Regulation of Vitamin K-dependent Protein S

INACTIVATION BY THROMBIN*

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Thrombin treatment of the vitamin K-dependent protein S resulted in the loss of the activated protein C cofactor activity associated with protein S. The addition of phospholipid vesicles inhibited the inactivation. Thrombin treatment did not alter the molecular weight of the native protein. However, upon reduction, a peptide of approximately 3000 daltons was released from the treated protein. The interaction between calcium and protein S was reduced by thrombin treatment. When the calcium interaction was determined by the quenching of the intrinsic fluorescence of protein S, thrombin treatment appeared to inhibit the interaction between calcium and the protein. When the calcium interaction was observed by measuring the effect on the electrophoretic mobility of the protein, thrombin treatment reduced the interaction between calcium and protein S. However, the effect of thrombin treatment on the interaction between calcium and protein S was less than observed by the fluorescent method. This observation suggests that fluorescence quenching may be a result of a structural change induced by calcium binding. Thrombin treatment of protein S appears to uncouple the calcium binding from the structural change. In addition, the interaction between protein S and phospholipid vesicles was reduced by thrombin treatment. These results suggest that the thrombin conversion of protein S into a two-chain protein causes the loss of a calcium-induced change in protein structure, loss of the lipid-binding properties, and the loss of cofactor activity.

Protein S is a vitamin K-dependent protein that is found in blood plasma (1, 2). Since it is a minor protein, relatively few studies have been carried out with respect to its structure and function. However, a number of properties of the protein observed in the past few years suggest that it may be a regulatory element with functions in both the coagulation and complement cascades. Dahlback has reported that human protein S forms a complex with the complement component C-4 binding protein (3–5). This observation, along with studies that demonstrated that protein S has an unusually high affinity for phospholipid vesicles (6), suggested that protein S might be involved in promoting the binding of C-4 binding protein to cell surfaces. Other studies have indicated that protein S is a cofactor for activated protein C-catalyzed inactivation of coagulation factor Va [7–9] and is required for the expression of the anticoagulant activity of activated protein C. Activated protein C is a serine protease derived from a vitamin K-dependent protein (10). Protein S appeared to enhance the binding of activated protein C to phospholipid vesicles (8) through the formation of a protein S-activated protein C-lipid complex that inactivates factor Va more rapidly than soluble activated protein C (8).

Dahlback and Stenflo (3) have reported that thrombin can cleave human protein S. Thrombin cleavage had no effect upon the native size of the protein but, following reduction, the result of an 8000-dalton fragment could be observed. This indicated that thrombin cleaved a region of the protein that was linked to the rest of the molecule by disulfide bonds (3). Dahlback also observed that thrombin treatment decreased the affinity between calcium ions and protein S.

In this paper the effects of treating bovine protein S with thrombin are reported. Thrombin was observed to inactivate the cofactor activity and decrease the interaction between protein S, calcium ions, and phospholipid vesicles.

EXPERIMENTAL PROCEDURES

Materials—Soybean trypsin inhibitor, QAE (quaternary aminoethyl)-Sephadex, phosphatidylyserine, phosphatidylcholine, blue dextran, and heparin were purchased from Sigma. Acrylamide was electrophoresis grade and purchased from Eastman. Dansyl amino acids were purchased from Pierce Chemical Co. Agarose-immobilized heparin and agarose-immobilized blue dextran were prepared by the cyanogen bromide method (11). Factor Va-deficient plasma was prepared from outdated human plasma by treatment with EDTA (12). All other reagents were of the highest grade commercially available.

Preparation of Proteins—Protein S, protein C, and factor V were prepared from bovine plasma as previously described (7). Protein C was activated with the factor X activator from Russell's viper venom (13) and subsequently purified by ion-exchange chromatography on QAE-Sephadex (13). Factor V was activated with thrombin and also purified by ion-exchange chromatography (14). Bovine thrombin was prepared by activating purified prothrombin with purified factor Xa, factor Va, phospholipids, and calcium as described by Owen and coworkers (15). Thrombin was separated from the activation components by chromatography on sulfopropyl-Sephadex (16). Purity of the various proteins was ascertained in at least two acrylamide gel electrophoresis systems.

