

Cleavage of Protein S by a Platelet Membrane Protease

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Abstract

Protein S is a vitamin K-dependent glycoprotein cofactor to the serine protease, activated protein C. In this study we demonstrate that ^{125}I -protein S bound to unstimulated platelets in a time- and calcium-dependent saturable reaction. Half-maximal binding occurred at a protein S concentration of 10 nM, with $\sim 1,100$ binding sites per platelet. The binding of protein S to platelets was followed by rapid cleavage of the protein mediated by a protease confined to the platelet membrane.

The membrane protease was Ca^{++} -dependent, inhibited by high concentrations of diisopropyl fluorophosphate, but was resistant to a variety of other protease inhibitors. Functional studies demonstrated that the cleavage of protein S was associated with complete loss of cofactor anticoagulant activity.

We conclude that protein S binds to platelets and is inactivated by a novel Ca^{++} -dependent membrane protease. This may represent a physiological reaction that regulates the activity of protein S.

Introduction

Protein S is a vitamin K-dependent glycoprotein cofactor to the serine protease activated protein C (APC)¹ (1). The latter is an anticoagulant, which inactivates Factors Va and VIIIa in a Ca^{++} - and lipid-dependent reaction (2). Deficiency of either protein C or S has recently been reported to be associated with recurrent venous thrombosis in humans (3, 4). Current evidence indicates that Protein S forms a 1:1 complex with APC that results in the increased affinity of APC for the platelet or phospholipid surface (5, 6).

The regulation of protein S activity in plasma is thought to be by thrombin, which cleaves the protein near the amino terminal end of the molecule, with subsequent loss of cofactor activity (7–9). There is some evidence that this event is unlikely to be a major control mechanism for the anticoagulant activity of protein S, because the reaction is inhibited by physiological Ca^{++} concentrations, and by the endothelial surface protein thrombomodulin (unpublished observation). The role of platelets in the modulation of protein S activity has yet to be fully elucidated.

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1. Abbreviations used in this paper: APC, activated protein C; AT III, antithrombin III; NEM, *N*-ethyl maleimide; PAGE, polyacrylamide gel electrophoresis; PGE_1 , prostaglandin E_1 ; PMSF, phenyl methyl sulphonyl fluoride.

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In this study we examined the binding of protein S to platelets and its effect on the anticoagulant activity of the protein. Our results indicate that the binding of protein S to platelets is rapidly followed by its proteolysis and the loss of cofactor activity.

Methods

All chemicals, were purchased from Sigma Chemical Co. (St. Louis, MO) except for the following: NaCl, Na_2HPO_4 , $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, EDTA, and glycine (Ajax Chemical Co., Sydney, Australia); sodium dodecyl sulphate (Pierce Chemical Co., Rockford, IL); KH_2PO_4 and D-glucose (Mallinckrodt Inc., St. Louis, MO); *N*-ethyl-maleimide, Di-n-butyl-phthalate, and dionylphthalate (BDH Chemical Co., Poole, England); and Trisacryl GF 2000 (LKB Instruments Inc., Ville Neuve, France).

Coagulation factors were of human origin. Protein C (10), protein S (7), thrombin (11), antithrombin III (ATIII) (12), prothrombin, and Factor X (13) were purified and activated as previously described. All proteins were shown to be homogenous as judged by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE). Protein S was labeled with ^{125}I Na using the Iodogen method (Pierce Chemical Co.), to a specific activity of 2,000 cpm/ng. The labeled protein retained functional anticoagulant activity as measured in the Factor Xa recalcification time described below.

SDS PAGE was performed according to Laemmli (14), using a 5% stacking and a 10% running gel. Before application to the gel, the samples were reduced by boiling in the presence of 0.5% mercapto-ethanol.

