The Activation of Bovine Protein C by Factor Xa*

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A complex composed of factor Xa and phospholipid vesicles assembled in the presence of calcium ions catalyzes a discrete cleavage of the heavy chain of bovine protein C that is indistinguishable from that produced by thrombin as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. This cleavage generates an active site capable of hydrolyzing small substrates and inactivating factor Va in the prothrombinase complex. Activation of protein C by factor Xa requires both calcium ions and phospholipid vesicles and proceeds at a rate an order of magnitude greater than that observed for α-thrombin in solution. γ-Carboxyglutamic acid-domainless protein C is not activated by factor Xa, consistent with the requirement for phospholipid and distinguishing this reaction from protein C activation by thrombin. Thrombomodulin serves as a cofactor for the factor Xa-catalyzed reaction, forming a 1:1 complex with factor Xa (apparent $K_a = 5.7 \times 10^{-10}$ M) and stimulating the saturated rate of protein C activation by factor Xa ($k_{cat} = 149 \text{ min}^{-1}$) to levels comparable to the thrombin-thrombomodulin complex. Protein C activation by factor Xa is not inhibited by the single thrombin inhibitor danyalyl-N-(3-ethyl-1,5-pentanediyl)amide but is inhibited by antithrombin III, tripeptide-chloromethyl ketones, and the monoclonal antibody α-BFX-2b that is highly specific for factor Xa. These data indicate that thrombomodulin is promiscuous in its role as a cofactor and suggest the existence of an alternative pathway for protein C activation in vivo.

Prothrombin is activated to thrombin by the prothrombinase complex that is composed of the protein cofactor Va and the serine protease factor Xa assembled in the presence of calcium ions on a membrane surface (2–8). This process is regulated in part through the actions of activated protein C (APC). A complex between thrombin and the endothelial cell surface protein thrombomodulin proteolytically converts the vitamin K-dependent zymogen protein C into the serine protease APC (9-13). In contrast to many of the other vitamin K-dependent coagulation factors, APC functions as an anticoagulant by inhibition of prothrombinase and factor Xase activity via limited proteolysis of proteins V (Va) and VIII (VIIIa) (14–19).

The only substantiated activator of protein C is α-thrombin (13, 20–25), which when bound to thrombomodulin has over a 1000-fold greater ability to activate protein C as compared with α-thrombin alone (10). In addition to augmenting the activation of protein C, the binding of thrombin to thrombomodulin drastically alters thrombin's procoagulant activities. When bound to thrombomodulin, thrombin no longer clots fibrinogen, activates factor V, inactivates protein S, or triggers platelet aggregation (12, 26–29). Thrombomodulin thereby imparts a natural anticoagulant surface on the endothelium and may serve to protect the microvasculature "downstream" from the site of injury by binding thrombin released at this site (13).

Recent studies have demonstrated that prothrombin activation intermediates (meizothrombin and meizothrombin(desF1)) can bind to thrombomodulin and activate protein C (30). The structural similarity between the catalytic subunit of factor Xa and the B-chain of α-thrombin (31) raises the question as to whether a similar anticoagulant mechanism exists for factor Xa as it does for thrombin. This idea is supported by studies aimed at investigating the thrombogenicity of a combination of factor Xa and agonist-active phospholipids in a normal canine model (32, 33). Infusion of factor Xa (6.5 × 10$^{-12}$ mol/kg) and phospholipid vesicles (4.0 × 10$^{-7}$ mol/kg) initiated a bleeding diathesis in normal dogs. Release of tissue plasminogen activator, activation of protein C, and inactivation of factors Va and VIIIa were observed. These results were attributed to secondary activation of protein C by thrombin generated by the Xa-PCPS combination. In this study we describe an alternative mechanism for the anticoagulant activity of the factor Xa-PCPS combination in which factor Xa and thrombomodulin activate protein C directly.

