Flory, P. D. (1936), *J. Am. Chem. Soc. 58*, 1877.
Fraenkel-Conrat, H. (1957), *Virology 4*, 1.
Fraenkel-Conrat, H., and Singer, B. (1964), *Virology 23*, 354.
Fraenkel-Conrat, H., and Williams, R. C. (1955), *Proc. Natl. Acad. Sci. U.S.A. 41*, 690.
Gilbert, P. F. C., and Klug, A. (1974), *J. Mol. Biol. 86*, 193.
Ginoza, W., Atkinson, D. E., and Wildman, S. G. (1954), *Science 119*, 269.
Haschemayer, R., and Bowers, W. (1970), *Biochemistry 9*, 435.
Jaenicke R., and Lauffer, M. A. (1969), *Biochemistry 8*, 3083.
Lonchampt, M., Lebeurier, G., and Hirth, L. (1972), *FEBS Lett. 22*, 297.
Roark, D. D., and Yphantis, D. A. (1969), *Ann. N.Y. Acad. Sci. 164*, 245.
Rodionova, N. P., Vesenina, N. E., Atabekova, T. I., Dzavakhia, V. G., and Atabekov, J. G. (1973), *Virology 51*, 24.
Sarkar, S. (1960), *Z. Naturforsch. B 15*, 778.
Scheele, R. B., and Lauffer, M. A. (1967), *Biochemistry 6*, 3076.
Schramm, G., and Zillig, W. (1955), *Z. Naturforsch. B 10*, 493.
Sophianopoulos, A. J., and Van Holde, K. E. (1964), *J. Biol. Chem. 239*, 2516.
Sperling, R., and Klug, A. (1975), *J. Mol. Biol. 96*, 425.
Stauffer, H., Srinivasan, S., and Lauffer, M. A. (1970), *Biochemistry 9*, 193.
Teller, D. C., Horbett, T. A., Richards, E. G., and Schachman, H. K. (1969), *Ann. N.Y. Acad. Sci. 164*, 66.
Timmermans, J. (1960), Physico-Chemical Constants of Binary Systems, Vol. III, New York, N.Y., Interscience.
Vogel, D., and Jaenicke, R. (1974), *Eur. J. Biochem. 41*, 607.
Weast, R. C., and Selby, S. M. (1966), in Handbook of Chemistry and Physics, 47th ed, Cleveland, Ohio, Chemical Rubber Co., p D 139.
Yphantis, D. A. (1964), *Biochemistry 3*, 297.

Anticoagulant Properties of Bovine Plasma Protein C following Activation by Thrombin†

Walter Kisiel,*,‡ William M. Canfield,§ Lowell H. Ericsson, and Earl W. Davie

ABSTRACT: Protein C is a vitamin K dependent glycoprotein which exists in bovine plasma as a precursor of a serine enzyme. Incubation of this plasma protein with α-thrombin at an enzyme-to-substrate weight ratio of 1:50 resulted in the cleavage of an Arg-Ile bond between residues 14 and 15 of the heavy chain of the molecule and the formation of activated protein C. The heavy chain of this serine enzyme contains the active-site sequence of -Leu-Cys-Ala-Gly-Ile-Leu-Gly-Asp-Pro-Arg-Asp-Ala-Cys-Gln-Gly-Asp-*SER*-Gly which is homologous with the corresponding regions of a number of plasma serine proteases. Activated protein C markedly prolongs the kaolin-cephalin clotting time of bovine plasma but not that of human plasma. The anticoagulant effect was totally obviated by prior incubation of the enzyme with diisopropyl phosphorofluoridate or phenylmethanesulfonyl fluoride. Incubation of activated protein C with bovine factor V resulted in a time- and temperature-dependent inactivation of this clotting factor, and this reaction was dependent on the presence of phospholipid and calcium ions. Activated protein C had no effect on the coagulant activity of factor XII, factor XI, factor X, factor IX, factor VII, or prothrombin. These data provide evidence for a mechanism for the activation of protein C in plasma and a potential role for this enzyme in blood coagulation and hemostasis.

Protein C is a vitamin K dependent protein present in bovine plasma (Stenflo, 1976). It is a glycoprotein composed of a heavy chain (mol wt 41 000) and a light chain (mol wt 21 000) held together by a disulfide bond(s). The light chain of protein C is homologous in its amino-terminal sequence with that of the four vitamin K dependent coagulation factors. The latter four proteins exist in plasma as zymogens and are converted to serine proteases by limited proteolysis (Davie and Fujikawa, 1975).

Present evidence indicates that protein C, like the four vitamin K dependent coagulation factors, exists in plasma as a precursor to a serine enzyme (Kisiel et al., 1976a; Esmon et al., 1976). Furthermore, protein C is converted to a serine amidase by a protease from Russell's viper venom or by trypsin. In this reaction, a tetradecapeptide is cleaved from the amino terminus of the heavy chain of the precursor protein with the concomitant formation of a diisopropyl phosphorofluoridate (iPr_2PF)[1] sensitive serine enzyme. This cleavage occurred at a specific Arg-Ile bond in the heavy chain of the molecule and resulted in the formation of a new amino-terminal sequence of Ile-Val-Asp-Gly-. Activated protein C did not exhibit any procoagulant activity. On the contrary, a significant inhibition of the partial thromboplastin time of bovine plasma was observed in the presence of activated protein C.

The goal of the present investigation was to determine

† From the Department of Biochemistry, University of Washington, Seattle, Washington 98195. *Received June 27, 1977*. This work was supported in part by Research Grants HL 16919 and GM 15731 from the National Institutes of Health.

‡ An Established Investigator of the American Heart Association.

§ Predoctoral Fellow, Medical Scientist Training Program, National Institutes of General Medical Science (GM 07266).

[1] Abbreviations used are: iPr_2PF, diisopropyl phosphorofluoridate; $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid.

whether protein C could be activated by one of the plasma serine proteases involved in blood coagulation and whether it might be involved in the inactivation of a specific coagulation factor(s). In the present communication, we report the activation of bovine plasma protein C by bovine α-thrombin, and the effect of activated protein C on several highly purified coagulation factors.

