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Proteolytic Activation of Protein C from Bovine Plasma†

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ABSTRACT: Protein C is a vitamin K dependent protein present in bovine plasma (Stenflo, J. (1976), *J. Biol. Chem. 251,* 355). It is a glycoprotein (mol wt approximately 62 000) composed of a heavy chain (mol wt 41 000) and a light chain (mol wt 21 000). The heavy chain has an amino-terminal sequence of Asp-Thr-Asn-Gln and contains nearly three-fourths of the carbohydrate. The light chain has an amino-terminal sequence of Ala-Asn-Ser-Phe. Incubation of protein C with either factor X activator from Russell's viper venom or trypsin resulted in the cleavage of an Arg-Ile bond between residues 14 and 15 of the heavy chain. Concomitant with this cleavage was the formation of a serine enzyme which was inhibited by diisopropyl phosphorofluoridate. Liberation of the tetradecapeptide decreased the molecular weight of the heavy chain from about 41 000 to 39 000 and resulted in the formation of a new amino-terminal sequence of Ile-Val-Asp-Gly in the heavy chain. No change in the molecular weight of the light chain was observed during the activation reaction. These results indicate that protein C, like the four vitamin K dependent coagulation proteins, exists in plasma in a precursor form and is converted to a serine protease by hydrolysis of a specific Arg–Ile peptide bond. The biological substrate for the enzymatic form of protein C and the physiological mechanism whereby protein C is converted to a serine enzyme are not known.

It is generally accepted that four coagulation proteins (prothrombin, factor VII, factor IX, and factor X) require vitamin K for their biosynthesis. Present evidence indicates that vitamin K functions in a posttranscriptional step which results in the carboxylation of specific glutamic acid residues to form γ-carboxyglutamic acid residues in the amino-terminal region of these proteins (Shah and Suttie, 1971; Johnson et al., 1972; Stenflo et al., 1974; Magnusson et al., 1974; Nelsestuen et al., 1974). The γ-carboxyglutamic acid residues are required for the binding of calcium to these proteins and their interaction with phospholipid during the coagulation process. The four vitamin K dependent coagulation factors are glycoproteins which exist in plasma as precursors of serine proteases (Davie and Fujikawa, 1975). Prothrombin, factor VII, and factor IX are single-chain proteins, while factor X has been isolated as a two-chain molecule.[1]

Recently, Stenflo (1976) has reported the isolation and characterization of a fifth vitamin K dependent protein from bovine plasma employing an immunochemical assay. This protein, designated protein C, was isolated by a combination of barium citrate adsorption and elution, ammonium sulfate fractionation, and DEAE-Sephadex A-50 chromatography. It is composed of a light chain and a heavy chain, and these chains are held together by a disulfide bond(s). The light chain was found to bind Ca^{2+} and showed considerable homology in its amino-terminal sequence with that of the four vitamin K dependent coagulation factors. In addition, γ-carboxyglutamic acid residues were also observed in the amino-terminal region of the light chain of protein C.

The goal of the present investigation was to determine if

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

protein C, like the other vitamin K dependent proteins, could be converted to a serine esterase by limited proteolysis and whether this enzyme would influence the coagulation system.

Experimental Section

Materials

Heparin sodium salt (grade I, 170 USP units/mg), soybean trypsin inhibitor (type II-S), bovine serum albumin, ovalbumin, carbonic anhydrase, dithiothreitol, lyophilized *Vipera russelli* venom, *N*-acetylneuraminic acid, thiobarbituric acid, and ϵ-aminocaproic acid were purchased from Sigma Chemical Co., St. Louis, Mo. The venom protease which activates factor X (RVV-X)[2] was purified to a homogeneous state as described by Kisiel et al. (1976). Benzamidine hydrochloride, cyclohexanone, and diisopropyl phosphorofluoridate (DFP) were obtained from Aldrich Chemical Co., Milwaukee, Wis. DEAE-Sephadex A-50, Sephadex G-150, and Sephadex G-25 were products of Pharmacia Fine Chemicals, Piscataway, N.J. Monoiodoacetic acid, 2-mercaptoethanol, and *N,N,N′,N′*-tetramethylethylenediamine were purchased from Eastman Kodak Co., Rochester, N.Y. Acrylamide and *N,N′*-methylenebisacrylamide were obtained from Bio-Rad Laboratories, Richmond, Calif. Guanidine hydrochloride was purchased from Heico, Inc., Delaware Water Gap, Pa. Diisopropyl-1-^{3}H-(*N*)-fluorophosphate (0.9 Ci per mmol) was obtained from New England Nuclear, Boston, Mass. Tritiated sodium borohydride was purchased from Amersham Searle Corp., Des Plaines, Ill. Tos-PheCH_2Cl-trypsin was a product of Worthington Biochemical Corp., Freehold, N.J. *N*-Benzoyl-L-phenylalanyl-L-valyl-L-arginine *p*-nitroanilide hydrochloride was obtained from AB Bofors, Mölndal, Sweden. Sodium dodecyl sulfate was purchased from British Drug House, Poole, England. Dialysis tubing was treated according to McPhie (1971) prior to use. The sequenator reagents were of Sequanol Grade (Pierce Chemical Co.). All other chemicals were commercial preparations of the highest quality available.