Platelet preparation. All manipulations were performed at room temperature. The washing buffer used in this procedure was 4.3 mM K_2HPO_4 , 4.3 mM Na_2HPO_4 , and 24.3 mM $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ (pH 6.5) containing 0.113 M NaCl and 5.5 mM glucose. To minimize platelet activation the buffer was adjusted to contain 5 mM benzamidine, 0.2 $\mu\text{g}/\text{ml}$ prostaglandin E_1 (PGE_1), 5 mM 1,3 dimethylxanthine, and 0.625 mM acetylsalicylic acid. The platelets were used within 4 h of preparation.

Blood was collected from normal human donors in 10-ml plastic tubes containing, to a final concentration, 2 mM EDTA, and the previously described inhibitors. Platelet-rich plasma, was prepared by centrifugation at 150 *g* for 15 min and the platelets washed as described by Baenziger and Majerus (15). Gel-filtered platelets were prepared as described by Comp and Esmon (16), except that trisacryl GF 2000 was used as the separating matrix instead of Sepharose 2B. Platelets were counted using a hemocytometer, and the results of duplicate determinations averaged.

Preparation of platelet membranes. Two different methods were used for the preparation of platelet membranes. The first was a minor modification of the procedure reported by Glanzmann et al. (17). In this method washed platelets were disrupted by three cycles of freeze thawing and the suspension subjected to maximum intensity sonication (Labsonic 1510, ultrasonic cell disruptor; B. Braun, Melsungen A.G., West Germany) for 45 s at 4°C. The disrupted platelets were centrifuged at 19,000 *g* for 25 min to remove intact platelets, platelet fragments, and granules. The membrane-rich supernatant was then centrifuged at 100,000 *g* for 60 min. The supernatant from this step was used as a cytosolic component. The pelleted membranes were washed twice in 20 mM Tris (pH 7.4) containing 0.15 M NaCl and 5 mg/ml bovine serum albumin (BSA), and finally resuspended in 500 μl of the same buffer. In other experiments the disrupted platelets were fractionated on a sucrose density gradient as reported by Chesney et al. (18).

Preparation of protein S cleaved by platelet membranes. Human protein S at a final concentration of 10 μM was incubated for 2 h at 37°C with buffer control or platelet membranes derived from 2.5×10^9 platelets in a reaction volume of 300 μl . The buffer used was 20 mM Tris (pH

7.4) containing 2.5 mM Ca^{++} , 0.15 M NaCl, and 5 mg/ml BSA. At the end of the incubation, the membranes were separated by centrifugation and the supernatant containing protein S was removed. A sample was boiled in SDS reducing buffer, and electrophoresed to confirm complete cleavage of the protein S. The remainder was used in the functional assay described below.

Preparation of protein S cleaved by intact platelets. Protein S (750 nM) containing ^{125}I -protein S (1 nM), was incubated in the presence of 1×10^{10} platelets in a reaction volume of 550 μl . The buffer used was 20 mM Tris (pH 7.4) containing 0.15 M NaCl, 5 mg/ml BSA, 2.5 mM Ca^{++} , 0.2 $\mu\text{g}/\text{ml}$ PGE_1 , 0.625 mM acetylsalicylic acid, and 5 mM 1,3 dimethylxanthine. At the following times (0, 30, 60, 120, 360, and 960 min), a 75- μl sample was withdrawn and the platelets pelleted by centrifugation at 100,000 g for 10 min. 25 μl of the supernatant was assayed for anticoagulant activity as described below, and the remainder boiled in SDS reducing buffer and electrophoresed.

Functional protein S assay. The assay is a Factor Xa recalcification time that relies upon the ability of protein S to enhance the anticoagulant activity of APC. To 50 μl of $\text{Al}(\text{OH})_3$ -adsorbed plasma was added 25 μl of 80 mM CaCl_2 , 25 μl phospholipid (platelin, general diagnostic, reconstituted according to the manufacturer's instructions), 25 μl APC or buffer control and 25 μl buffer, protein S, or protein S preincubated with platelet membranes. Clotting was initiated by addition of 50 μl of 1 μM prothrombin and 50 μl of 2 nM Factor Xa. The time was recorded in triplicate using a fibrometer coagulation machine (BBL Microbiology Systems, Cockeysville, MD).