Experimental Section

Materials

Urease, γ-globulin, lyophilized *Vipera russelli* venom, soybean trypsin inhibitor (type II-S), bovine serum albumin, ovalbumin, carbonic anhydrase, myoglobin, imidazole (grade I), *p*-aminobenzamidine hydrochloride, heparin, dithiothreitol, phenylmethanesulfonyl fluoride ($PhCH_2SO_2F$), prothrombin deficient plasma, and factor X deficient plasma were obtained from Sigma Chemical Co., St. Louis, Mo. Benzamidine hydrochloride, cyclohexylmorpholinoethylcarbodiimide, iPr_2PF, and 4-vinylpyridine were purchased from Aldrich Chemical Co., Milwaukee, Wis. DEAE-Sephadex A-50, SP-Sephadex C-50, Sephadex G-25, G-75, G-150, and G-200 were products of Pharmacia Fine Chemicals, Piscataway, N.J. 2-Mercaptoethanol and *N,N,N',N'*-tetramethylethylenediamine were purchased from Eastman Kodak Co., Rochester, N.Y. Acrylamide, *N,N'*-methylenebis(acrylamide), and Bio-Gel A-15m (agarose) were obtained from Bio-Rad Laboratories, Richmond, Calif. Diisopropyl *N*-[1-^{3}H]fluorophosphate (0.9 Ci/mmol) was obtained from New England Nuclear, Boston, Mass. Sodium dodecyl sulfate was purchased from British Drug House, Poole, England. Urea (Ultra Pure) was obtained from Schwarz/Mann, Van Nuys, Calif. Chromogenic amidase substrates *N*-benzoyl-L-phenylalanyl-L-valyl-L-arginyl-*p*-nitroanilide (S-2160), *N*-benzoyl-L-isoleucyl-L-glutamylglycyl-L-arginyl-*p*-nitroanilide (S-2222), L-glutamylglycyl-L-arginyl-*p*-nitroanilide (S-2227), D-phenylalanyl-L-pipecolyl-L-arginyl-*p*-nitroanilide (S-2238), D-valyl-L-leucyl-L-lysyl-*p*-nitroanilide (S-2251), D-valyl-L-leucyl-L-arginyl-*p*-nitroanilide (S-2266), and D-prolyl-L-phenylalanyl-L-arginyl-*p*-nitroanilide (s-2302) were obtained from Kabi Diagnostica, Stockholm, Sweden. Dialysis tubing was treated according to McPhie (1971) prior to use. Cyanogen bromide and the sequenator reagents (Sequanol grade) were obtained from Pierce Chemical Co., Rockford, Ill. Purified soybean trypsin inhibitor was prepared according to Frattali and Steiner (1969) and bovine brain cephalin was prepared according to Bell and Alton (1954). Bovine brain thromboplastin and bovine factor VII deficient plasma were prepared as previously described (Kisiel and Davie, 1975). Factor V deficient plasma was prepared according to Lewis and Ware (1953). In this procedure, oxalated, human plasma was incubated at 37 °C until the one-stage prothrombin time was greater than 100 s. The venom protease (RVV-X) which activates factor X,[2] factor IX (Lindquist et al., 1978), and protein C was purified as previously described (Kisiel et al., 1976b). Plasminogen was isolated from the barium sulfate eluate of bovine plasma essentially according to Walther et al. (1974) employing benzamidine and $PhCH_2SO_2F$ throughout the isolation procedure. Highly purified bovine factor V was prepared according to Smith and Hanahan (1976) and was kindly provided by Dr. D. J. Hanahan, San Antonio, Tex. Bovine α-thrombin was isolated essentially according to Lundblad et al. (1975) following activation of prothrombin by a factor X_a-Ca^{2+}-phospholipid mixture (Esmon et al., 1974). The specific activity of the thrombin preparation was 2750 NIH units/mg of protein. By sodium dodecyl sulfate-polyacrylamide gel electrophoresis, this preparation appeared to contain approximately 95% α-thrombin and 5% β-thrombin. The following proteins were isolated from bovine plasma to a homogeneous state according to the respective reference: prothrombin and protein C (Kisiel et al., 1976a), fibrinogen (Matusda et al., 1972), antithrombin III (Kurachi et al., 1976), factor X_1 (Fujikawa et al., 1972), factor IX (Fujikawa et al., 1973), factor XI (Koide et al., 1977), factor XII (Fujikawa et al., 1977), and factor VII (Kisiel et al., 1977).

Methods

Activated protein C and α-thrombin concentrations were determined from the absorbance at 280 nm using $E_{280}^{1\%}$ values of 13.7 (Kisiel et al., 1976a) and 19.5 (Winzor and Scheraga, 1964), respectively. The protein concentrations of all other proteins used in this study were determined from the absorbance at 280 nm assuming an $E_{280}^{1\%} = 10.0$.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed as previously described (Kisiel et al., 1976a) employing 10% polyacrylamide gels.

The heavy and light chains of activated protein C were prepared in the following way. Activated protein C (80 mg) was reduced and S-pyridylethylated according to Friedman et al. (1970). A portion of the activated protein C sample (approximately 10%) had previously been treated with tritiated iPr_2PF to label the active-site serine (Kisiel et al., 1976a). Salts and excess reagents were removed by gel filtration in a Sephadex G-25 column (2.6 × 65 cm) previously equilibrated with 2.3 M formic acid. The heavy and light chains were separated by gel filtration in a Sephadex G-150 column (2.6 × 95 cm) previously equilibrated with 2.3 M formic acid containing 3 M urea. Salt-free protein was obtained by gel filtration in Sephadex G-25 equilibrated with 1.2 M formic acid followed by lyophilization.

Amino-terminal sequence analyses were performed with a Beckman sequencer (Model 890B) utilizing the dimethylbenzylamine (DMBA) system developed by Hermodson et al. (1972). Phenylthiohydantoin amino acids were quantitated either directly by high-pressure liquid chromatography (Bridgen et al., 1976) or by gas chromatography after silylation. Amino-terminal analysis of the heavy chain of activated protein C was carried out on a single preparation of the *S*-pyridylethyl peptide. Approximately 100 nmol of the heavy chain (assuming a mol wt of 35 000) was subjected to analysis. The analysis of the cyanogen bromide fragment containing the active site of activated protein C was carried out on two different preparations of the *S*-pyridylethyl peptide. In each case, approximately 150 nmol of the peptide (assuming a mol wt of 6000) was subjected to sequence analysis.

Cyanogen bromide digestion of the heavy chain of activated protein C was carried out by dissolving 60 mg of salt-free pyridylethylated heavy chain and 120 mg of cyanogen bromide in 60 mL of 18.7 M formic acid and incubating the reaction mixture at 4 °C for 24 h. The reaction was terminated by a tenfold dilution with water and subsequently lyophilized. The cyanogen bromide fragments of the pyridylethylated heavy chain of activated protein C were separated by gel filtration in a Sephadex G-75 column (1.6 × 95 cm) previously equilibrated with 2.3 M formic acid containing 3 M urea. The active-site cyanogen bromide peptide was monitored in the column effluent by following the presence of radioactivity. The peptides were desalted by gel filtration in a Sephadex G-25

[2] The nomenclature for the various coagulation factors is that recommended by an international nomenclature committee (Wright, 1959).