EXPERIMENTAL PROCEDURES

Materials—1-Palmitoyl-2-oleyl-phosphatidylserine (PS), 1-palmitoyl-2-oleyl-phosphatidylcholine (PC), chymotrypsin, Russell's viper venom, Reactive Blue 2-Sepharose CL-4B, benzamidine-Sepharose 6B, QAE-Sepharose Q-50, SP-Sepharose C-50, Sepharose CL-4B, phenyl-Sepharose, Sephadex G-50, and Sephadex G-25 were from Sigma. Heparin-Sepharose (34) and dextran sulfate-agarose (23) were prepared by cyanogen bromide activation of Sepharose CL-4B. FPR-thrombin-agarose was prepared by coupling bovine α-thrombin to Affi-Gel 10 (Bio-Rad) as per the manufacturer's instructions. The active site of thrombin was then blocked by reaction with a 10-fold molar excess of α-phenylalan-L-prolyl-l-arginine chloromethyl ketone (FPR-ck) (Calbiochem). DEAE-cellulose (DE52) was purchased from Whatman. The chromogenic substrates S2222 and S2328 were

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1 The abbreviations used are: APC, activated protein C; IgG, immunoglobulin G; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HBS, HEPES-buffered saline; FPR-ck, D-phenylalanyl-L-prolyl-L-arginyl chloromethyl ketone; PS, 1-palmitoyl-2-oleyl-phosphatidylserine; PC, 1-palmitoyl-2-oleyl-phosphatidylcholine; PCPS, vesicles composed of 75% PC and 25% PS; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DAPA, dansylarginine-N-(3-ethyl-1,5-pentanediyl)amide; Gla, γ-carboxyglutamic acid.

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from Helena Laboratories. Rabbit lungs were obtained from Bel-Freeze. The specific thrombin inhibitor dapsonearginine-N-(3-ethyl-1,5-pentanediyl)amide (DAPA) was prepared as described (35). Phospholipid vesicles (PCPs) composed of 75% PC and 25% PS were prepared as described (36).

Results—All proteins were of bovine origin except as noted. Factors X (37), Xa (38), Va (17), prothrombin (37), thrombin (39), APC (23), Gla-domainless protein C (40), and human antithrombin III (41) were prepared by previously reported methods. Protein C was prepared essentially as described in the figure legends. Prior to chromatography on heparin-Sepharose and dextran sulfate-agarose, the protein C pool from the QAE-Sephadex column was dialyzed into 0.025 M sodium citrate, pH 6.0, 1 mM benzamidine, 0.1 M NaCl and applied to a Whatman DE52 column (2.0 × 20 cm) equilibrated in the same buffer. The column was washed with 100 ml of the equilibration buffer to remove contaminating prothrombin. Protein C was then eluted with an 800-ml linear gradient from 0.1 to 0.4 M NaCl in 25 mM sodium citrate, pH 6.0, 1 mM benzamidine. The peak containing the protein C activity was then chromatographed on heparin-Sepharose and dextran sulfate-agarose as described (42). The protein C sample was then dialyzed into 0.02 M Tris-Cl, pH 7.4, 0.15 M NaCl and sequentially passed over Reactive Blue 2-Sepharose (2.5 × 20 cm) to remove potential contaminating prothrombin and then over a benzamidine-Sepharose column (2.5 × 20 cm) to remove any traces of APC or other active serine proteases. Prior to use in activation experiments, protein C samples were reacted with a 10-fold molar excess of FPR-ck to block any otherwise undetectable serine protease activity. The samples were subsequently dialyzed against a Sephacryl G-25 column equilibrated in 0.02 M HEPES, pH 7.4, 0.15 M NaCl.

Rabbit lung thrombomodulin was isolated by the method of Galvin et al. (11) with the following changes. The eluate from QAE-Sephadex was dialyzed for 5 h against 0.02 M Tris-Cl, pH 7.5, 0.1 M NaCl, 0.5 mM CaCl2, 0.5% Lubrol-PX, 1 mM benzamidine with two changes of buffer. Thrombomodulin was then added to an FPR-thrombin Affi-Gel 10 column (2.5 × 30 cm containing 120 mg of thrombin) equilibrated in the same buffer. The column was washed with 200 ml of 0.02 M Tris-Cl, pH 7.5, 0.5 mM CaCl2, 0.5% Lubrol, 0.4 M NaCl, 1 mM benzamidine followed by 100 ml of 0.02 M Tris-Cl, pH 7.5, 1 mM EDTA, 0.4 M NaCl, 1 mM benzamidine. Thrombomodul in was then eluted with 0.02 M Tris-Cl, pH 7.5, 1 mM EDTA, 0.1% Lubrol, 2 M NaCl, 1 mM benzamidine. The thrombomodulin was bound to a 0.1 × 10-cm column of Phenyl-Sepharose equilibrated in the same buffer. Thrombomodulin was eluted with 0.02 M Tris-Cl, pH 7.5, 0.1 M NaCl, 0.02% Lubrol and concentrated in a Centricon 30 microconcentrator. Traces of degradation products were removed by gel filtration on a Superose-12 FPLC column equilibrated in 0.02 M Tris-Cl, pH 7.5, 0.1 M NaCl, 0.02% Lubrol, 0.02% NaN3.