Measurement of binding of ^{125}I -protein S to platelets. Binding studies were performed in Eppendorf tubes using 5×10^7 washed platelets in a final volume of 200 μl . The buffer used was 20 mM Tris (pH 7.4) containing 0.15 M NaCl, 2.5 mM Ca^{++} , and 5 mg/ml BSA.

Platelets were incubated with ^{125}I -protein S (3–40 nM), in the presence of 0.2 $\mu\text{g}/\text{ml}$ PGE_1 , 0.625 mM acetylsalicylic acid, and 5 mM 1,3 dimethylxanthine. Binding studies were performed in the presence or absence of APC (25 nM) and Factor V_a (2.5 nM). After 20 min at 37°C the incubation mixture was layered onto 0.5 ml of oil (eight parts di-n-butylphthalate and two parts dinonylphthalate) in 1.5-ml Eppendorf tubes and centrifuged at 20,000 g for 2 min. The supernatant and the oil were then removed, taking care not to dislodge the pellet. The tip of the tube containing the pellet was cut and counted in 1250 Rack Gamma 11 counter (LKB Instruments Inc., Bromma, Sweden). Nonspecific binding was determined by adding 100 M excess of unlabeled protein S and

specific binding calculated by subtracting nonspecific counts from the total counts obtained. The nonspecific binding ranged between 45 and 65% of the total radioactivity bound to the platelet surface.

Results

Experiments were undertaken to determine whether protein S binds to unstimulated platelets. These studies were performed using gel-filtered platelets or platelets washed as described in Methods. In addition benzamidine, PGE_1 , acetylsalicylic acid, and 1,3 dimethylxanthine were used throughout the experiments to minimize platelet activation. On phase-contrast microscopy the platelets appeared discoid and unstimulated.

Fig. 1 represents a time course of specific binding of ^{125}I -protein S to unstimulated platelets. Binding was specific, time dependent, and equilibrium was reached after 15 min. No binding occurred in the absence of Ca^{++} .

Fig. 2 shows the specific binding of increasing concentrations of ^{125}I -protein S to 5×10^7 platelets, which represents the mean of five separate binding experiments. The binding was saturable. Double reciprocal plot analysis of the data revealed maximal binding of 90 fmol/ 5×10^7 platelets, which represents 1,100 binding sites per platelet, with an affinity constant of 10 nM. Similar results were obtained if the inhibitors PGE_1 , benzamidine, acetylsalicylic acid, and 1,3 dimethylxanthine were excluded from the incubation reaction, or if the platelets were pretreated with 10 nM thrombin for 1 min before the addition of ^{125}I -protein S.

The inclusion of Factor V_a (2.5 nM) and or APC (25 nM) did not alter the results of the binding studies.

After binding experiments were performed the platelet pellet containing the bound protein S and the supernatant (containing the unbound protein S), were resuspended and boiled in reducing buffer and run on SDS PAGE. The unbound protein S in the supernatant appeared as a doublet identical to the starting material, whereas protein S bound to the platelet surface demonstrated cleavage of the upper band to the lower molecular weight peptide. When electrophoresis was performed under nonreducing