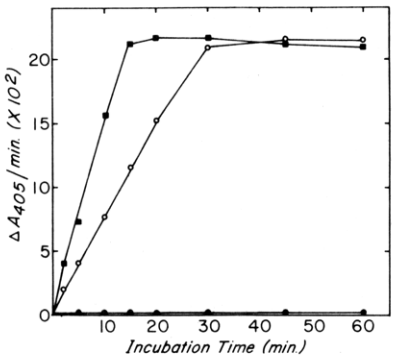

FIGURE 1: Time curve for the conversion of protein C to an enzyme by thrombin. Protein C (5 mg) was activated at 37 °C with 100 μg of thrombin in the presence of 0.05 M Tris-HCl (pH 8.0) in a final volume of 5 mL. At the desired time, 0.1 mL of the reaction mixture was transferred to a test tube containing 10 μg of bovine antithrombin III and 10 μg of heparin in 10 μL of 0.05 M Tris-HCl–0.1 M NaCl (pH 7.5). Amidase activity was measured as described under Methods: (■) complete reaction; (●) iPr_2PF-treated sample; (○) activation of protein C (5 mg) by 125 μg of RVV-X at 37 °C in the presence of 0.05 M Tris-HCl–5 mM $CaCl_2$ (pH 8.0) in a final volume of 5 mL. In the protein C–RVV-X reaction mixture, 0.1-mL aliquots were withdrawn and made 20 mM in EDTA prior to assay.

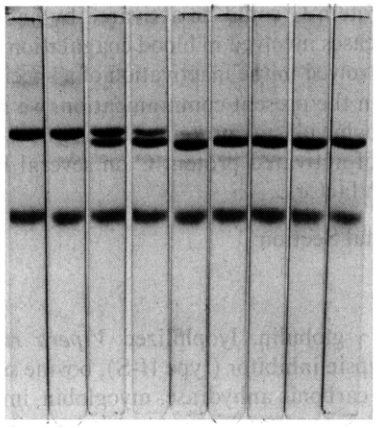

FIGURE 2: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of protein C following activation by thrombin. Each sample (20 μL) containing 20 μg of protein was removed from the reaction mixture corresponding to that shown in Figure 1 and incubated at 100 °C for 2 min with 5% sodium dodecyl sulfate in the presence of 5% 2-mercaptoethanol. Electrophoresis was carried out as described under Methods. The numbers at the bottom of the tubes represent incubation times. The anode was at the bottom of the gel.

column previously equilibrated with 1.2 M formic acid, and the salt-free peptides were subsequently lyophilized.

Factor VII was assayed as previously described (Kisiel and Davie, 1975). Prothrombin and factor X were assayed by one-stage methods according to Hjort et al. (1955) and Bachmann et al. (1958), respectively. Factor X_a was assayed by the same procedure as factor X except RVV-X was omitted from the assay. Factor IX and factor IX_a were measured by a one-stage assay as described by Fujikawa et al. (1973). The kaolin–cephalin clotting time was determined by a slight modification of the procedure described by Biggs (1976). In this assay, 0.1 mL of pooled, bovine plasma was incubated with a 0.1-mL solution of kaolin (5 mg/mL) in cephalin for 5 min at 37 °C followed by the successive additions of 0.1 mL of test sample and 0.1 mL of 0.025 M calcium chloride. Factor V activity was determined according to the procedure of Kappeler (1955). The assay was standardized with citrated pooled bovine plasma and test samples were diluted to produce clotting time in the 18–35-s range of the calibration curve. One unit of factor V activity is arbitrarily defined as the amount present in 1 mL of normal bovine plasma.

The amidase activity of activated protein C was measured according to Svendsen et al. (1972) by a fixed-time assay with various chromogenic substrates. In the assay, 10 μL of activated protein C containing 5–30 μg of protein was added to 2.0 mL of 0.10 mM substrate–0.05 M Tris-HCl–0.05 M imidazole-HCl buffer (pH 8.3) containing 1 mM $CaCl_2$ and 0.1 M NaCl. The reaction mixture was incubated at 37 °C for 1–5 min and the reaction was stopped by the addition of 0.3 mL of glacial acetic acid. The absorbance was then read at 405 nm.

Isolation of Activated Protein C. The isolation of protein C following proteolysis by thrombin was carried out as follows. Protein C (100 mg) was incubated for 1 h at 37 °C with 2–4 mg of α-thrombin in the presence of 0.05 M Tris–H_3PO_4 (pH 8.0) in a final volume of 40 mL. Following a 1-h incubation, the reaction mixture was made 1 mM in benzamidine and the pH of the solution reduced to 6.5 with 1 M H_3PO_4. The incubation mixture was applied to a SP-Sephadex C-50 column (2.6 × 30 cm) previously equilibrated at room temperature with 0.05 M sodium phosphate (pH 6.5) containing 1 mM benzamidine. After application of the sample, the column was washed with 1–2 column volumes of the equilibration buffer. Activated protein C appeared in the void volume and was subsequently concentrated by ultrafiltration and stored at −20 °C. No detectable thrombin activity (as measured by its ability to clot a 0.4% fibrinogen solution) was observed in the nonadsorbed fraction containing the activated protein C. Thrombin could, however, be eluted quantitatively from the SP-Sephadex column by elution with 0.3 M sodium phosphate buffer (pH 6.5).

Results

Activation of Protein C by Thrombin. Incubation of protein C with purified α-thrombin at an enzyme-to-substrate weight ratio of 1:50 resulted in the rapid conversion of protein C to activated protein C. This conversion was conveniently monitored by following the appearance of amidase activity toward Bz-Phe-Val-Arg-*p*-nitroanilide as well as analysis by sodium dodecyl sulfate–polyacrylamide gel electrophoresis following reduction with 2-mercaptoethanol. A time curve for the conversion of protein C to activated protein C is shown in Figure 1. In this experiment, protein C was incubated with thrombin at pH 8.0 at a weight ratio of substrate-to-enzyme of 50:1. Aliquots were removed at various times and the activation terminated by the addition of bovine antithrombin III and heparin. In control experiments, it was observed that bovine antithrombin III, in the presence or absence of heparin, had little effect on the amidase activity of activated protein C (K. Kurachi, unpublished data), while greater than 95% of the thrombin activity was inhibited. Amidase activity reached a maximum in approximately 20 min (solid squares, Figure 1) and iPr_2PF treatment completely inhibited this activity (solid circles, Figure 1). The activation of protein C by RVV-X is also shown in figure 1 for comparison (open circles). In the latter experiments, the reaction was complete in approximately 30 min employing a weight ratio of substrate-to-enzyme of 40:1.

