, which will implicitly alter any quantitative interpretations. Quantitation of protein S cofactor activity has been elusive, to say the least.

A study of the binding and inactivation of factor Xa by protein S indicated a Ca^{2+}-dependent nature to this interaction (*24*). The protein S concentration needed for 50% inhibition of factor Xa activity was 1.6-fold higher in the presence of EDTA than in the presence of divalent metal ions. Protein S−C4BP binding interactions have also been reported to be influenced significantly by the Ca^{2+} ion. This process has been studied by sedimentation, gel filtration, electrophoresis, and plate binding assays with somewhat variable results. Published gel filtration experiments show clear evidence of increased hydrodynamic volume in the absence of Ca^{2+} consistent with the self-association process demonstrated here.

Although it has been reported that cleavage within the thrombin-sensitive loop decreases the Ca^{2+} binding capacity for protein S (*16*), this cleavage does not affect the quality of self-association. There is no significant difference in sedimentation behavior observed throughout the Ca^{2+}-driven transformation from oligomeric protein S to monomeric protein S between the cleaved protein S and the intact protein S. This suggests that the conformational change induced by Ca^{2+} binding that is lost through cleavage in the thrombin sensitive loop does not play a role in the self-association of protein S.

We extended our studies to evaluation of potential heterodimer formation between protein S and prothrombin. While it is clear that prothrombin fragment 1 self-associates in the presence of Ca^{2+} (*38*), the most complete study revealed that prothrombin does not exhibit significant association in the presence or absence of Ca^{2+} thus creating the need for alternative explanations for the reports of self-association of this protein (*32*). Protein S is a common contaminant of prothrombin preparations and comigrates with prothrombin on detergent polyacryimide gels. Theoretically a prothrombin dimer, a protein S dimer, or the heterodimer would behave similarly in sedimentation experiments on the basis of the similarity of their molecular weights. Our data show that prothrombin and protein S do not associate with each other in either the presence of Ca^{2+} or EDTA. Our data do not provide an explanation for the self-association reports for some prothrombin samples, but they do rule out the possibility of a protein S−prothrombin dimer as an explanation.

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