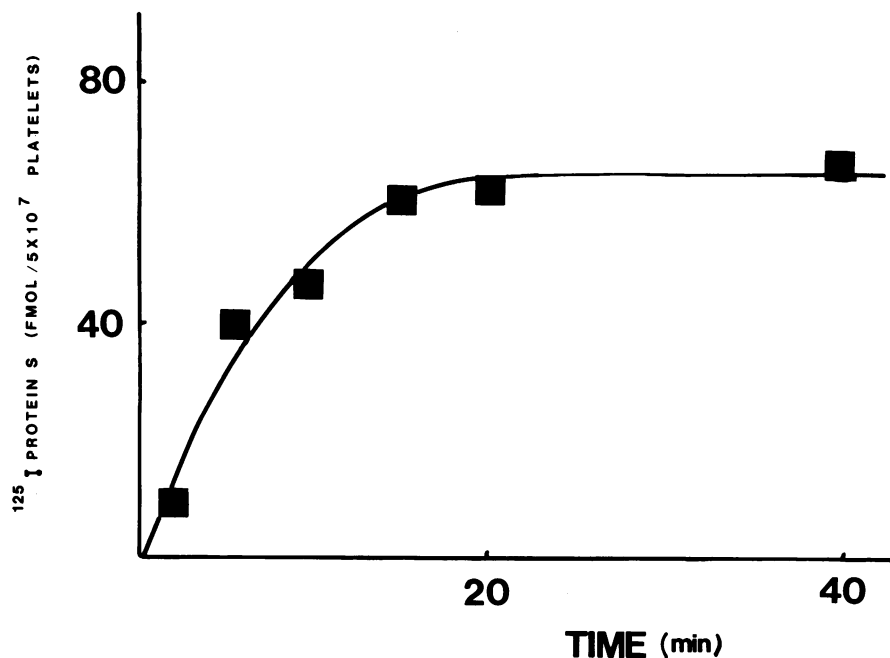


Figure 1. Time course of binding of ^{125}I -protein S to platelets. Binding studies were performed using 5×10^7 platelets and 20 nM ^{125}I -protein S. Samples were removed at the indicated times, and the amount of ^{125}I -protein S bound to the platelet surface determined as described in Methods.

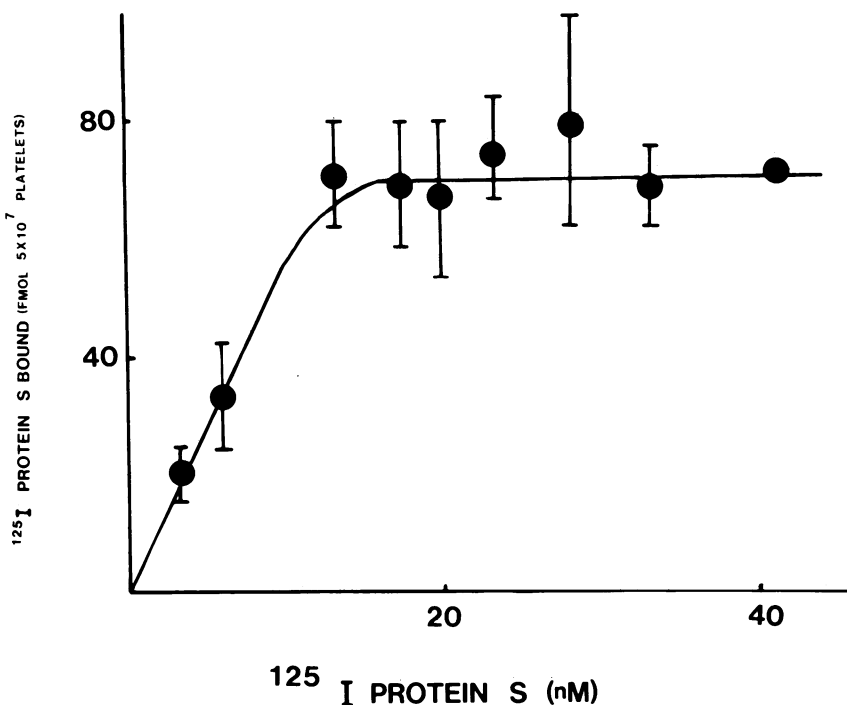


Figure 2. Binding isotherm of ¹²⁵I-protein S to platelets. ¹²⁵I-protein S (3–40 nM) was incubated with 5×10^7 platelets for 20 min at 37°C. At the end of this period the samples were processed as described in Methods. These results represent the mean values of five separate binding experiments \pm SD. At the highest concentration of protein S, insufficient data points were available for calculation of a meaningful standard deviation.

conditions protein S appeared as a single band with an estimated molecular mass of 72 kD, which was identical to the starting material. These observations suggest that the cleaved portion of protein S remains attached to the remainder of the molecule by disulphide bonds.