Methods

Protein C concentrations were determined from the absorbance at 280 nm using an $E_{280}^{1\%} = 13.7$ and correcting for Rayleigh scattering according to Shapiro and Waugh (1966). The $E_{280}^{1\%}$ value was determined in the analytical ultracentrifuge according to Babul and Stellwagen (1969). For carbohydrate analyses, protein was determined by amino acid analysis after hydrolysis in 6 N HCl for 24 h at 110 °C in evacuated tubes.

Amino acid analyses were performed according to the methods of Moore and Stein (1963) and Spackman et al. (1958) employing a Durrum Model D 500 amino acid analyzer. Samples were hydrolyzed in 6 N HCl at 110 °C for 24, 48, and 72 h in evacuated tubes. Threonine and serine content was determined by extrapolation to zero-time hydrolysis on semilogarithmic paper. Valine, isoleucine, and leucine values were those determined for the 72-h hydrolysis. Tryptophan was determined by the procedure of Hugli and Moore (1972), and half-cystine was determined either as cysteic acid by the method of Hirs (1967) or as carboxymethylcysteine according to Crestfield et al. (1963).

Neutral sugar was determined by the anthrone procedure according to Spiro (1966) using a 1:1 mixture of mannose and galactose as a standard. Neuraminic acid was determined by the thiobarbituric acid method of Warren (1959) using *N*-acetylneuraminic acid as a standard. Hexosamine was determined with the amino acid analyzer on samples previously hydrolyzed in 2 N HCl for 20 h at 110 °C.

The carbohydrate content of the heavy and light chains of protein C was also determined by tritiating the glycoprotein according to the method of Van Lenten and Ashwell (1971) and determining the distribution of the radiolabel in the two chains after separation by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. In these experiments, the sodium dodecyl sulfate gels were sliced into 1-mm sections, digested with 0.5 ml 30% H_2O_2 at 100 °C, and solubilized in 10 ml of Bray's solution (Bray, 1960). The radioactivity in each slice was counted in a Beckman liquid scintillation counter (Model LS-100C).

Sodium dodecyl sulfate gel electrophoresis was performed by the method of Weber and Osborn (1969). Samples were subjected to electrophoresis at room temperature for 3–4 h in 10% polyacrylamide gels at 5 mA/gel. The electrophoresis buffer was 0.1 M Tris-H_3PO_4 (pH 7.0) containing 0.1% sodium dodecyl sulfate. Gels were stained for protein with Coomassie brilliant blue R according to Fairbanks et al. (1971), or for carbohydrate by the procedure of Glossmann and Neville (1971). The molecular weights were estimated by interpolation from a linear semilogarithmic plot of apparent molecular weight vs. distance of migration using the following proteins as standards: bovine serum albumin (68 000), ovalbumin (45 000), bovine carbonic anhydrase (29 000), and myoglobin (17 000).

Ultracentrifugation was performed with a Beckman Model E analytical ultracentrifuge equipped with an electronic speed control. Sedimentation equilibrium experiments were performed according to Yphantis (1964) employing a six-channel Kel-F centerpiece. Rayleigh patterns were recorded on Kodak II-G photographic plates and the plates analyzed on a modified Nikon microcomparator as described by DeRosier et al. (1972). Point-by-point molecular weight averages were calculated utilizing a computer program developed by Teller (1973).

Sedimentation equilibrium studies were carried out in 0.05 M sodium acetate buffer (pH 5.5) containing 6 M guanidine hydrochloride. Analyses were performed at three different protein concentrations at 20 °C at a rotor speed of 24 000 rpm. A partial specific volume of 0.707 was determined from the amino acid analyses and corrected for 18% carbohydrate content (Gibbons, 1966; Lee and Timasheff, 1974). The solvent density at 20 °C was obtained from its index of refraction at 23.5 °C measured with an Abbe refractometer (Kielley and Harrington, 1960).

The carboxymethylated heavy and light chains of protein C were also subjected to sedimentation equilibrium analysis in 0.05 M Tris-HCl buffer (pH 7.5) containing 6 M guanidine hydrochloride. Ultracentrifugation was carried out at 20 °C at rotor speeds of 30 000 and 36 000 rpm for the heavy and light chains, respectively. Partial specific volumes for the heavy (0.702) and light (0.681) chains were calculated from their respective amino acid and carbohydrate contents employing carbohydrate contents of 18 and 15% for the heavy and light chains, respectively.

Automated Edman degradations were performed with a

[2] Abbreviations used are: RVV-X, the protease from Russell's viper venom that activates factor X; DFP, diisopropyl phosphorofluoridate; DIP, diisopropyl phosphoryl; Tos-PheCH_2Cl, L-1-tosylamido-2-phenylethyl chloromethyl ketone; EDTA, ethylenediaminetetraacetic acid; DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane; Bz, benzoyl.

Beckman sequenator Model 890A employing 2–10-mg samples of carboxymethylated heavy or light chains. The operation of the instrument and the methods of sequenator analysis are modifications (Hermodson et al., 1972) of the techniques of Edman and Begg (1967).