Thrombin-treated protein S was prepared by incubation of protein S (4.6 μM) with thrombin (0.054 μM) for 2 h at 37 °C. Following incubation thrombin was isolated from protein S by chromatography on a Pharmacia Mono Q column using a 0.1 to 0.4 M NaCl gradient in 0.02 M Tris-HCl, pH 7.5, and 0.001 M benzamidine hydrochloride. The heavy chain of thrombin-treated protein S was isolated by treating the protein for 2 h with 35 mM dithiothreitol and then for 2 h with 80 mM iodoacetate. The sample was then gel filtered on a column (0.9 x 30 cm) of Sephadex G-50. The peak protein sample

1 The abbreviations used are: dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; SDS, sodium dodecyl sulfate.
was collected. When electrophoresed on SDS-polyacrylamide gels the protein ran as a single band. The apparent molecular weight of the band was not altered by reducing agents.

Carboxypeptidase-treated protein S was prepared by incubating protein S (1.0 ml, 8 μM) with a mixture of carboxypeptidase A (1 μM) and carboxypeptidase B (1 μM) for 2 h at 37°C. The reaction mixture was then chromatographed on a Pharmacia Mono Q column with a 0.1 to 0.4 M NaCl gradient (0.02 M Tris-HCl, pH 7.5, 1 mM benzamidine hydrochloride). The treated protein S eluted as a single peak at 0.31 M NaCl. When the material was analyzed on an SDS-polyacrylamide gel system a single band of protein was observed both in the presence and absence of reducing agents. The apparent molecular weight was 60,000.

Protein concentrations were monitored by measuring the absorbance at 280 nm. The molecular weights and extinction coefficients used for all protein components were as follows: factor Vα, 180,000 (14), E280 = 1.0; protein S, 64,000 (1); E280 = 10.0; activated protein C, 50,000, E280 = 13.7 (7); thrombin, 57,000, E280 = 21.4 (15).

Electrophoresis—Sodium dodecyl sulfate-gel electrophoresis was performed by the method of Laemmli (17). Gels were stained with Coomassie Brilliant Blue R. The effect of calcium ions on the electrophoretic mobility of protein S was measured by using 7.5% polyacrylamide gels prepared by the method of Davis (18) and stained with Coomassie Brilliant Blue G-250. Appropriate calcium ion concentrations were maintained by polymerizing the gels with calcium and then maintaining that calcium ion concentration in the running buffers. The Rf for protein S samples was determined by measuring the distance of migration of protein S divided by the distance of migration of the tracking dye.

End Group Analysis—Amino-terminal amino acids were determined by the method of Gray (19). In this method the protein was labeled with dansyl chloride for 2 h, separated from reagents by acetone precipitation, and hydrolyzed for 16 h at 110°C in 6 N HCl. Samples were then dried. Dansyl-labeled amino acids were extracted with ethyl acetate and run in the two-dimensional thin layer chromatography system described by Gray (19). Spots were compared with standard dansyl-amino acids.

Determination of Dissociation Constants for Calcium to Protein S—The dissociation constant for the interaction of calcium with protein S and thrombin-treated protein S was determined by the method described by Myrml and co-workers (20). In this method the total concentration of protein S, PS, and calcium, Ca2+, must be known. In order to calculate the dissociation constant one must measure the effect of calcium on the electrophoretic mobility of protein S. The ratio of liganded protein S to free protein S, R, is the observed mobility divided by the maximum mobility.

\[
\frac{1}{R} = \frac{K \times Ca^{2+}}{R} - \frac{1}{K} \times PS
\]

From this equation (20), a plot of 1/(1 - R) versus Ca2+/R gives a slope of Kd. Thus from this plot the association constant between calcium and protein S can be obtained.