As Fig. 3 shows, 60% of the cleaved protein S bound to the platelet surface was displaced within 20 min of the addition of 100 M excess unlabeled native protein S.

To determine whether protein S was cleaved by a surface or an internalized protease, released during activation of the platelet, platelet membranes and cytosol were prepared as described in

Methods. The time course of incubating ¹²⁵I-protein S with washed platelet membranes (Fig. 4) demonstrated that complete cleavage of protein S occurred within 20 min. In contrast, no cleavage of ¹²⁵I-protein S was observed when the protein was incubated with the cytosolic platelet components despite prolonged incubation (up to 2 h). These studies were performed in the presence and absence of saturating levels of phospholipids (platelet), with identical results obtained. When platelets were subfractionated on a sucrose density gradient as described in Methods, the protease activity resided maximally in the membrane fraction. When ¹²⁵I-protein S (30 nM) was incubated for 2 h with alpha granules derived from 2×10^9 /ml platelets, no cleavage was observed. Under similar conditions, platelet membranes derived from the same number of platelets resulted in cleavage of protein S after 10 min which was complete by 20 min. A concentration ten times higher of alpha granules resulted in nearly complete cleavage of the same concentration of protein S within 2 h.

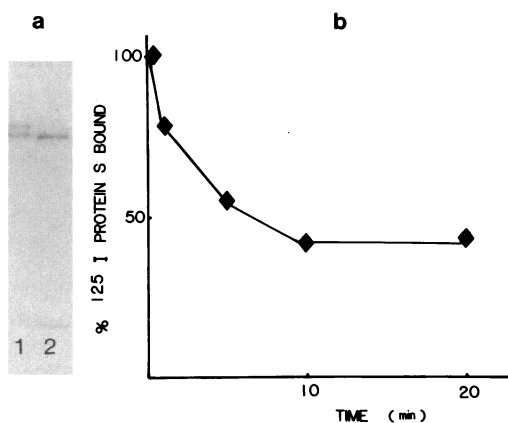


Figure 3. Displacement of cleaved ¹²⁵I-protein S from platelets. Binding studies were performed as detailed in Methods, using 5×10^7 platelets and 25 nM ¹²⁵I-protein S incubated for 20 min. Complete cleavage of protein S was documented by SDS PAGE (a). The displacement of bound cleaved protein S by 100-fold molar excess native protein S is shown in b. The results are presented as the percentage protein S that remains bound at different times after the addition of the excess protein S. (a) Lane 1, unbound protein S (supernatant); lane 2, bound protein S.

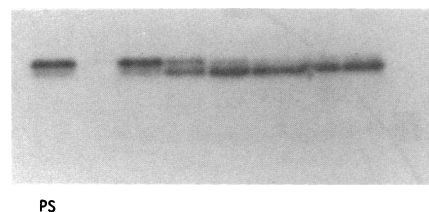


Figure 4. The time-course of cleavage of protein S by isolated platelet membranes. Reaction mixtures contained ¹²⁵I-protein S (45 nM) and platelet membranes derived from 3×10^7 platelets in a total volume of 30 μ l. The buffer used was 20 mM Tris (pH 7.4) containing 0.15 M NaCl, 5 mg/ml BSA, and 2.5 mM Ca⁺⁺. Samples were removed at 0, 20, 40, 80, and 120 min and electrophoresed as described in Methods. Lane PS, ¹²⁵I-protein S.

The incubation of ^{125}I -protein S with red cell membranes (prepared as for platelet membranes), resulted in no cleavage of protein S, suggesting that the observed cleavage was not secondary to a nonspecific membrane effect.

To rule out the possibility that the membrane protease represented a plasma protein adsorbed to the platelet surface, we studied the ability of recalcified plasma (in the presence and absence of saturating concentrations of phospholipid) to proteolyze protein S. After 2 h incubation no cleavage of protein S could be demonstrated.