Evidence of a change in the molecular weight of protein C during its activation by thrombin was obtained by sodium dodecyl sulfate–polyacrylamide gel electrophoresis following reduction with 2-mercaptoethanol (Figure 2). The zero-time sample showed two bands, corresponding to the heavy and light

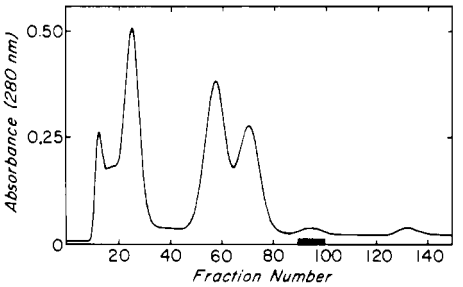

FIGURE 3: Gel filtration of a cyanogen bromide digest of the S-pyridylethylated heavy chain of activated protein C. The lyophilized digest (60 mg) was dissolved in 5 mL of 2.3 M formic acid containing 3 M urea and applied to a Sephadex G-75 column (2.6 × 95 cm) previously equilibrated with the same solution. The cyanogen bromide fragments were eluted with 2.3 M formic acid in 3 M urea at a flow rate of 45 mL/h. Fractions were collected in 3 mL/tube. The fraction shown by the solid bar was pooled, lyophilized, and subjected to sequence analysis.

chains of protein C. With time, a reduction in the molecular weight of the heavy chain of protein C from 41 000 to 39 000 was observed. Under these conditions, the conversion was essentially complete in 15 min. No change in the molecular weight of the light chain was observed during the activation reaction. The activation of protein C by thrombin, as monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in the presence or absence of 2-mercaptoethanol, appeared indistinguishable from that observed following the activation of protein C by RVV-X (Kisiel et al., 1976a). In order to more definitively demonstrate that protein C activated by thrombin was indeed identical with that obtained by RVV-X activation, a preparative scale activation of protein C was performed with thrombin and the activated protein C separated from thrombin by SP-Sephadex C-50 chromatography. By this procedure, activated protein C appeared in the void volume while thrombin remained tightly bound to the ion exchange resin. The activated protein C possessed significant amidase activity toward Bz-Phe-Val-Arg-*p*-nitroanilide, and this activity was readily inhibited by 5 mM iPr_2PF or $PhCH_2SO_2F$. Incubation of activated protein C with tritiated iPr_2PF followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in the presence of reducing agent revealed that the radiolabeled inhibitor was present exclusively in the heavy chain of the enzyme. These experiments demonstrate that α-thrombin converts protein C to a serine enzyme which is essentially identical with that formed after activation by trypsin or RVV-X (Kisiel et al., 1976a).

Amino-Terminal and Active-Site Sequences of Activated Protein C. Amino-terminal analysis of the heavy chain of protein C obtained after thrombin proteolysis was carried out in a Beckman sequenator. The amino-terminal sequence for the first eight residues was Ile-Val-Asp-Gly-Gln-Glu-Ala-Gly. This sequence is identical with the amino-terminal sequence of the heavy chain of protein C obtained after activation by either RVV-X or trypsin (Kisiel et al., 1976a). These data indicate that thrombin cleaves a peptide bond between Arg-14 and Ile-15 of the heavy chain of protein C with the concomitant formation of activated protein C.

It was also of interest to examine the amino acid sequence of activated protein C in the active-site region. Amino acid analyses of the heavy chain of protein C indicated the presence of four methionine residues (Kiseil et al., 1976a). Thus, the heavy chain should yield five peptides upon digestion with cyanogen bromide. Accordingly, the *S*-pyridylethyl heavy chain of activated protein C, which had been previously labeled (approximately 10%) with radioactive iPr_2PF, was digested with cyanogen bromide and the peptide fragments were then fractionated by gel filtration on a Sephadex G-75 column (Figure 3). Six ultraviolet-absorbing peaks were obtained. The active-site fragment (shown by the solid bar) was identified in the eluent by its radioactivity. Analysis of the active-site peptide by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 10% polyacrylamide gels revealed a radioactive band with a mobility corresponding to an apparent mol wt of about 6000. This band did not stain, however, with Coomassie brilliant blue. Amino-terminal analyses of the active-site peptide indicated leucine as the amino-terminal residue (approximately 0.75 equiv per 6000 g of protein). The sequence of the first 18 residues of this polypeptide is shown in Figure 4 along with the reactive site sequences of thrombin, factor IX_a, factor VII, and factor X_a. The repetitive yields for the degradations were about 95%. The active-site serine corresponding to serine-195 in chymotrypsin appeared in position 17 of the peptide. These data indicate that activated protein C contains an active-site amino acid sequence which is homologous with that of the four vitamin K dependent serine proteases involved in blood coagulation.

Amidase Activity of Activated Protein C. Activated protein C, free of any detectable thrombin activity, exhibited significant amidase activity toward the chromogenic substrate Bz-Phe-Val-Arg-*p*-nitroanilide (S-2160). The specific activity of the enzyme for this substrate increased about threefold in the presence of 1-10 mM calcium chloride. Prior treatment of the enzyme with 1 mM ethylenediaminetetraacetic acid (EDTA) to remove endogenous calcium ions resulted in virtually a complete loss of amidase activity. The amidase activity, however, was restored by the addition of calcium ions. Accordingly, a study was initiated to measure the effect of calcium ion concentration on activated protein C amidase activity toward this substrate. Activated protein C was incubated with 1 mM EDTA for 60 min and the EDTA-protein sample was dialyzed against 0.05 M Tris-HCl-0.1 M NaCl (pH 7.5) overnight at 4 °C. Activated protein C was then assayed for amidase activity at several calcium ion concentrations ranging from 0 to 10 mM. The results of these experiments revealed that increasing the calcium ion concentration had a hyperbolic effect on the amidase activity of activated protein C. A maximum rate stimulation of about 20-fold was observed between 0 and 0.5 mM $CaCl_2$. When phospholipid (bovine brain cephalin) was added to the assay system at several concentrations (6-60 μg/mL), no significant change of amidase activity was observed in the presence of 1 mM $CaCl_2$.

Activated protein C was also tested for its amidase activity toward several other chromogenic amidase substrates. The enzyme used in these studies was initially treated with 1 mM EDTA followed by dialysis against 0.05 M Tris-HCl-0.1 M NaCl (pH 7.5). The enzyme was tested with each substrate in the presence of 1 mM $CaCl_2$ employing a substrate concentration of 0.1 mM (Table I). No hydrolysis was observed when activated protein C was tested with S-2227 and S-2251. These substrates are particularly good substrates for urokinase and plasmin, respectively (manufacturer's data sheet, Kabi Diagnostica). Weak amidase activity was observed with the factor X_a specific substrate, S-2222, while the remaining three substrates were all hydrolyzed by activated protein C almost as well or better than that observed with S-2160. These latter substrates are also readily hydrolyzed by thrombin at approximately equal rates. These data indicate that activated protein C has a substrate specificity more similar to that of thrombin than factor X_a or plasmin.