Factor VII was assayed as previously described (Kisiel and Davie, 1975). Factor X and prothrombin were assayed by one-stage methods according to Bachmann et al. (1958) and Hjort et al. (1955), respectively. Factor IX was measured by a one-stage assay as described by Fujikawa et al. (1973). The aggregation of human platelets was examined by the method of Ardlie et al. (1970) using a Payton aggregation module.

The esterase activity of activated protein C was measured according to Svendsen et al. (1972) by a fixed-time assay with the chromogenic substrate, Bz-Phe-Val-Arg-*p*-nitroanilide hydrochloride (S-2160). In the assay, 100 μl of activated protein C containing 50 to 150 μg of protein was added to 2.25 ml of 0.11 mM substrate–0.05 M Tris-HCl–0.05 M imidazole buffer (pH 8.3) containing 0.1 M NaCl. The reaction mixture was incubated at 37 °C for 5–10 min, the reaction was stopped by the addition of 0.3 ml of glacial acetic acid, and the absorbance was read at 405 nm.

Reduced and alkylated heavy and light chains of protein C were prepared according to the procedure described by Stenflo (1976). The isolation of the heavy and light chains of protein C following proteolysis by trypsin or RVV-X was carried out as follows. Protein C (40 mg) was incubated at 37 °C with either 200 μg of Tos-PheCH_2Cl-trypsin or 2–4 mg of purified RVV-X in the presence of 5 mM $CaCl_2$ 0.1 M NaCl, and 0.05 M Tris-HCl (pH 7.5) in a final volume of 20 ml. The reaction was terminated after 30 min by the addition of 1 ml of 1 M DFP in the experiments employing trypsin. Experiments employing RVV-X were terminated after 2 h by the addition of solid EDTA to a final concentration of 20 mM. Reaction mixtures were then concentrated to about 5 ml by ultrafiltration and desalted by gel filtration in a Sephadex G-25 column (2.6 × 50 cm) previously equilibrated with 0.1 M NH_4HCO_3. Salt-free protein was obtained by lyophilization. Carboxymethylated heavy and light chains of either trypsin- or RVV-X-treated protein C were prepared by the same procedures employed for the untreated protein C pre paration.

Purification of Bovine Protein C. Protein C was purified from bovine plasma essentially according to Stenflo (1976). In initial experiments, the position of elution for protein C was determined by an immunochemical assay using rabbit anti-bovine protein C (Stenflo, 1976). In later experiments, protein C was determined qualitatively by sodium dodecyl sulfate gel electrophoresis.

Protein C obtained from DEAE-Sephadex A50 chromatography contained approximately 20% prothrombin in addition to small amounts of factor IX and factor VII. Accordingly, the sample from the DEAE-Sephadex column was made 10 mM in DFP and subjected to rechromatography on DEAE-Sephadex under the same conditions as the initial chromatography. Protein C subjected to rechromatography was essentially free of factor IX but still contained appreciable amounts of prothrombin. Prothrombin was removed by preparative discontinuous electrophoresis at 2 °C in a Buchler Poly-Prep 200 apparatus where protein C elutes ahead of prothrombin. The buffer compositions and electrophoretic conditions were identical with those previously described for bovine factor VII (Kisiel and Davie, 1975). Prior to electrophoresis in a 7% acrylamide resolving gel–4% acrylamide concentrating gel system, samples of protein C were dialyzed at 4 °C against 0.025 M Tris-H_3PO_4 buffer (pH 7.5) containing 1 mM benzamidine hydrochloride. Optimal recovery of protein C in preparative electrophoresis was a function of the applied load, and at relatively high loads (75–150 mg of protein) molecular aggregates of protein C were formed in the concentration process. Accordingly, sample loads of 50 mg of DEAE-Sephadex protein C were routinely subjected to preparative electrophoresis. The pooled samples from the preparative electrophoresis were routinely made 5 mM in DFP and either stored at −20 °C or desalted by gel filtration through a Sephadex G-25 column (2.6 × 50 cm) equilibrated with 0.1 M NH_4HCO_3. Salt-free protein was then obtained by lyophilization.

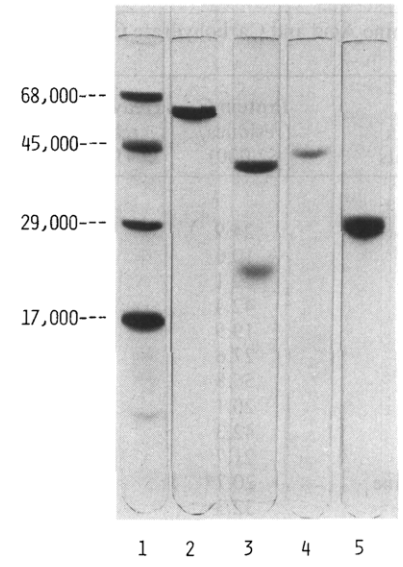

FIGURE 1: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of bovine protein C. Sample 1 contains a mixture of reduced standard proteins which include bovine serum albumin (68 000), ovalbumin (45 000), bovine carbonic anhydrase (29 000), and myoglobin (17 000); sample 2, protein C; sample 3, reduced protein C; sample 4, carboxylated heavy chain of protein C; sample 5, carboxylated light chain of protein C. All samples were stained for protein with Coomassie brilliant blue R. The anode was at the bottom of the gel.