We incubated platelet membranes with protein S in the presence of APC to investigate whether APC protected its cofactor from proteolysis. The results in the first half of Fig. 5 demonstrate that APC (20 nM) neither enhanced nor inhibited the reaction. APC (20 nM) in the absence of platelet membranes did not cleave protein S under the experimental conditions used.

Human protein S is very sensitive to proteolytic degradation by thrombin. Because the cleavage pattern of protein S produced by the platelet membrane protease resembles thrombin-induced cleavage as judged by SDS PAGE criteria, it was essential to exclude the presence of thrombin as a possible contaminating protease in our platelet membrane preparations. To this end we studied a time course of the cleavage of ^{125}I -protein S by the membrane protease, in the presence of 700 nM ATIII and 0.2 U/ml heparin. Cleavage of protein S again occurred within 20 min and was not impaired by the presence of the antithrombin heparin complex (second half of Fig. 5).

The nature of the platelet membrane protease was further investigated by the use of specific protease inhibitors. Fig. 6 represents the cumulative results of these experiments. The protease activity was inhibited by both EGTA and EDTA and by di-isopropyl fluorophosphate (50 mM), suggesting the protease is a calcium-dependent serine protease. Other agents, such as *N*-ethylmaleimide (NEM) (1 mM), leupeptin (1.5 mM), benzamidine (5 mM), phenyl methyl sulphonyl fluoride (PMSF) (100 nM), and the specific thrombin inhibitor, hirudin (1 U/ml), failed to inhibit the activity of the platelet membrane protease.

Activity of platelet membrane-cleaved protein S. This was assessed using a Factor Xa recalcification time of $\text{Al}(\text{OH})_3$ plasma, as described in Methods. The protein S content of the absorbed plasma was < 0.3 nM as determined by a radioimmunoassay for protein S. The mean control clotting time in the absence of APC or protein S was 40 ± 1 s. In the presence of 10

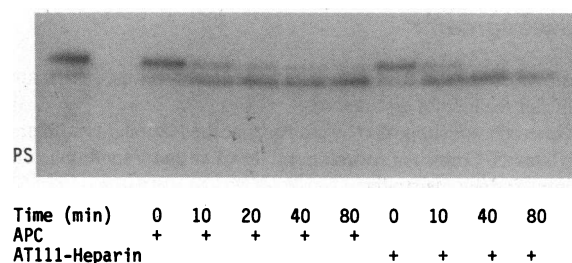


Figure 5. Effect of activated protein C and ATIII-heparin complex on the cleavage of protein S by the platelet membrane protease. ^{125}I -protein S (45 nM) was incubated with membranes derived from 3×10^7 platelets, in the presence of either APC (20 nM), or ATIII (700 nM) and heparin (0.2 U/ml). The reaction volume was 30 μl . At 0, 10, 20, 40, and 80 min, a sample was withdrawn, boiled in SDS reducing buffer and processed for electrophoresis as described in Methods. Lane PS, ^{125}I -protein S.

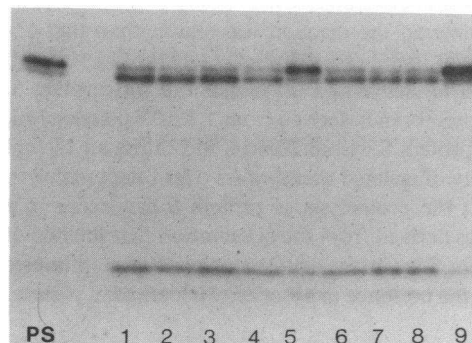


Figure 6. The effect of protease inhibitors on the platelet membrane protease. Platelet membranes from 3×10^7 platelets in a volume of 30 μl , were preincubated for 2 h at 37°C with: control buffer (lane 1), 1.5 mM leupeptin (lane 2), 1 mM NEM (lane 3), 100 nM PMSF (lane 4), 50 mM di-isopropyl fluorophosphate (lane 5), 1 U/ml hirudin (lane 6), 5 mM benzamidine (lane 7), ATIII 700 nM, and heparin 0.2 U/ml (lane 8), and 5 mM EGTA (lane 9). At the end of this time, 45 nM ^{125}I -protein S was added and the incubation continued for a further 120 min. The samples were then boiled in SDS reducing buffer and processed as described in Methods. Lane PS, ^{125}I -protein S.