As observed in a previous study (Kisiel et al., 1976a), the amidase activity of activated protein C was readily inhibited

Protein C	Leu	Cys	Ala	Gly	Ile	Leu	-	Gly	Asp	Pro	-	Arg	-	Asp	Ala	Cys	Glu	Gly	Asp	SER	Gly
Thrombin[a]	Phe	Cys	Ala	Gly	Tyr	Lys	Pro	Gly	Glu	Gly	Lys	Arg	Gly	Asp	Ala	Cys	Glu	Gly	Asp	SER	Gly
Factor IX_a[b]	Phe	Cys	Ala	Gly	Tyr	His	-	Glu	Gly	Gly	Lys	-	-	Asp	SER	Cys	Gln	Gly	Asp	SER	Gly
Factor VII[c]	Phe	Cys	Ala	Gly	Tyr	Thr	-	Asp	Gly	Thr	Lys	-	-	Asp	Ala	Cys	Lys	Gly	Asp	SER	Gly
Factor X_a[d]	Phe	Cys	Ala	Gly	Tyr	Asp	-	Thr	Gln	Pro	Glu	-	-	Asp	Ala	Cys	Gln	Gly	Asp	SER	Gly

FIGURE 4: Active-site sequence of activated protein C and the four vitamin K dependent coagulation proteins. Amino acid residues in protein C that are identical with the coagulant proteins are shown in blocks. Dashes refer to spaces that have been inserted to bring the five proteins into alignment for better homology. The active-site serine analogous to serine-195 in chymotrypsin is shown in capital letters: (a) from Magnusson et al., 1975; (b) from Enfield et al., 1974; (c) from Kisiel et al., 1977; (d) from Titani et al., 1975.

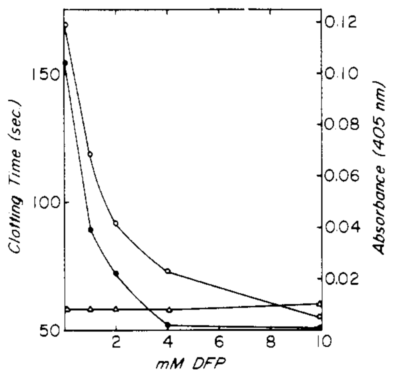

FIGURE 5: The effect of various concentrations of iPr_2PF on the anticoagulant and amidase activities of activated protein C. Activated protein C (500 μg) was incubated at room temperature for 2 h with various concentrations of iPr_2PF in the presence of 0.05 M Tris-HCl-0.1 M NaCl (pH 7.5) in a final volume of 1 mL. In addition, 1-mL quantities of buffer were treated with the same concentrations of iPr_2PF to serve as controls. The anticoagulant activity of activated protein C (or buffer control) following iPr_2PF treatment was measured in the kaolin-cephalin clotting assay as described under Methods after a 100-fold dilution with 0.05 M Tris-HCl-0.1 M NaCl (pH 7.5) containing 1 mg/mL bovine serum albumin. Amidase activity was measured as described under Methods employing 10 μg of iPr_2PF-treated activated protein C. The reaction mixture was incubated at 37 °C for 3 min and the reaction was stopped by the addition of acetic acid: (O) kaolin-cephalin clotting time of iPr_2PF-treated activated protein C samples; (●) amidase activity of iPr_2PF-treated activated protein C samples; (Δ) kaolin-cephalin clotting time of iPr_2PF-treated buffer control samples following 100-fold dilution.

TABLE I: Amidase Activity of Activated Protein C.

Substrate	Designation	Sp act.[a]	Rel act.
Bz-Phe-Val-Arg-pNA	S-2160	3.57	1.00
Bz-Ile-Glu-Gly-Arg-pNA	S-2222	0.35	0.10
L-Glu-Gly-Arg-pNA	S-2227	0.06	0.02
D-Phe-Pip-Arg-pNA	S-2238	4.21	1.18
D-Val-Leu-Lys-pNA	S-2251	0.00	0.00
D-Val-Leu-Arg-pNA	S-2266	4.38	1.23
D-Pro-Phe-Arg-pNA	S-2302	2.45	0.68

[a] μmol hydrolyzed min^{-1} mg^{-1}. Amidase activity was determined as described under Methods employing 10 μg of activated protein C and 0.1 mM substrate. The reaction mixture was incubated at 37 °C for 2 min and the reaction terminated by the addition of glacial acetic acid.

by iPr_2PF. In addition, when activated protein C (1.5 mg/mL) was incubated at room temperature for 2 h in the presence of 8 mM $PhCH_2SO_2F$, about 80% of the amidase activity was lost when compared to a control sample. Purified soybean trypsin inhibitor, at a tenfold molar excess to enzyme, had no effect on the amidase activity of activated protein C.

Anticoagulant Properties of Activated Protein C. Earlier work indicated that activated protein C, but not its precursor, markedly prolonged the partial thromboplastin time of bovine plasma (Kisiel et al., 1976a). Accordingly, experiments were initiated to determine if the apparent anticoagulant activity of activated protein C was dependent in part or totally on its enzymatic activity, and to determine what protein(s) participating in blood coagulation was affected. Initial studies indicated that as little as 0.1 μg of bovine activated protein C increased the kaolin-cephalin clotting time of bovine plasma about threefold. Activated protein C employed in these experiments was completely free of thrombin. In striking contrast, as much as 10 μg of bovine activated protein C had no significant effect on the kaolin-cephalin clotting time of human plasma. When activated protein C was treated with various concentrations of iPr_2PF for a fixed time and subsequently assayed for amidase and anticoagulant activity, a parallel decline of amidase and anticoagulant activity was observed (Figure 5). Essentially the same results were observed when $PhCH_2SO_2F$ was used instead of iPr_2PF. In these experiments, however, the rate of inhibition of activated protein C at comparable inhibitor concentrations was significantly lower. Nonetheless, these data definitely demonstrate that the apparent anticoagulant effect of activated protein C is totally dependent on its amidase activity.

In order to investigate at which point in the coagulation scheme activated protein C exerted its inhibitory effect, the enzyme was incubated with a number of purified coagulation proteins in the presence and absence of 2 mM $CaCl_2$ and phospholipid. The various incubations were carried out in 0.05 M Tris-HCl-0.1 M NaCl (pH 7.5) in plastic tubes at 37 °C employing a weight ratio of activated protein C to coagulation factor of 1:10. Aliquots of the incubation mixtures were then removed at 5-min intervals for a total period of 1 h. Under these conditions, activated protein C had no detectable effect on the coagulant activities of factor XII or factor XII_a, factor XI or factor XI_a, factor X or factor X_a, factor IX or factor IX_a, or factor VII. In addition, no change in the primary structures of prothrombin, thrombin, factor X, factor X_a, factor IX, or factor IX_a was detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis following incubation with activated protein C. Moreover, activated protein C had no effect on bovine plasminogen or fibrinogen as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis following reduction with 2-mercaptoethanol.