Protein C obtained from preparative electrophoresis was completely free of factor VII, factor IX, and prothrombin activity when 100 μg (in 0.1 ml) was employed in each assay. Trace amounts of factor X activity (less than 0.005 U/ml) were consistently observed in purified preparations of protein C. Approximately 100 mg of purified protein C was obtained from 50 l. of bovine plasma.

Results

Molecular Weight of Protein C by Sodium Dodecyl Sulfate Gel Electrophoresis and Sedimentation Equilibrium. A single protein band was observed by sodium dodecyl sulfate gel electrophoresis for protein C (Figure 1, sample 2) and a molecular weight of 58 000 ± 1000 was estimated by this technique for the unreduced protein. Upon reduction with 2-mercaptoethanol, a heavy chain (mol wt 41 000 ± 1500) and a light chain (mol wt 21 000 ± 1000) were observed (Figure 1, sample 3). The apparent molecular weights of unreduced protein C and its heavy and light chains were independent of gel concentration when electrophoresis was carried out in polyacrylamide gels ranging from 7 to 15% (Segrest and Jackson, 1972).

The apparent molecular weights of the carboxymethylated heavy and light chains of protein C were estimated as 44 000 and 28 000, respectively (Figure 1, samples 4 and 5). The in-

TABLE I: Amino Acid and Carbohydrate Compositions of Bovine Protein C.

Components	Protein C (residues/ 62 000)	Heavy Chain (residues/ 41 000)	Light Chain (residues/ 21 000)
Amino acid			
Lys	24.9	17.4	7.6
His	10.6	6.0	4.4
Arg	29.1	15.4	9.1
Asp	42.4	29.6	26.3
Thr	19.9	16.3	4.5
Ser	27.6	19.9	10.4
Glu	56.8	31.8	26.1
Pro	20.1	13.9	6.8
Gly	42.3	25.0	16.8
Ala	21.7	16.3	7.3
Half-Cystine	20.7[a]	1.8[b]	8.5[b]
Val	32.3	27.6	2.9
Met	7.7	3.5	2.7
Iso	17.2	14.8	2.3
Leu	36.9	27.4	9.7
Tyr	9.9	7.3	2.6
Phe	16.1	9.6	9.1
Trp[c]	14.5	11.6	2.1
Carbohydrate			
Hexose	28.9	20.6[d]	8.3[d]
N-Acetylglucosamine	9.7	6.9[d]	2.8[d]
N-Acetylneuraminic acid	14.0	10.0[d]	4.0[d]
Protein (%)	82	82	85
Carbohydrate (%)	18	18[d]	15[d]

[a] Determined as cysteic acid. [b] Determined as carboxymethylcysteine. [c] Determined by the method of Hugli and Moore (1972). [d] Calculated from the distribution of radiolabeled carbohydrate according to the method of Van Lenten and Ashwell (1971).

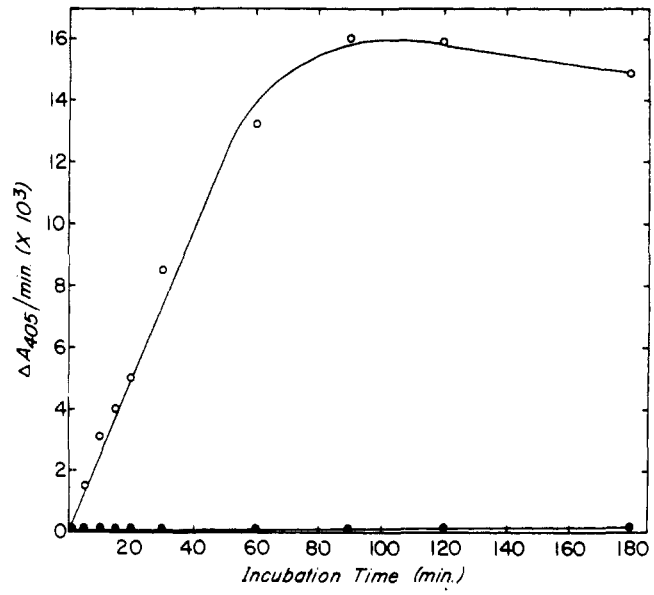

FIGURE 2: Time curve for the conversion of protein C to an enzyme by RVV-X. Protein C (40 mg) was incubated at 37 °C with 2 mg of RVV-X in the presence of 5 mM $CaCl_2$, 0.1 M NaCl, and 0.05 M Tris-HCl (pH 7.5) in a final volume of 20 ml. At the desired time, 0.1 ml of the reaction mixture was withdrawn and made 20 mM in EDTA. Amidase activity was measured as described in Methods after a 1:1 dilution of the sample with 0.05 M Tris-HCl-0.02 M EDTA (pH 7.5). (O—O) Complete reaction; (●—●) DFP-treated sample.

crease in the apparent molecular weight of the light chain following carboxymethylation presumably is related to its relatively high content of half-cystine. Anomalously high molecular weight values have been observed for other proteins following the introduction of negatively charged groups into the molecule (Arndt and Berg, 1970; Tung and Knight, 1971) and apparently result from decreased binding of sodium dodecyl sulfate to the derivatized protein (Tung and Knight, 1972).