Molecular Weights and Extinction Coefficients for Proteins—The molecular weights and extinction coefficients (ε_{max}) of the various proteins were as follows: factor Xa, 45,300 and 1.24 (44); protein C, 58,000 and 1.37 (14); activated protein C, 54,200 and 1.37 (14); Gla-domainless protein C, 54,000 and 1.37 (14, 40); thrombin, 37,400 and 1.95 (49); thrombomodulin, 74,000 and 0.88 (10); factor Va, 150,000 and 1.74 (46); prothrombin, 72,000 and 1.44 (47); antithrombin III, 150,000 and 1.40 (48).

Electrophoresis—Protein C cleavage by factor Xa or thrombin was monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 15-18% gradient polyacrylamide gels. Protein C samples (20 μM) were incubated for 3 h at 37 °C in the presence or absence of calcium, PCPs, factor Va, and factor Xa as indicated in the figure legends. Samples were then placed on ice until ready for use. Immunoassays of activated protein C were performed on 15% acrylamide gels by following the hydrolysis of the chromogenic substrate S2238. The assay was performed at 25 °C in a buffer containing 0.02 M Tris-Cl, pH 7.4, 0.15 M NaCl, 0.2 M S2238. The absorbance change at 405 nm was monitored in a Molecular Devices Vmax spectrophotometer. Protein C samples were treated with a 10-fold molar excess of FPR-ck and desalted on a Sephadex G-25 column equilibrated with HBS prior to use to eliminate the possibility that contaminating serine proteases may be interfering with activation experiments.

The activation of Protein C by factor Xa—Inactivation of factor Va by thrombin-activated protein C and factor Xa-activated protein C was evaluated by use of a prothrombinase assay as described previously (7, 35, 49). This assay utilizes a reconstituted system of purified components composed of prothrombin (1.4 μM), PCPs (15 μM), Ca2+ (2.0 mM), factor Xa (14 nM), factor Va (2.0 μM), and the specific thrombin inhibitor, DAPA (3.0 μM), in HBS. Thrombin generation was measured by following the increase in fluorescence intensity as DAPA binds to thrombin. Fluorescence intensity was measured with a Perkin-Elmer MFP-44A spectrofluorometer. The excitation wavelength was 354 nm, the emission wavelength was 565 nm, and a 430-nm cut-off filter was used in reference beam.

Activated protein C was prepared by incubating protein C (10 μM) with either factor Xa (10 nM) or thrombin (10 nM) in the presence of thrombomodulin (15 nM) and PCPs (600 μM) in HBS containing 5 mM CaCl2. The progress of the reaction was followed by measuring the activated protein C activity of aliquots removed with time, as has been previously described. When the reaction was complete, samples were placed on ice until ready for use.

The ability of these activated protein C samples to inactivate factor Va was assessed as follows: factor Va (0.2 μg/ml) was incubated with APC (10 μg/ml) at 22 °C in a buffer containing 0.02 M HEPES, pH 7.4, 0.15 M NaCl, 0.02% Lubrol, and 0.02% NaN3. After various reaction times, aliquots (50 μl) were removed and added to 0.45 ml of HBS containing 0.01 M sodium citrate at 0 °C for subsequent assay of factor Va activity. The assay was performed by pipeting 10 μl of the diluted sample into a cuvette containing 2 ml of the assay mixture previously described. The reaction was initiated by addition of factor Xa (14 nM). Under these conditions, the initial rate of thrombin generation is directly proportional to the concentration of functionally active factor Va.