nM APC, the clotting time was prolonged by 10 s. The addition of increasing concentrations of protein S resulted in a linear prolongation of the clotting time with saturation achieved in the presence of 60 nM protein S. When protein S cleaved by the platelet membrane protease (as confirmed by SDS PAGE) was substituted for intact protein S, the anticoagulant function of activated protein C was not enhanced, implying that in this form, protein S was devoid of anticoagulant activity (Fig. 7). In other studies the loss of cofactor activity was correlated to the cleavage of protein S as assessed by SDS PAGE.

Protein S was also cleaved and inactivated by intact platelets. In these studies the platelets were separated and washed as described in Methods, and incubated with protein S in the presence of 0.2 $\mu\text{g}/\text{ml}$ PGE_1 , 5 mM 1,3 dimethylxanthine, and 0.625 mM

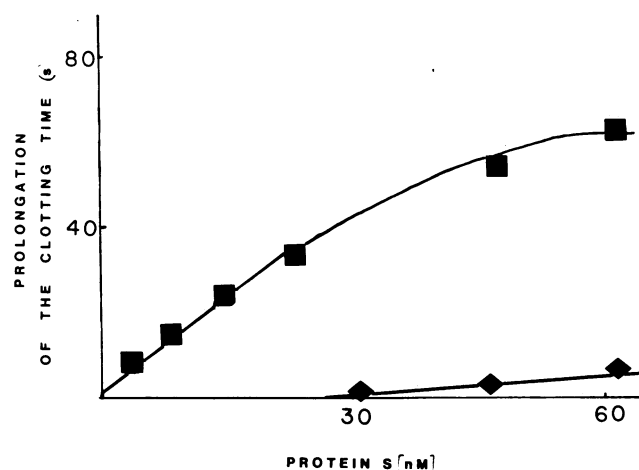


Figure 7. Functional activity of membrane-cleaved protein S. Functional activity was measured using a Factor Xa recalcification time as described in Methods. The prolongation of the clotting time represents the contribution of protein S minus the effect of APC alone. Protein S was cleaved by incubation with platelet membranes for 2 h at 37°C as described in Methods. Solid diamonds, protein S cleaved by the platelet membranes. Solid squares, protein S.

salicylic acid. However, the reaction was slower than that observed using platelet membranes. Intact platelets (1×10^{10} /ml) required 360 min to cleave and inactivate 750 nM protein S, whereas platelet membranes derived from 1×10^{10} platelets/ml, cleaved 750 nM protein S within 20 min, indicating an 18-fold increase in activity of isolated membranes over intact platelets.

Evidence that the proteolysis of protein S can occur in a plasma milieu was derived from the observation that the rate of cleavage of protein S by intact platelets or platelet membranes was unaltered by the presence or absence of heparinized plasma.

Discussion

The results of this study demonstrate that protein S binds specifically to unstimulated platelets, with half-maximal binding at 10 nM, and an estimated 1,100 binding sites per platelet. The binding was not enhanced by prior incubation of platelets with thrombin, APC and Factor V_a , alone or in combination. Harris and Esmon (19), using the bovine system, demonstrated that binding of protein S to platelets did not require platelet activation but was dependent on the presence of Factor V_a and APC. In these studies bovine protein S bound to platelets with a K_d of 31 nM, and 1,500 binding sites per platelet. Schwartz et al. (20) studied the binding of human protein S to platelets and reported the presence of 400 binding sites per platelet. In their experiments binding was unaffected by APC, but maximum binding required the presence of 0.2 U/ml thrombin.