Sedimentation equilibrium experiments with protein C in 6 M guanidine hydrochloride indicated the protein behaved either nonideally or heterogeneously at all concentrations tested. A minimal molecular weight of 62 000 was calculated by the method of Teller (1973). The carboxymethylated heavy and light chains of protein C also exhibited heterogeneity in 6 M guanidine hydrochloride in the ultracentrifuge and minimal molecular weight values of 35 000 and 18 000 were calculated for the heavy and light chains, respectively. The tendency of intact protein C and its heavy and light chains to associate in 6 M guanidine hydrochloride precluded an accurate calculation of its molecular weight by sedimentation equilibrium techniques, and the values reported here are presented only as approximate values. The minimal molecular weight of the intact protein C is in good agreement with the sum of the heavy and light chain molecular weights (i.e., 62 000) determined by sodium dodecyl sulfate gel electrophoresis. The heavy chain of protein C also migrates slightly ahead of the heavy chain of bovine factor X_1 in sodium dodecyl sulfate gel electrophoresis. This polypeptide has an apparent molecular weight of 42 000–43 000 by sodium dodecyl sulfate gel electrophoresis and 39 100 calculated from amino acid sequence and carbohydrate content (Titani et al., 1975). The molecular weights observed for intact protein C and the light chain by sodium dodecyl sulfate gel electrophoresis are in good agreement with those reported by Stenflo (1976). The apparent molecular weight of the reduced heavy chain (41 000), however, is significantly higher than that of 35 000 reported by Stenflo (1976) by this technique.

Amino Acid and Carbohydrate Compositions of Bovine Protein C. The amino acid and carbohydrate compositions of protein C are reported in Table I. Protein C contains approximately 18% carbohydrate by weight and this includes hexose, glucosamine, and neuraminic acid. Thus, protein C is composed of about 11 100 g of carbohydrate and 50 900 g of protein per 62 000 g of glycoprotein.

The amino acid and carbohydrate compositions for the heavy and light chains of protein C are aslo shown in Table I. The amino acid composition of the intact protein is in reasonably good agreement with that obtained by summation of the heavy and light chains. Some amino acids, such as aspartic acid and half-cystine, however, show poor agreement. The rather large discrepancies in half-cystine values may be due in part to the fact that cystine was determined as cysteic acid in the intact protein and *S*-carboxymethylcysteine in the heavy and light chains.

The carbohydrate contents of the heavy and light chains were estimated by the tritiating method of Van Lenten and Ashwell (1971). These studies indicate that about 75% of the radiolabel was located in the heavy chain and 25% in the light chain.

Conversion of Protein C to a Serine Enzyme. A time curve for the conversion of protein C to an enzyme is shown in Figure 2. In this experiment, protein C was incubated with RVV-X at pH 7.5 in the presence of 5 mM $CaCl_2$. The weight ratio of

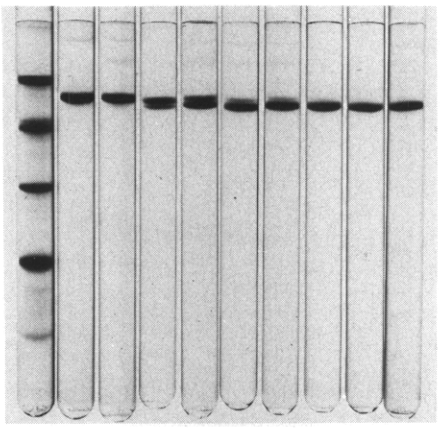

FIGURE 3: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of protein C following activation by RVV-X. Each sample (10 μl) containing 20 μg of protein was removed from the reaction mixture corresponding to that shown in Figure 2 and incubated at 100 °C for 5 min with 5% sodium dodecyl sulfate in the absence of reducing agents. Electrophoresis was carried out as described in Methods. The numbers at the bottom of the tubes represent incubation times; sample labeled Stand contains a mixture of reduced standard proteins which include bovine serum albumin (68 000), ovalbumin (45 000), bovine carbonic anhydrase (29 000), and myoglobin (17 000).