**Stoichiometry of the Factor Xa-Thrombomodulin Complex**—Protein C samples (2.5 μM) in HBS, pH 7.4, 0.005% Lubrol-PX, 5 mM CaCl2 were incubated at 22 °C with thrombomodulin (10 nM) and incremental amounts of factor Xa or α-thrombin (0–20 nM) in the presence or absence of phospholipid vesicles (200 μM). Initial rates of protein C activation were determined as described under "Experimental Procedures." Increasing levels of factor Xa (or α-thrombin) produced an increase in the initial rate of protein C activation until saturation was reached at an apparent 1:1 molar ratio of thrombomodulin to factor Xa. An apparent dissociation constant was determined from the data obtained under the conditions described by Gutfreund (50).

**RESULTS**

**Protectolytic Cleavage of Protein C by Factor Xa**—Electrophoretic analysis of the factor Xa cleavage of bovine protein C is shown in Fig. 1. Protein C samples (10 μM) were incubated with factor Xa (200 nM) in the presence or absence of PCPs vesicles (200 μM), calcium ions (5 mM), and factor Va (200 nM) as indicated in the figure legends. Lanes 1–8 represent intact protein C samples treated as indicated, and lanes 9–11 are samples of thrombin-generated APC which were subjected to identical treatment as protein C. These results demonstrate that factor Xa is able to catalyze a single cleavage in the heavy chain of protein C in the presence of PCPs and Ca2+ and in the presence of PCPs, Ca2+, and factor Va. No cleavage of the light chain of protein C or APC was observed. In fact, proteolysis of protein C by factor Xa in the presence of PCPs and Ca2+ yields an SDS-gel pattern that is identical to that of thrombin-generated APC.

**Activation of Protein C by the Factor Xa-PCPS-Ca2+ Complex and α-Thrombin**—Due to the similarity in the products of factor Xa-cleaved protein C and thrombin-generated APC, it was of interest to see whether this cleavage was capable of generating an active site. Bovine protein C samples were incubated with factor Xa in the presence of PCPS vesicles
Protein C Activation by Factor Xa

Inhibition of Prothrombinase Activity by Activated Protein C

The conversion of prothrombin to thrombin by the prothrombinase complex is inhibited by activated protein C via proteolytic inactivation of factor Va. The ability of factor Xa-activated protein C to inhibit prothrombinase activity relative to thrombin-generated APC was determined. Thrombin-activated protein C and factor Xa-activated protein C were prepared as described under “Experimental Procedures.” Samples of factor Va (1.3 μM) were incubated with APC (10 nM) at 22 °C in HBS, pH 7.4, containing 2 mM CaCl₂ and 15 μM PCPS. Aliquots were removed with time and made 10 mM in sodium citrate and assayed for factor Xa cofactor activity in a prothrombinase assay as described under “Experimental Procedures.” Assay conditions were such that the initial rate of thrombin generation was directly proportional to the concentration of functionally active factor Va. A comparison of thrombin and factor Xa-activated protein C inhibition of prothrombinase activity is shown in Fig. 3. The closed symbols represent inhibition of prothrombinase activity by thrombin-generated APC, and the open symbols represent inhibition by factor Xa-generated APC. Both showed virtually identical rates of factor Va inactivation.

Effect of Various Treatments on the Activation of Protein C by Factor Xa—The initial observation that protein C could be activated by factor Xa raised the question as to whether a contaminant (thrombin, prothrombin, Russell’s viper venom, or some unknown protease) was responsible for this activation, especially in light of the reports by other investigators that this reaction does not occur (9, 12, 20–25). Table I summarizes the results of a series of experiments that demonstrate that the activation of protein C by the Xa-PCPs complex is indeed specific for factor Xa and not the result of contamination.

A protein C activation system composed of protein C (4 μM), factor Xa (100 nM), and saturating levels of phospholipid (400 μM) and calcium ions (5 mM) was incubated at 37 °C for various periods of time. Initial rates of protein C activation were determined as described under “Experimental Procedures.” Parallel experiments in which α-thrombin replaced factor Xa were also performed. Incubation times were adjusted so that less than 15% of substrate cleavage was observed. All incubations were run at 10 nM and then assayed for its ability to function in the prothrombinase complex as described under “Experimental Procedures.”