When activated protein C was incubated with a highly purified preparation of bovine factor V, however, a rapid decrease of factor V activity was noted. The inactivation of factor V by activated protein C was found to show an absolute dependence on the addition of phospholipid and calcium ions. Incubation of factor V with activated protein C and calcium ions or with activated protein C and phospholipid at 37 °C had no significant effect on factor V activity over a 30-min incubation period. Moreover, incubation of factor V with phospholipid and cal-

cium ions in the absence of activated protein C caused no detectable change in factor V activity in a comparable time period. Figure 6 shows the effect of increasing concentrations of activated protein C (15 to 300 ng/mL) on factor V activity at approximately physiological concentrations of factor V (1 unit/mL). In these experiments, activated protein C was preincubated at 37 °C for 1 min at the designated concentrations with 2 mM $CaCl_2$ and phospholipid in 0.05 M Tris-HCl-0.1 M NaCl (pH 7.5) containing 0.1% bovine serum albumin. The phospholipid concentration in the incubation mixture was approximately 60 μg/mL. Factor V was then added to the preincubation mixture and aliquots were removed at various times and assayed for factor V activity. As shown in Figure 6, factor V activity rapidly decreased employing concentrations of activated protein C as low as 15 ng/mL. In the absence of activated protein C, the factor V activity remained essentially unchanged. The ability of activated protein C to inactivate factor V was found to be totally dependent on its enzymatic activity since iPr_2PF-treated preparations of activated protein C, devoid of amidase activity, had no effect on factor V activity at 1000 ng/mL final concentration in the presence of phospholipid and calcium ions.

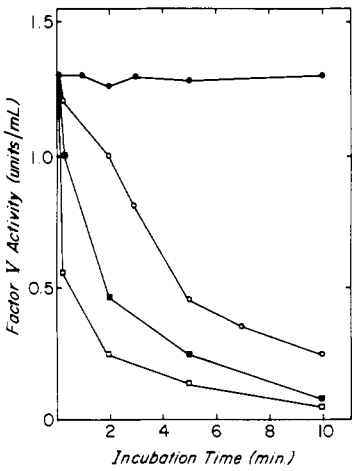

FIGURE 6: Effect of increasing concentrations of activated protein C on factor V activity. Factor V (16 μg) was incubated at 37 °C with increasing concentrations of activated protein C in the presence of 2 mM $CaCl_2$, 60 μg of phospholipid (cephalin), 0.1 M NaCl, 1 mg of bovine serum albumin, and 0.05 M Tris-HCl (pH 7.5) in a final volume of 1 mL. At the desired time, 100 μL of the reaction mixture was withdrawn, diluted approximately five- to tenfold with 0.05 M Tris-HCl-0.1 M NaCl (pH 7.5) containing 1 mg/mL bovine serum albumin and assayed for factor V as described under Methods. The final concentration of activated protein C in the incubation mixture was: (●) 0 ng/mL; (○) 15 ng/mL; (■) 75 ng/mL; (□) 300 ng/mL.

Discussion

The present data demonstrate that bovine protein C is readily converted to a serine enzyme by α-thrombin employing an enzyme-to-substrate weight ratio of 1:50. In this reaction, thrombin cleaves an Arg-Ile bond between residues 14 and 15 in the amino-terminal region of the heavy chain of protein C. Cleavage of the tetradecapeptide by thrombin results in the formation of a new amino-terminal sequence of Ile-Val-Asp-Gly in the heavy chain of activated protein C. This cleavage is highly specific as no degradation of activated protein C was observed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis after 3 h of incubation. The mechanism of activation of protein C by thrombin appears to be indistinguishable from that observed in a previous study employing trypsin or RVV-X as activators of protein C (Kisiel et al., 1976a).

The amino acid sequence in the active site of activated protein C is homologous with that found in the active-site regions of the four vitamin K dependent coagulation factors (Figure 4). Consequently, it seems highly probable that the basic mechanism for the proteolytic activation of protein C is similar to that observed in the activation of these coagulation factors. Activation of these proenzymes also involves the cleavage of a peptide bond in the amino-terminal portion of the polypeptide chain containing the active serine. In the course of the activation of these proteins, a new amino-terminal isoleucine or valine is generated and this residue probably forms an ion pair with the carboxyl group of the aspartic acid adjacent to the active serine analogous to the pancreatic serine proteases (Sigler et al., 1968; Stroud et al., 1975). These reactions lead to the formation of the "charge relay system" which is typical of serine proteases (Matthews et al., 1967; Sigler et al., 1968; Blow et al., 1969; Shotton and Watson, 1970). Activated protein C contains an aspartic acid residue at position 189. In trypsin, this residue forms an ion pair with a basic amino acid residue in the substrate (Mares-Guia and Shaw, 1965; Ruhlmann et al., 1973; Blow et al., 1974; Sweet et al., 1974; Krieger et al., 1974). Presumably, the aspartic acid residue at position 189 in activated protein C also confers this enzyme with specificity toward basic amino acids.

In relation to the vitamin K dependent coagulation proteins, the highest degree of homology in the active-site region of activated protein C is found with that of thrombin. This is particularly interesting in view of the similarities observed in the substrate specificity of these two enzymes using several tripeptide *p*-nitroanilides as substrates. The amidase activity of activated protein C was found to be markedly affected by calcium ions. Preincubation of activated protein C with EDTA resulted in only marginal amidase activity of the enzyme which was restored to a maximal level by the addition of 0.5–1 mM $CaCl_2$. This observation suggests that protein C was apparently isolated as a metalloprotein complex. Preliminary results of calcium binding experiments with activated protein C by the equilibrium dialysis technique indicate that at 0.5 mM $CaCl_2$ approximately 1–2 mol of calcium ions is bound per mol of protein (W. Kisiel, unpublished data). This suggests that the binding of as little as 1–2 mol of calcium per mol of activated protein C is sufficient for maximal amidase activity as measured with the chromogenic substrate S-2160. Whether this degree of calcium binding promotes maximal biological activity remains to be shown.