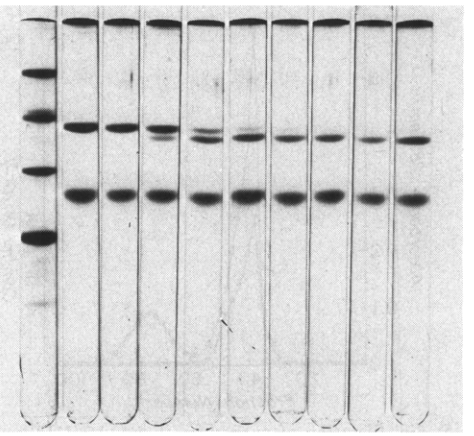

FIGURE 4: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of reduced protein C following activation by RVV-X. The samples and conditions are identical with those described in Figure 3, except the protein was incubated with 5% sodium dodecyl sulfate and 10% 2-mercaptoethanol before electrophoresis.

substrate to enzyme was 20:1. Aliquots of the reaction mixture were removed at various times and the activation terminated by the addition of EDTA to a final concentration of 20 mM. A portion of each aliquot was then made 5 mM in DFP while the remainder of each aliquot was treated with isopropyl alcohol to serve as controls. Both DFP-treated and control samples were then assayed for amidase activity using Bz-Phe-Val-Arg-*p*-nitroanilide as substrate. As shown in Figure 2, control samples rapidly developed amidase activity and reached a maximum in approximately 60 min (open circles). DFP treatment of aliquots of the reaction mixture (solid circles) completely inhibited the enzymatic activity. The experiments with DFP indicate that the amidase formed in the presence of RVV-X is a serine enzyme. A similar time curve was observed for reaction mixtures of protein C and trypsin. In these experiments, however, the reaction was essentially complete in 15 min, employing a weight ratio of substrate to enzyme of 200:1.

Evidence of a change in the molecular weight of protein C during its conversion to an enzyme by RVV-X was shown by sodium dodecyl sulfate gel electrophoresis (Figure 3). In these experiments, aliquots were removed at various times from a reaction mixture corresponding to that shown in Figure 2 and analyzed by sodium dodecyl sulfate gel electrophoresis. At zero time, a single protein band was observed for protein C. Progressively with time, a new faster moving band appeared and, after 60 min, nearly all the protein C was converted to this new species. The apparent molecular weight of protein C decreased from 58 000 to about 56 000 and indicated that the activation of protein C was coincident with the release of a relatively small peptide (or peptides) from the molecule.

Since protein C is composed of two polypeptide chains, the reaction mixture was examined by sodium dodecyl sulfate gel electrophoresis after reduction with 2-mercaptoethanol to determine whether the heavy or light chain was cleaved during the activation reaction. The results of these experiments are shown in Figure 4. The zero-time sample showed two bands, corresponding to the heavy and light chains of protein C. With time, a new band with an apparent molecular weight of 39 000 was observed and its appearance corresponded to a decrease in the amount of the heavy chain of the precursor protein. In addition, the appearance of the new heavy chain coincided with the formation of enzymatic activity. No change in the molecular weight of the light chain was observed during the activation reaction. Sodium dodecyl sulfate gel electrophoresis experiments of protein C activated with trypsin revealed a similar proteolysis of the heavy chain coincident with enzyme formation. In contrast to the activation of protein C by RVV-X, additional proteolysis of the light chain was observed in the presence of trypsin. This proteolysis, however, was unrelated to the activation reaction. These data indicate that protein C is converted to a serine enzyme by RVV-X or trypsin, and this conversion occurs coincident with the release of a low molecular weight peptide(s) (mol wt approximately 2000) from the heavy chain of protein C. The enzyme formed, called activated protein C, was readily inhibited by DFP, and covalent binding of this inhibitor to the heavy chain of activated protein C was shown by employing [^{3}H]DFP. In these experiments, protein C was activated with RVV-X and treated with [^{3}H]DFP. The tritium-labeled protein was then reduced, carboxymethylated, and subjected to gel filtration on Sephadex G-150 (Figure 5). Two protein peaks were observed. The first peak contained the heavy chain of activated protein C and virtually all of the radioactivity. Similar results were observed when protein C was activated with trypsin and treated in the same manner. Moreover, the apparent molecular weights of the carboxymethylated heavy chain of activated protein C obtained by either trypsin or RVV-X were found to be identical (Figure 6). These experiments indicate the binding site for the diisopropyl phosphoryl group in activated protein C is present in the heavy chain of the enzyme.

Amino-Terminal Sequence. Amino-terminal analyses of the heavy chains of protein C and activated protein C were carried out in a Beckman sequenator. The amino-terminal sequence for the first 28 residues of the heavy chain of protein C was as follows: Asp-Thr-Asn-Gln-Val-Asp-Gln-Lys-Asp-Gln-Leu-Asp-Phe-Arg-Ile-Val-Asp-Gly-Gln-Glu-Ala-Gly-Trp-Cys-Glu-Ser-Pro-Trp-. The yield of amino-terminal aspartic acid was approximately 0.65 equiv/mol of protein assuming 33 600 g of protein/mol of heavy chain. Amino-terminal analyses of the heavy chains of protein C obtained after