![Fig. 1. Proteolytic cleavage of bovine protein C by factor Xa.](image)

![Fig. 2. Protein C activation by thrombin and factor Xa. Bovine protein C (5 μM) was incubated at 37 °C with either thrombin (100 nM) (○) or factor Xa (100 nM) (●) in HBS, pH 7.4, containing 400 μM PCPS and 5 mM CaCl₂. At timed intervals, samples were removed and initial rates of protein C activation were determined as described under “Experimental Procedures.”](image)

and Ca²⁺ as indicated in the legend to Fig. 2. The phospholipid concentration was chosen so that all components would be bound to the vesicle surface. Aliquots were removed at timed intervals and assayed for their ability to hydrolyze the chromogenic substrate S2238 as described under “Experimental Procedures.” Parallel experiments in which factor Xa was replaced by an equal concentration of α-thrombin were performed. The results of these experiments are presented in Fig. 2. Not only did factor Xa activate protein C as measured by hydrolysis of S2238, but it did so at a rate 10–15-fold greater than an equivalent concentration of α-thrombin under the same conditions. Control experiments in which Ca²⁺ or phospholipid was omitted from the factor Xa reaction mix resulted in no detectable protein C activation. In addition, the initial rate of protein C activation by factor Xa was saturable with respect to PCPS and was maximal at concentrations of PCPS which would result in all the protein C being bound to the membrane (43). Thus, it would appear that Ca²⁺-dependent binding of protein C to PCPS vesicles is required for activation by factor Xa.

![Fig. 3. Inhibition of prothrombinase activity by APC. Factor Va (1.3 μM) was incubated with either thrombin-generated APC (10 nM) (○) or factor Xa-generated APC (10 nM) (●) and then assayed for its ability to function in the prothrombinase complex as described under “Experimental Procedures.”](image)
The addition of antithrombin III and heparin to the reaction mixture at concentrations which would rapidly inhibit either thrombin or factor Xa resulted in 100% inhibition of the Xa-catalyzed activation of protein C. Since the Russell's viper venom X activator is not inhibited by antithrombin III, this rules out a role for contaminating Russell's viper venom. Also in support of this finding is the fact that no Russell's viper venom X activator could be detected in the factor Xa samples by a two-stage factor X clotting assay (data not shown) and that treatment with D-glutamylglycylarginyl chloromethyl ketone (which does not inhibit Russell's viper venom X activator) prior to incubation with protein C yields 100% inhibition of protein C activation.

2) DAPA is a specific thrombin inhibitor ( \( K_d = 3 \times 10^{-8} \) M) which at a concentration of 10 M would be in vast excess of that amount needed to inhibit any thrombin that could conceivably be in the system. DAPA had no effect on the Xa-catalyzed activation of protein C. In control experiments, factor Xa was replaced by an identical concentration of thrombin, and 100% inhibition of the a-thrombin-catalyzed protein C activation was observed. These results rule out the possibility that contaminating thrombin is a factor in this system.

3) The monoclonal antibody \( \alpha \)-BFX-2b is highly specific for factor Xa and does not cross-react with the other vitamin K-dependent proteins (51). Incubation of factor Xa with a 5-fold molar excess of this monoclonal antibody for 15 min prior to addition to the protein C/phospholipid/Ca\(^{2+}\) mixture resulted in 90% inhibition of protein C activation, indicating directly the absolute specificity of this reaction for factor Xa.

4) Previously described experiments (Figs. 1 and 2) demonstrated that factor Xa-catalyzed activation of protein C requires both calcium ions and phospholipid. This is characteristic of the reversible Ca\(^{2+}\)-dependent lipid binding of other vitamin K-dependent proteins. In support of this mechanism are the following results. When EDTA (10 mM) was added at time 0 to the factor Xa-dependent protein C activation system described above (Table I), 95% inhibition was observed. In contrast, the thrombin-catalyzed activation of protein C is inhibited by Ca\(^{2+}\). The addition of EDTA to the thrombin activation reaction reverses the Ca\(^{2+}\) inhibition. Gla-domainless protein C is activated very poorly (~5% of intact protein C) in the factor Xa system. These data suggest that Gla domain-dependent protein C binding to phospholipid vesicles is an important accessory for activation by factor Xa. In contrast, thrombin activates Gla-domainless protein C at a rate comparable with that observed for intact protein C, indicating no fundamental requirement of phospholipid binding in this reaction.