Phospholipid Preparation—Phospholipids dissolved in chloroform were dried under nitrogen onto the wall of a glass tube. Vesicles were prepared by adding buffer to the tube and sonicating for approximately 10 min. The temperature of the tubes during sonication was kept below 37°C. Following sonication, the lipid preparation was centrifuged for 30 min at 20,000 × g to remove large particles and metal fragments generated during the sonication. Phospholipid concentrations were estimated by measuring the organic phosphorus by the method of Chen et al. (21) and by using a weight conversion factor of 25 (phospholipid/phosphorus). It was assumed that the mole fraction of phospholipids in the vesicles was the same as the mole fraction of phospholipids in the starting material.

Interaction between Protein S and Phospholipid Vesicles—The interaction between protein S and phospholipid vesicles was determined by the method described by Nelsestuen and Lim (22). Relative 90° light-scattering measurements were made in a Farand Mark I spectrophotometer at room temperature. All experiments were carried out in a 2.0-m1 final volume of 0.1 M NaCl, 0.02 M Tris-HCl, pH 7.5, 2 mM CaCl2, and 0.9 μg/ml of phospholipid. Protein samples were added to the cuvette, mixed, and relative scattering was measured. Scattering was compared to a solution that contained no protein but did contain phospholipid. Data were analyzed for bound and free protein as previously described.

Inactivation of Factor Va—Time courses of factor Va inactivation were carried out in a standard 0.200 ml reaction mixture at 37°C. The reaction mixtures contained activated protein C (0.5 μg), CaCl2 (0.005 M), phospholipid, protein S, and factor Va as indicated in the figure legends and brought to the final 0.2-m1 volume with buffer (0.1 M NaCl, 0.02 M Tris, pH 7.5, and 1 mg/ml of bovine serum albumin). Reactions were initiated by the addition of factor Va. Starting at 0.5 min and continuing until 5 min after the reaction was initiated, samples were removed and assayed for factor Va activity. For each course the apparent first-order rate constant (kapp) was calculated from the slope of a plot of the log factor Va activity versus time. Factor Va was assayed by a one-stage clotting assay as described previously (7).

Assay of Protein S—The blank included 0.1 ml of human plasma, 0.1 ml of rabbit brain thromboplastin, 0.1 ml of activated protein C (10 μg/ml in 0.1 M NaCl, 0.02 M Tris-HCl, pH 7.5, and 1 mg/ml of bovine serum albumin) and 0.1 ml of 0.025 M CaCl2 which initiated clotting. The addition of protein S lengthened the clotting time. Purified protein S was used to construct a standard curve and arbitrary units were assigned.

RESULTS

When thrombin was incubated with protein S, a time-dependent loss of activated protein C cofactor activity was observed (Fig. 1). The loss of activity did not appear to be due to the conversion of protein S into an inhibitor of factor Va inactivation. First, the rate of factor Va inactivation following complete inactivation of protein S was identical to the rate observed in the absence of protein S. Second, the addition of thrombin-inactivated protein S to a factor Va inactivation reaction, either in the presence or absence of native protein S, had no effect on the rate of factor Va inactivation (Fig. 2). This observation indicates that the thrombin-treated protein did not slow the rate of the reaction by forming an inactive complex with activated protein C. Rather it suggests that thrombin-treated protein S does not form a kinetically significant complex with the enzyme.
labeled and compared with native protein S by SDS-polyacrylamide gel electrophoresis. Protein S appears as a tightly spaced doublet in this electrophoretic system (Fig. 4). Though this has been observed by others (1) the reason for this heterogeneity is unknown. In the absence of reducing agents no change in the apparent molecular weight of protein S (64,000) was observed (Fig. 4). However, upon reduction, the molecular weight of the thrombin-treated protein was reduced to 61,000 (Fig. 4) suggesting that the 3000-dalton peptide residues in the carboxyl end of the protein. Since the dramatic effect of thrombin on the activity of protein S did not appear to be correlated with a major change in the protein, i.e. the size of the native protein remained unchanged by the cleavage, it seemed necessary to examine other physical parameters of the protein in order to provide an explanation for the loss of activity. One parameter that was examined was the quenching of the intrinsic fluorescence of protein S by calcium ions. When native protein S is treated with calcium ions, the intrinsic fluorescence is quenched (Fig. 5). When thrombin-treated protein S was titrated with calcium only a very limited amount of quenching could be observed. This may be due to a small contamination of the two proteins was similar. As the calcium concentration increased the mobility of native protein