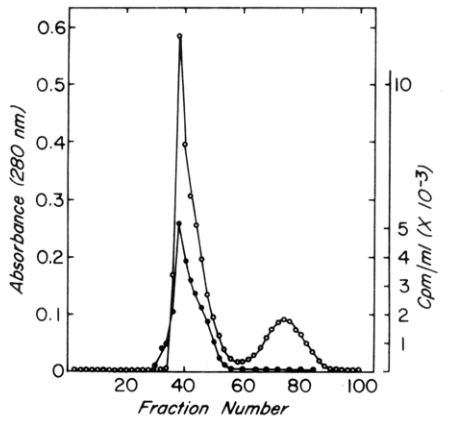

FIGURE 5: Gel filtration of carboxymethylated [^{3}H]diisopropylphosphoryl-activated protein C on a Sephadex G-150 column. Protein C was activated as described in Figure 2. Following activation, the reaction mixture was treated with 225 nmol of [^{3}H]Dip-F (total counts 4.4×10^8 cpm). The protein was reduced and carboxymethylated as described in Methods and applied to a Sephadex G-150 column (2.6 × 95 cm) which was previously equilibrated with 0.2 M NH_4HCO_3. Elution was carried out with 0.2 M NH_4HCO_3 and samples were assayed for protein and radioactivity. (●—●) Radioactivity; (○—○) absorbance.

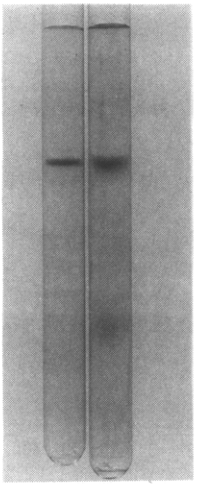

FIGURE 6: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the carboxymethylated heavy chain of activated protein C. Sample 1 is 10 μg of the heavy chain obtained after activation by RVV-X. Sample 2 is 10 μg of the heavy chain obtained after activation by trypsin.

activation by either RVV-X or trypsin revealed an amino-terminal sequence of Ile-Val-Asp-Gly-Gln-Glu-Ala-Gly-Trp-Cys-Glu-Ser-Pro-Trp. This sequence coincides exactly with that observed in residues 15 through 28 in the heavy chain of the precursor molecule. These data indicate that the conversion of protein C to an enzyme by either RVV-X or trypsin is due to the cleavage of an Arg-Ile peptide bond in positions 14 and 15 of the heavy chain. Furthermore, the molecular weight of the released tetradecapeptide (mol wt 1700) is in good agreement with the difference observed by sodium dodecyl sulfate gel electrophoresis between the heavy chain of protein C and the heavy chain of activated protein C.

The light chain of protein C was shown to contain the amino-terminal sequence of Ala-Asn-Ser-Phe-Leu. This sequence is identical with that previously reported by Stenflo (1976).

Effect of Protein C and Activated Protein C on the Coagulation System. One of the major objectives of this investigation was to determine whether protein C or activated protein C participates in blood coagulation. Accordingly, experiments were designed to test (1) the potential clot-promoting effects of activated protein C in normal plasma, (2) whether the structure of protein C was changed during coagulation of whole plasma by either the intrinsic or extrinsic pathways, and (3) whether activated protein C would aggregate platelets.

Initial studies indicated that protein C activated with trypsin contained some clot-promoting activity when tested in normal plasma in the presence of $CaCl_2$ and phospholipid. This clot-promoting activity, however, was due to the presence of trace amounts of factor X in the protein C preparation. This was shown by passing a solution of protein C through a column of anti-factor X_1–agarose to adsorb trace amounts of contaminating factor X. Protein C eluted from this column was completely free of factor X and, when activated with trypsin, no clot-promoting activity was detected. Indeed, activated protein C at a concentration of 50 μg/ml actually prolonged the clotting time of normal bovine plasma in the presence of phospholipid and $CaCl_2$.

The possibility that the molecular structure of protein C was affected by the coagulation process was examined in two different ways. In the first series of experiments, bovine blood (4 l.) was collected in the absence of anticoagulants and allowed to clot at 4 °C. Protein C was then isolated from the serum by the same procedure used for its isolation from plasma. The serum preparation was found to be indistinguishable from that of plasma as judged by sodium dodecyl sulfate gel electrophoresis. Moreover, the yield of protein C from serum was essentially the same as that from plasma. In the second set of experiments, protein C (5 mg) was labeled with fluorescein isothiocyanate (10% on Celite) according to Rinderknect (1962) and the unreacted dye and salts were removed by gel filtration on a Sephadex G-25 column (2.6 × 50 cm). The column was previously equilibrated with 0.05 M Tris-HCl buffer (pH 7.5) containing 0.1 M NaCl. The fluorescein-labeled protein (150 μg in 0.1 ml) was then mixed with 0.1 ml of pooled bovine plasma, 0.1 ml of bovine brain thromboplastin, and 0.1 ml of 0.025 M $CaCl_2$, and a clot was formed in 17 s. The clot was centrifuged in a Beckman microfuge and the serum (0.3 ml) was treated with sodium dodecyl sulfate (final concentration of 10%) and boiled for 5 min. This procedure was repeated using 0.1 ml of a kaolin–phospholipid mixture in place of the thromboplastin. In this case, a clot was observed in about 75 s. A sample consisting of 0.1 ml of bovine plasma, 0.1 ml of labeled protein C, and 0.2 ml of 0.05 M Tris-HCl buffer (pH 7.5) containing 0.1 M NaCl served as a control. When 20-μl aliquots of each sample were subjected to sodium dodecyl sufate gel electrophoresis following reduction with 10% 2-mercaptoethanol, no difference in the mobilities of fluorescein-labeled heavy and light chains of protein C were observed between serum samples and the plasma control. While these experiments do not rule out the possibility that a small amount of protein C had undergone some molecular change, it is clear that no major change occurred as a result of coagulation via either the intrinsic or extrinsic pathways.