The anticoagulant property of activated protein C observed in a previous study (Kisiel et al., 1976a) has been confirmed and investigated further in the present study. Activated protein C markedly prolongs the kaolin-cephalin clotting time of bovine plasma but was totally ineffective when human plasma was employed. When activated protein C was incubated with several highly purified preparations of bovine coagulation factors in the presence of phospholipid and calcium ions, only the activity of factor V was affected. The inactivation of factor V by activated protein C occurred at extremely low concentrations of the enzyme, in the presence of phospholipid and calcium ions, and rapidly inactivated factor V at physiological concentrations. Also, activated protein C was at least 100 times more effective than thrombin on a weight basis in the inactivation of factor V (W. Kisiel, unpublished data). These experiments suggest that activated protein C effects a change in the factor V molecule by minor proteolysis resulting in its loss of activity. It cannot be ruled out, however, that activated protein C acts indirectly in this process by activating some other protein in the factor V preparation which in turn inactivates factor V. The ability of activated protein C to inactivate factor V was absolutely dependent on its enzymatic activity

as iPr_2PF-treated preparations of activated protein C had no effect on factor V activity in the presence of phospholipid and calcium ions.

Factor V is a plasma glycoprotein which is an important component of the prothrombinase complex. Current evidence indicates that factor V has no apparent enzymatic activity and exerts its effect by augmenting the proteolytic activity of factor X_a. Furthermore, evidence from several studies suggests that factor V circulates in an inactive, high molecular weight form and must be activated by limited proteolysis to a lower molecular weight form prior to its participation in the prothrombinase complex (Smith and Hanahan, 1976). From the results of this study, it is unclear whether high molecular weight factor V is affected by activated protein C. The factor V preparation employed in this study, when gel filtered through a calibrated column of Sephadex G-200, eluted in two activity peaks with K_d values of 0.035 and 0.157. These distribution coefficients correspond to molecular weights of 450 000 and 210 000, respectively. The majority of the factor V activity (ca. 80%) was associated with the lower molecular weight protein. Thus, the possibility that activated protein C either specifically inactivates the low molecular weight form of factor V (i.e., factor V_a) or modifies the high molecular weight factor V rendering it incapable of activation will require further investigation.

In view of the anticoagulant nature of activated protein C, a major objective of this investigation was to determine whether protein C could be activated by one of the serine proteases involved in coagulation. The present data clearly demonstrate the activation of protein C by α-thrombin by limited proteolysis. In contrast, incubation of protein C at pH 7.5 with either a complex of factor X_a-phospholipid-calcium ions or factor VII-tissue factor-calcium ions revealed little (ca. 10%) activation of protein C in 60 min as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. In these experiments, the weight ratios of protein C to factor X_a and factor VII were 10:1 and 25:1, respectively. Thus, it would appear that neither factor X_a nor factor VII is physiologically capable of activating protein C. It remains to be shown whether other activated coagulation factors, particularly those involved in the early phase of intrinsic coagulation, are capable of activating protein C.

The primary physiological role of protein C in bovine plasma is not known. The present data suggest one possibly unique role for protein C as it relates to the coagulation system. Assuming a physiological concentration of 5000 ng of protein C per mL of blood (Stenflo, 1976) and a minimal concentration of 40–50 ng of thrombin per mL of blood to effect clot formation (Shuman and Majerus, 1976), it is possible that at this substrate-to-enzyme weight ratio a small percentage of protein C is converted to activated protein C. Furthermore, the activation of about 1% of protein C (i.e., 50 ng/mL) would result in a sufficient concentration of activated protein C to rapidly inactivate factor V at physiological concentrations. Additional experiments are required, however, to support this hypothesis.

The mechanism whereby activated protein C is neutralized and cleared from the blood stream is particularly enigmatic. The anticoagulant potency of activated protein C decreased very slowly upon incubation in bovine plasma suggesting that it is inactivated very slowly by proteolysis or by plasma serine protease inhibitors. As previously noted, purified bovine antithrombin III in the presence or absence of heparin had little effect on the enzyme. Thus, other mechanisms may exist for the neutralization of activated protein C under physiological conditions.

Acknowledgments

The authors express their sincere thanks to Drs. K. Fujikawa and K. Kurachi of this laboratory for providing highly purified preparations of bovine factor XII and factor XI, respectively. Thanks are also due to Dr. D. J. Hanahan for the preparation of bovine factor V, and to Dr. Kenneth Walsh for valuable discussions. The excellent technical assistance of Karen Lebens, Maurene Cronyn, and Lynn Schrader was also greatly appreciated. The authors are indebted to the P. D. and J. Meats Company of Kent, Wash., for kindly supplying the bovine blood employed in these experiments.

References

Bachmann, F., Duckert, F., and Koller, F. (1958), *Thromb. Diath. Haemorrh. 2*, 24.

Bell, W. N., and Alton, H. G. (1954), *Nature (London) 174*, 880.

Biggs, R. (1976), in Human Blood Coagulation, Hemostasis and Thrombosis, Biggs, R., Ed., Oxford, Blackwell Scientific Publications, p 677.

Blow, D. M., Birktoft, J. J., and Hartley, B. S. (1969), *Nature (London) 249*, 54.

Blow, D. M., Janin, J., and Sweet, R. M. (1974), *Nature (London) 249*, 54.

Bridgen, P. J., Cross, G. A. M., and Bridgen, J. (1976), *Nature (London) 263*, 613.

Davie, E. W., and Fujikawa, K. (1975), *Annu. Rev. Biochem. 44*, 799.

Enfield, D. L., Ericsson, L. H., Fujikawa, K., Titani, K., Walsh, K. A., and Neurath, H. (1974), *FEBS Lett. 47*, 132.

Esmon, C. T., Owen, W. G., and Jackson, C. M. (1974), *J. Biol. Chem. 249*, 7798.

Esmon, C. T., Stenflo, J., Suttie, J. W., and Jackson, C. M. (1976), *J. Biol. Chem. 251*, 3052.

Frattali, V., and Steiner, R. F. (1969), *Anal. Biochem. 27*, 285.

Friedman, M., Krull, L. H., and Cavins, J. F. (1970), *J. Biol. Chem. 245*, 3868.

Fujikawa, K., Legaz, M. E., and Davie, E. W. (1972), *Biochemistry 11*, 4882.

Fujikawa, K., Thompson, A. R., Legaz, M. E., Meyer, R. G., and Davie, E. W. (1973), *Biochemistry 12*, 4938.

Fujikawa, K., Walsh, K. A., and Davie, E. W. (1977), *Biochemistry 16*, 2270.

Hermodson, M. A., Ericsson, L. H., Titani, K., Neurath, H., and Walsh, K. A. (1972), *Biochemistry 11*, 4493.

Hjort, P., Rapaport, S. I., and Owren, P. A. (1955), *J. Lab. Clin. Med. 46*, 89.

Kappeler, R. (1955), *Z. Klin. Med. 153*, 103.

Kisiel, W., and Davie, E. W. (1975), *Biochemistry 14*, 4928.

Kisiel, W., Ericsson, L. H., and Davie, E. W. (1976a), *Biochemistry 15*, 4893.