Thus we can conclude from the results of the current study and those of Schwartz et al., that in the human system, unlike the bovine, protein S is not dependent on APC or Factor V_a for the binding reaction to occur. Whether platelet activation is a prerequisite for binding cannot be fully resolved, because minor degrees of activation are difficult to prevent during platelet preparation. We have attempted to minimize this event throughout the experiment, by the inclusion of the protease inhibitor benzamidine and the platelet release inhibitors PGE_1 , acetylsalicylic acid, and 1,3 dimethylxanthine. Furthermore, in all experiments the platelets were used within 4 h of blood collection.

Once bound to the platelets, protein S was rapidly cleaved and inactivated by a platelet protease, in spite of the presence of inhibitors of the platelet release reaction. This observation suggested that the responsible protease was located on the platelet surface. When platelets were fractionated into separate components significant protease activity was only noted in association with the platelet membranes. The small amount of activity detected in the alpha granule component of the sedimentation gradient most probably represents trapped platelet membranes in that fraction.

In direct displacement studies, labeled fully cleaved protein S was displaced from its platelet binding site upon the addition of excess molar native protein S. In other studies the incubation of intact platelets with protein S resulted in progressive inactivation and cleavage of the protein, confirming exchange of bound and free protein S. These results suggest that the platelets are involved in a continuous turnover of protein S. The observation that isolated platelet membranes cleaved protein S more readily than intact platelets could represent increased fluidity of isolated membranes and thus a more efficient exchange of protein S or possibly the exposure of more protease sites on the membranes compared with intact platelets.

The cleavage of protein S by platelet membranes was Ca^{++} dependent because the activity was inhibited by either EDTA

or EGTA. Our studies do however indicate that this protease differs from other Ca^{++} -dependent platelet proteases. The protease reported by Phillips et al. (21) was primarily cytosolic, was destroyed by freeze thawing and was inhibited by the sulfhydryl blocker NEM, and partially by PMSF. These characteristics contrast with the calcium-dependent protease described in this paper, which is expressed on the surface of intact platelets, is completely absent from the cytosolic compartment, and is resistant to freeze thawing, PMSF, and NEM. Tracy et al. (22), reported a calcium-dependent protease that cleaves Factor V_a , however this also appears different from the one that cleaves protein S, in that its expression was inhibited by PGE_1 , suggesting that it was released from internal platelet granules, and it was sensitive to the protease inhibitor, leupeptin.

By SDS PAGE criteria the cleavage of protein S by the membrane protease is similar to that induced by thrombin. That ATIII-heparin complex, hirudin and benzamidine did not inhibit the cleavage of protein S by the platelet membranes suggest that thrombin is unlikely to be the responsible protease. Furthermore, thrombin-induced cleavage of protein S is inhibited by calcium (reference 7, and our unpublished observations), whereas the platelet-associated protease is calcium dependent.

We have some evidence to indicate that both thrombin and the platelet-associated protease cleave protein S at a similar or closely associated site. When protein S cleaved by platelet membranes was incubated with thrombin, no further proteolysis could be observed. Similarly, thrombin-cleaved protein S was not susceptible to further degradation by the platelet protease (data not shown). However until the N-terminal amino acid sequence of platelet membrane-cleaved protein S is determined, comparison with the thrombin cleavage site must remain speculative.

Unlike other procoagulant and anticoagulant proteins, protein S circulates in plasma in an active form and does not require prior activation to partake in the coagulation reaction. A possible mechanism for the control of its activity has been attributed to thrombin, which cleaves protein S with subsequent loss of its anticoagulant activity. The observation that the effects of thrombin are inhibited by calcium and the endothelial cell surface protein thrombomodulin, suggests that this event is unlikely to be a major physiological control. The results of this study suggest that the platelet surface contains a novel calcium-dependent protease that may act to regulate the activity of protein S. The nature of the membrane-associated activity is the subject of our current studies.

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