Thus, all observations support the conclusion that the observed reaction is due to factor Xa and not due to thrombin, Russell's viper venom, or extraneous protease contamination.

**Stimulation of Factor Xa-catalyzed Protein C Activation by Thrombomodulin**—The potential stimulation of factor Xa-catalyzed protein C activation by thrombomodulin was suggested by analogy to thrombin-catalyzed activation and indications from other groups that factor Xa binds thrombomodulin (52). Solutions of protein C (5 \( \mu \)M) in HBS, pH 7.4, 5 mM Ca\(^{2+}\) were incubated at 37 °C with factor Xa (10 nM) in the presence of either PCPs (400 \( \mu \)M) or thrombomodulin (10 nM) or both PCPs (400 \( \mu \)M) and thrombomodulin (10 nM). Aliquots were withdrawn with time, and initial rates of S2238 hydrolysis were determined. The lower curve (open triangles) in Fig. 4 represents protein C activation in the presence of saturating levels of phospholipid and Ca\(^{2+}\). Protein C activation in the presence of thrombomodulin at concentration equimolar to factor Xa (closed circles) shows a modest increase in the activation rate, whereas the presence of both thrombomodulin and phospholipid (open circles) dramatically increased the rate of protein C activation as compared with phospholipid alone. These data indicate that thrombomodulin can serve as a cofactor for factor Xa as well as for thrombin in the activation of protein C.

A comparison of the kinetic constants determined by Galvin and co-workers (11) for the thrombin-catalyzed protein C activation with those determined for factor Xa-catalyzed protein C activation are shown in Table II. The factor Xa-thrombomodulin-phospholipid complex has a \( K_m \) for protein C of 10 \( \mu \)M and \( k_{cat} \) of 149 min\(^{-1}\). The latter value is comparable with protein C activation by the thrombin-thrombomodulin complex in the presence and absence of phospholipids.

**Stoichiometry of the Factor Xa-Thrombomodulin Complex**—

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**Table I**

| Treatment | Residual activity | Xa | IIa |
|-----------|------------------|----|-----|
| No addition\(^a\) | 100 | 100 |
| AT IP (1 \( \mu \)M) + heparin (3 units/ml) | 0 | 0 |
| DAPA (10 \( \mu \)M) | 100 | 0 |
| \( \alpha \)-BFX-2b (500 \( \mathrm{nm} \)) | 9 | 100 |
| \( \alpha \)-FV-7 (500 \( \mathrm{nm} \)) | 100 | 100 |
| EDTA (10 mM) | 5 | 350 |
| Gla-domainless protein C\(^a\) | 5 | 100 |

\(^a\) Protein C samples (4 \( \mu \)M) in the presence of PCPs (400 \( \mu \)M) and calcium ions (5 mM) were incubated with either factor Xa (100 nM) or IIa (100 nM), and initial rates of protein C activation were determined.

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**Table II**

| | \( K_m \) | \( k_{cat} \) |
|---|---|---|
| IIa | 60 | 1.21 |
| IIa/TM\(^a\) | 8 | 250* |
| IIa/TM/PCPs | 0.1 | 214* |
| Xa/TM/PCPs | 10 | 149 |

\(^a\) From Ref. 11.

\(^*\) TM, thrombomodulin.
A fixed concentration of protein C (2.5 μM) in HBS, pH 7.4, 0.005% Lubrol-PX, 5 mM CaCl₂ was incubated at 22 °C with thrombomodulin (10 nM) and incremental amounts of α-thrombin (panel A), factor Xa (panel B), α-thrombin in the presence of PCPS vesicles (200 μM) (panel C), or factor Xa in the presence of PCPS (200 μM) (panel D). Initial rates of protein C activation were determined as described under “Experimental Procedures.” The insets show a linearization of the activity saturation isotherm used to determine the apparent dissociation constants and binding stochiometries as described under “Experimental Procedures.”