Kisiel, W., Fujikawa, K., and Davie, E. W. (1977), *Biochemistry 16*, 4189.

Kisiel, W., Hermodson, M. A., and Davie, E. W. (1976b), *Biochemistry 15*, 4901.

Koide, T., Kato, H., and Davie, E. W. (1977), *Biochemisty 16*, 2279.

Krieger, M., Kay, L. M., and Stroud, R. M. (1974), *J. Mol. Biol. 83*, 203.

Kurachi, K., Schmer, G., Hermodson, M. A., Teller, D. C., and Davie, E. W. (1976), *Biochemistry 15*, 368.

Lewis, M. L., and Ware, A. G. (1953), *Proc. Soc. Exp. Biol.*

Med. 84, 640.
Lindquist, P. A., Fujikawa, K., and Davie, E. W. (1978), *J. Biol. Chem.* (in press).
Lundblad, R. L., Uhteg, L. C., Vogel, C. N., Kingdon, H. S., and Mann, K. G. (1975), *Biochem. Biophys. Res. Commun. 66,* 482.
Magnusson, S., Petersen, T. E., Sottrup-Jensen, L., and Claeys, H. (1975), *Cold Spring Harbor Conf. Cell Proliferation 2,* 123.
Mares-Guia, M., and Shaw, E. (1965), *J. Biol. Chem. 240,* 1579.
Matsuda, M., Iwanaga, S., and Nakamura, S. (1972), *Thromb. Res. 1,* 619.
Matthews, B. W., Sigler, P. B., Henderson, R., and Blow, D. M. (1967), *Nature (London) 214,* 652.
McPhie, P. (1971), *Methods Enzymol. 22,* 23.
Ruhlmann, A., Kulka, D., Schwager, P., Bartels, K., and Huber, R. (1973), *J. Mol. Biol. 77,* 417.
Shotton, D. M., and Watson, H. C. (1970), *Nature (London) 225,* 811
Shuman, M. A., and Majerus, P. W. (1976), *J. Clin. Invest. 58,* 1249.
Sigler, P. W., Blow, D. M., Matthews, B. W., and Henderson, R. (1968), *J. Mol. Biol. 35,* 143.
Smith, C. M., and Hanahan, D. J. (1976), *Biochemistry 15,* 1830.
Stenflo, J. (1976), *J. Biol. Chem. 251,* 355.
Stroud, R. M., Krieger, M., Koeppe, R. E., Kossiakoff, A. A., and Chambers, J. L. (1975), *Cold Spring Harbor Conf. Cell Proliferation 2,* 13.
Svendsen, L., Blomback, B., Blomback, M., and Olsson, P. I. (1972), *Thromb. Res. 1,* 267.
Sweet, R. M., Wright, H. T., Janin, J., Chothia, C. H., and Blow, D. M. (1974), *Biochemistry 13,* 4212.
Titani, K., Fujikawa, K., Enfield, D. L., Ericsson, L. H., Walsh, K. A., and Neurath, H. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 3082.
Walther, P. J., Steinman, H. M., Hill, R. L., and McKee, P. A. (1974), *J. Biol. Chem. 249,* 1173.
Winzor, D. J., and Scheraga, H. A. (1964), *Arch. Biochem. Biophys. 104,* 202.
Wright, I. (1959), *J. Am. Med. Assoc. 170,* 325.

Activation of Human Factor XI (Plasma Thromboplastin Antecedent) by Factor XII_a (Activated Hageman Factor)†

Kotoku Kurachi and Earl W. Davie*

ABSTRACT: Factor XI (plasma thromboplastin antecedent) is a plasma protein that participates in the early phase of blood coagulation. It was isolated from human plasma by barium citrate adsorption of the vitamin K dependent proteins followed by ammonium sulfate fractionation, heparin-agarose, carboxymethyl-Sephadex, and benzamidine-agarose column chromatography. Factor XI is a glycoprotein containing 5% carbohydrate and has a molecular weight of about 124 000. It is composed of two identical polypeptide chains (mol wt about 60 000), and these two chains are held together by a disulfide bond(s). Its amino-terminal sequence is Gly-Cys-Val-Thr-Gln-Leu-Leu-Lys-Asp-Thr-Gln-Phe-Glu-Gly-Gly-. Factor XI is converted to an enzyme, factor XI_a, by factor XII_a (activated Hageman factor). This reaction readily occurs with an enzyme-to-substrate ratio of 1 to 50. Factor XI_a is composed of a pair of heavy chains (mol wt approximately 35 000) and a pair of light chains (mol wt approximately 25 000). These four chains are held together by disulfide bonds. The amino-terminal sequence of the heavy chains is Gly-Cys-Val-Thr-Gln-Leu-Leu-Lys-Asp-Thr-Gln-Phe-Glu-Gly-Gly-Asp-Ile- which is identical with that found in the precursor molecule. The amino-terminal sequence of the light chains is Ile-Val-Gly-Gly-Thr-Val-Ala- ? -His-Gly-Glu-Trp-Pro-Trp-Gln-Val-. This chain also contains the active site sequence of Ile-Cys-Ala-Gly-Tyr-Arg-Glu-Gly-Gly-Lys-Asp-Ala-Cys-Lys-Gly-Asp-SER-Gly-Gly-Pro-. The active site serine is shown in capital letters. These data indicate that factor XI is converted to factor XI_a by the cleavage of an internal peptide bond in each of the two precursor chains. Human factor XI_a is inhibited by diisopropyl phosphorofluoridate and antithrombin III. The inhibitor in each case is bound to the light chain of factor XI_a which contains the active site serine residue. The stoichiometry of the complex formed between factor XI_a and antithrombin III was shown to be 1 mol of enzyme combined with 2 mol of inhibitor. These data further support the conclusion that factor XI_a is a serine protease containing two active sites per mole of enzyme.

Factor XI (plasma thromboplastin antecedent)[1] is a coagulation protein present in plasma in a precursor form. It participates in the early phase of the coagulation process (Davie and Fujikawa, 1975). Individuals with factor XI deficiency often bleed excessively after injury and minor surgery (Rosenthal et al., 1953). Factor XI deficiency has also been reported in a cow by Kociba et al. (1969).

The isolation and detailed characterization of bovine factor XI has been reported recently from our laboratory (Koide et al., 1977a). It is a glycoprotein (mol wt 124 000) composed of two similar or identical polypeptide chains held together by a disulfide bond(s). It contains an active site serine sequence which is homologous to that of other serine proteases (Koide

† From the Department of Biochemistry, University of Washington, Seattle, Washington 98195. *Received October 5, 1977.* This work was supported in part by Research Grant HL 16919 from the National Institutes of Health.

[1] The nomenclature for the various coagulation factors is that recommended by an international nomenclature committee (Wright, 1959).