![Figure 2](image2.png)

**Fig. 2.** Effects of thrombin-inactivated protein S on factor Va inactivation. Factor Va (0.15 unit) was treated with activated protein C (0.5 μg) and phospholipid (12.5 μg/ml), as described under “Experimental Procedures.” Thrombin-inactivated protein S was prepared by treating protein S (15 μM) for 2 h with thrombin (0.5 μM) in a volume of 0.1 ml. Factor Va inactivation time courses were carried out with no addition (○), thrombin-inactivated protein S (80 nM) (●), protein S (80 nM) (□) and protein S (80 nM) with thrombin-inactivated protein S (80 nM) (■).

![Figure 3](image3.png)

**Fig. 3.** Inhibition of protein S inactivation by phospholipids. Protein S (15.6 μM) was treated with thrombin (0.5 μM) in the presence of 0 μg (○), 2.5 μg (□), 10 μg (△), and 20 μg (●) of phospholipid vesicles (20% phosphatidylserine, 80% phosphatidylcholine) in a final volume of 0.200 ml. At the indicated times a sample was removed and assayed for protein S as described under “Experimental Procedures.”

**Fig. 4.** SDS-polyacrylamide gel electrophoresis of protein S and thrombin-treated protein S. Thrombin-treated protein S was prepared as described under “Experimental Procedures.” Protein S and thrombin-treated protein S were run in lanes 1 and 2, respectively. Lanes 3 and 4 are the same samples of protein S and thrombin-treated protein S which had been reduced with 2-mercaptoethanol and boiled for 1 min prior to electrophoresis.

portion of protein S that is located in the carboxyl-terminal end of the protein.

When protein S or thrombin-treated protein S were electrophoresed on polyacrylamide gels in the absence of calcium ions, the observed mobility or R f of each of the proteins was the same (Fig. 6). That is, a mixture of the two could not be resolved in the absence of calcium ions. The indicated that the total charge on the two proteins was similar. As the calcium concentration increased the mobility of native protein
Calcium-dependent quenching of the fluorescence of protein S and thrombin-inactivated protein S. The fluorescence of protein S (○) in the presence of the indicated calcium concentrations was determined in a Farand Mark I fluorometer with the excitation monochromator set at 295 nm and the emission monochromator set at 340 nm. Protein S samples were prepared in 0.1 M NaCl, 0.02 M Tris-HCl, pH 7.5. Thrombin-inactivated protein S was prepared by incubating protein S (15.6 μM) with thrombin (0.5 μM) for 2 h. Thrombin was then removed by chromatography on QAE-Sephadex and dialyzed into the same buffer used with the untreated protein S. No cofactor activity could be detected in the treated protein S sample. Protein S was then treated with the indicated calcium concentrations (△), and the fluorescence was determined.

Calcium effects on the electrophoretic mobility of protein S and thrombin-inactivated protein S. Protein S (15.6 μM) was treated with thrombin (0.5 μM) in a total volume of 0.200 ml for 2 h. No detectable cofactor activity was present by this time. Samples (0.040 ml) were electrophoresed on 7.5% acrylamide gels in the presence of the calcium concentrations indicated in the figures. After staining, the R\textsubscript{x} of thrombin-treated protein S (■) and untreated protein S (○) was determined by measuring the mobility of the protein and the tracking dye.

Determination of dissociation constant for calcium interaction with protein S and thrombin-inactivated protein S. The data in Fig. 6 were replotted by the method described under "Experimental Procedures." The concentrations of protein S used are indicated in the figure.