**DISCUSSION**

The protein C zymogen is a disulfide-linked heterodimer (Fig. 6). A single cleavage by thrombin at Arg₁₄ of the heavy chain converts protein C into APC. The light chain contains two epidermal growth factor-like domains and the lipid binding portion of the molecule, which contains 11 γ-carboxyglutamatic acid residues. In bovine protein C residues 85-87 of the light chain are composed of a sequence of three amino acids (Glu-Gly-Arg) that are identical to the three amino acids preceding the factor Xa cleavage sites in prothrombin. This cleavage site homology between thrombin and protein C was the initial source of our interest. We anticipated that factor Xa would produce a proteolytic modification of the light chain of protein C. We were surprised by the finding that factor Xa was unable to cleave the light chain of protein C and that in the presence of phospholipid and Ca²⁺ factor Xa catalyzed protein C activation since previous studies have reported that, apart from thrombin, none of the other vitamin K-dependent coagulation factors were able to activate protein C (10, 13, 20-25).

The results of this study demonstrate that a complex composed of factor Xa, phospholipid vesicles, and calcium ions catalyzes a discrete cleavage of the heavy chain of protein C that is indistinguishable from that produced by thrombin as judged by SDS-PAGE. Thrombomodulin serves as a cofactor for this reaction and stimulates the rate of protein C activation to levels comparable with the thrombin-thrombomodulin complex. Similar results have been reported independently in a human system (53) except that no stimulation by thrombomodulin was observed, and inhibition studies indicated that the proteins were contaminated with thrombin. Esmon and Eson (53) had also observed protein C activation in the presence of factor Xa and thrombomodulin, but later attributed this activation to contaminating thrombin, since immunochemically purified proteins did not exhibit this activity.²

² N. L. Esmon and C. T. Esmon, personal communication.
We have tested for interference by prothrombin and thrombin by several means. First is the homogeneity of the proteins used in this study as judged by SDS-PAGE (Fig. 7): no thrombin was detectable in either the protein C or factor Xa. If trace contaminants were present and responsible for protein C activation in the systems described in this paper, they would most likely be present in the protein C since only catalytic amounts of factor Xa were employed. If traces of thrombin (undetectable by silver staining) were present in the lane 7, bovine activated protein C (IIa-generated); lane 8, molecular weight standards.

Fig. 7. SDS-PAGE. Samples were prepared for SDS-PAGE as described under “Experimental Procedures.” 30 μg of each protein were loaded onto the gel. Panel A shows the pattern under nonreducing conditions. Panel B is the electrophoretic pattern in the presence of 100 mM dithiothreitol. Lane 1, molecular weight standards; lane 2, bovine factor X; lane 3, bovine factor Xa; lane 4, bovine prothrombin; lane 5, human prothrombin; lane 6, bovine protein C; lane 7, bovine activated protein C (IIa-generated); lane 8, molecular weight standards.

In addition to the perceived purity of the reagents used, inhibition studies confirmed the specificity of this reaction for factor Xa (Table I). The inhibition of factor Xa-catalyzed activation of protein C by a monoclonal antibody (α-BFX-2b) which is highly specific for factor Xa demonstrates the dependence of this reaction for factor Xa. The possibility that factor Xa is activating a contaminating zymogen is ruled out based on the fact that initial rates of protein C activation by factor Xa do not exhibit a lag (Figs. 2 and 4). If prothrombin were a contaminant in this system, addition of factor Va to the Xa-Ca²⁺-PCPS complex would result in a dramatic acceleration in thrombin conversion to thrombin and therefore protein C activation. This, however, is not observed. Reports by Thompson and Salem (52) that factor Xa bound to thrombomodulin does not activate prothrombin also cast doubt on a role for prothrombin in this system. The most convincing evidence that thrombin or its derivatives are not contaminants of the factor Xa-mediated protein C activation comes from protein C activation experiments done in the presence of the specific thrombin inhibitor DAPA. Concentrations of DAPA (10 μM), which are in vast excess of the amount required to inhibit any thrombin in the system (K_a = 3 × 10⁻⁸ M), had no effect on protein C activation by factor Xa.

An apparent 1:1 stoichiometry between factor Xa and thrombomodulin was observed in protein C activation studies (Fig. 5). The dissociation constant is essentially the same in both the presence and absence of phospholipid. This apparent 1:1 stoichiometry is also indicative of the specificity of the reaction for factor Xa.

In retrospect, the observation that factor Xa activates protein C and thus the existence of an additional, potentially relevant mode of protein C activation is not surprising. Several lines of evidence suggest this outcome. First, the rate of protein C activation by the thrombin-thrombomodulin complex is relatively slow. Although the rate of activation of protein C by thrombin is accelerated 1000-fold (kcat = 250 min⁻¹) upon binding to thrombomodulin, it is still relatively low when compared with prothrombin conversion to thrombin by the prothrombinase complex (kcat = 2100 min⁻¹) (7) or the rate of factor X activation by the intrinsic factor Xase (kcat = 500 min⁻¹) (56). This, in conjunction with the observation that vascular endothelial cells (in the presence of exogenous factor Xa and Ca²⁺) possess prothrombinase activity (57–59), suggests that perhaps an additional regulatory mechanism is necessary to avoid excess thrombin generation. Second, our recent demonstration that prothrombin activation intermediate meizothrombin and meizothrombin(desF1) can bind to thrombomodulin and activate protein C suggests that thrombomodulin is not fastidious in its role as cofactor for thrombin. In light of these findings and structural similarities between the catalytic subunit of factor Xa and the B-chain of thrombin, it is not surprising that factor Xa has been shown to bind to thrombomodulin.

Although the rate of protein C activation by the Xa-thrombomodulin complex (kcat = 149 min⁻¹) is comparable with the thrombin-thrombomodulin-PCPS complex (kcat = 250 min⁻¹), it is still relatively low. A variety of explanations may be considered. The low rate of protein C activation may be due to the necessity to maintain a subtle control of the coagulation process and that in conjunction with other anticoagulants (i.e., tissue plasminogen activator release, heparin-antithrombin III, etc.) it constitutes a significant anticoagulant potential yielding environmentally sensitive hemostasis.

The relatively high K_a for protein C (10 μM) displayed by the Xa-thrombomodulin complex raises the question of the physiological importance of this reaction. While local concentrations of protein C may be quite high in comparison with bulk plasma concentrations, it is unlikely that it would approach 10 μM. The possibility exists that an unidentified
additional cofactor may exist for this reaction which could lower the $K_m$ as well as accelerate the reaction. The high $K_m$ determined for protein C may also be the result of an in vitro experiment associated with isolated thrombomodulin. In our experiments, thrombomodulin was reconstituted with phospholipids by mixing thrombomodulin with preformed phospholipid vesicles. This may result in improper orientation of the thrombomodulin receptor on the surface and disrupt the functioning of this enzyme complex. The composition of the lipid bilayer may also be of importance. A less likely possibility is that the species specificity and the integrity of the isolated thrombomodulin may be a factor in the unusually high $K_m$. All of the proteins in this study were of bovine origin with the exception of thrombomodulin, which was from rabbit lung. Species variability of thrombomodulin has been reported (13, 27). Isolated thrombomodulin is exceedingly stable under a variety of conditions. This, however, does not preclude the possibility that the initiation of extraction of the thrombomodulin may act as a scavenger for activated coagulation factors in vitro. Preparations of thrombomodulin have been shown to be stable to extraction by detergents and proteases (13, 27), indicating that protein C activation by the factor Xa-thrombomodulin-PCPS complex. In the present study we show that a potential alternative or complementary mechanism for this anticoagulant activity in which factor Xa and thrombomodulin directly activate protein C may exist.

Preliminary in vitro experimentation indicates that protein C activation by the factor Xa-thrombomodulin-PCPS complex also occurs in the human system. Due to the promiscuous nature of thrombomodulin as a cofactor for thrombin, we are currently investigating the possibility that thrombomodulin may act as a scavenger for activated coagulation factors (i.e. VIIa, IXa, etc.). In this role, the binding of proteases to thrombomodulin may not only play a role in protein C activation but also function in the modulation of the activities of these enzymes in the coagulation cascade.

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