Discussions

Thrombin catalyzes a cleavage of a peptide bond in protein S which converts it from a single-chain protein to a two-chain disulfide-linked protein. Though this change in structure appears minor, it resulted in the complete loss of activated protein C cofactor activity. The ability of protein S to interact with activated protein C, either to stimulate factor Va activation or to form a nonproductive complex, appeared to be lost. In addition to the loss of functional activity, there was also a change in the interaction between protein S and calcium ions. Studies of fluorescence quenching and the effect of calcium on electrophoretic mobility indicated that thrombin treatment resulted in the loss of some of the calcium binding sites. Thrombin modification of protein S had a greater effect on the fluorescence quenching by calcium than on the calcium inhibition of electrophoretic mobility. This might indicate that the calcium-induced structural changes that result in tryptophan quenching might have become uncoupled by the thrombin cleavage. In addition to the modification of some of the calcium binding sites thrombin-modified protein S lost...
most of its phospholipid binding properties. Therefore, it is apparent that the modification in structure that is induced by thrombin cleavage alters calcium binding sites in such a way that the calcium interaction neither induces a fluorescence change nor allows the protein to interact with phospholipid vesicles, two properties that are closely associated with function.

Calcium interaction with the vitamin K-dependent proteins is required in order for these proteins to be able to interact with membranes or phospholipid vesicles. Calcium also quenches the intrinsic fluorescence of several of these proteins, including prothrombin (23, 24), factor X (24), and protein C (25). It is thought that the fluorescence quenching is due to a conformational change in the protein that is induced by calcium binding. This change in conformation, which is slow at 0 °C, appears to be required in order for the proteins to interact with phospholipid vesicles and for the expression of functional properties (24). The observations made with protein S appear to support the idea that the calcium-induced change in conformation that is monitored by fluorescence quenching is essential for function.

The thrombin-induced change in the bovine protein S-calcium interaction was also observed with human protein S. Dahlback observed that calcium did not alter the electrophoretic mobility of the human protein following thrombin treatment to the same degree observed with the native protein (26). The thrombin effect does not appear to have precedent among other vitamin K-dependent proteins. Thrombin cleaves a 14-amino acid peptide from the heavy chain of protein C, which results in its activation to a serine protease (27). This does not alter the calcium binding region of the protein, which is located on the light chain (27, 28). Thrombin cleavage of prothrombin reduces calcium binding of the zymogen by the removal of prothrombin fragment 1 (a 24,000-dalton peptide) from the amino-terminal portion of the protein. Fragment 1 contains all of the γ-carboxyglutamic acid residues found in prothrombin and most of the calcium binding sites (29). The resulting protein, prothrombin 1, has a low affinity for calcium. The finding that calcium binding is reduced without release of the peptide that contains the γ-carboxyglutamic acid residues seems to suggest that there is a requirement for the existence of a specific secondary structure for the complete calcium-protein S interaction. Thrombin treatment must cause a disruption of this structure which results in a loss of binding sites and the uncoupling of the conformational change associated with calcium quenching of tryptophan fluorescence.

The observation that thrombin inactivates protein S suggests that thrombin, the product of the coagulation cascade, is able to regulate its own formation through a complex series of reactions. Thrombin is able to enhance the rate of prothrombin activation through a number of mechanisms including the activation of coagulation factors V (14, 30–32) and VIII (33, 34). It is also able to inhibit prothrombin activation through the activation of protein C (35, 36) which can inactivate factors Va (13, 37, 38) and VIIa (33, 34, 38). The effect of thrombin on protein S is suggestive of another mechanism by which thrombin can affect the rate by which it is formed. Protein S inactivation would lead to enhanced thrombin formation since this effect would reduce the effectiveness of activated protein C as an inhibitor of coagulation. At present, however, the relative contribution of each of these reactions on the overall rate of thrombin formation is unclear, as there may be other factors that regulate thrombin activity that are yet to be elucidated.

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