Activated protein C was also examined for its effect on the aggregation of human platelets under conditions similar to those employed for aggregation by thrombin. At concentrations as high as 100 μg/ml, activated protein C had no effect on platelet aggregation.

Amidase Activity of Activated Protein C. Activated protein C, purified by preparative electrophoresis, has significant amidase activity toward the chromogenic substrate Bz-Phe-Val-Arg-*p*-nitroanilide and the specific activity at 37 °C was

0.5 μmol per min per mg of protein. The K_m for activated protein C was 1.4×10^{-4} M as determined from Lineweaver–Burk plots. Benzamidine hydrochloride was a competitive inhibitor for activated protein C with a K_i of 7.5×10^{-4} M. As previously noted, the amidase activity of activated protein C was also readily inhibited by DFP.

Discussion

The present data indicate that bovine protein C is a glycoprotein which exists in plasma as a two-chain molecule. These experiments confirm the initial data reported by Stenflo (1976). The carbohydrate present in this protein includes hexose, glucosamine, and neuraminic acid. Radiolabeling experiments suggest that the molecule contains five carbohydrate chains linked to the heavy chain and two carbohydrate chains linked to the light chain. This calculation assumes that the carbohydrate chains in protein C are branched and similar to those observed in bovine prothrombin (Nelsestuen and Suttie, 1972). Accordingly, the 14 neuraminic acid residues in protein C would be equivalent to 7 carbohydrate chains. From the distribution of radiolabel, it would appear that 5 carbohydrate chains are linked to the heavy chain and 2 carbohydrate chains are linked to the light chain. In this situation, the theoretical distribution of radiolabel would be 71 and 29% in the heavy and light chains, respectively. Additional experiments are necessary, however, to confirm this suggestion.

Like the four vitamin K dependent coagulation factors, protein C exists in plasma as an inactive precursor to a serine enzyme.[3] The present experiments indicate that the hydrolysis of a specific Arg–Ile bond in the heavy chain of protein C by either RVV-X or trypsin is responsible for the conversion of this plasma protein to an enzyme. These results are summarized in Figure 7 which shows the partial structure of bovine protein C and the location of the peptide bond split during the activation reaction. Cleavage of the tetradecapeptide results in the formation of a new amino-terminal sequence of Ile-Val-Asp-Gly in the heavy chain of activated protein C. This amino-terminal sequence is homologous to that found in the amino-terminal end of bovine trypsin (Walsh and Neurath, 1964), bovine thrombin (Magnusson, 1971), bovine chymotrypsin A (Hartley, 1964), bovine plasmin (Nagasawa and Suzuki, 1970), bovine factor X_a (Fujikawa et al., 1972), and bovine factor IX_a (Fujikawa et al., 1974). Despite the similarities of structure and mechanism of activation observed for bovine protein C and bovine factor X_1, no apparent homology was observed in the sequence of the peptide released from the heavy chains of these two proteins during the activation reaction (Titani et al., 1975).

The amino acid sequence surrounding the active site in the heavy chain in activated protein C has not been determined. It is very probable, however, that it will be homologous with that of factor X_a and other serine proteases. Thus, the critical event in the activation of protein C may be the formation of an ion pair between the amino-terminal isoleucine and an aspartic acid residue adjacent to the active center serine. This could lead to the charge relay network characteristic of the pancreatic proteases (Matthews et al., 1967; Sigler et al., 1968; Blow et al., 1969; Shotton and Watson, 1970).

The physiological function of protein C remains unknown. The experiments described here to test the possible clot-promoting effect of activated protein C have been negative thus far. Indeed, a significant inhibition of coagulation was noted in the presence of activated protein C. Whether this was due simply to a competitive binding of activated protein C with the other vitamin K dependent coagulation factors for phospholipid surfaces or the degradation of certain coagulation factors by activated protein C is not known. The possible involvement of activated protein C in other physiological processes, such as the fibrinolytic, kinin, or complement systems, will require additional studies.

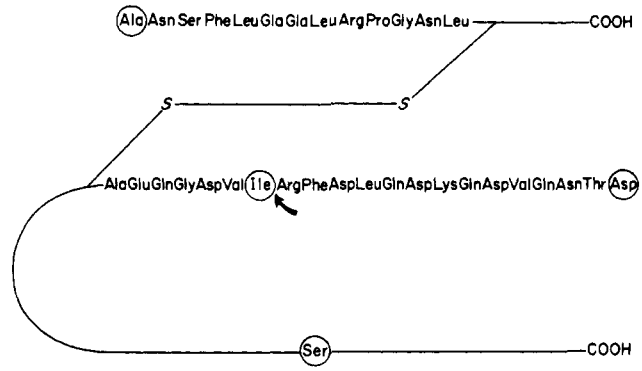

FIGURE 7: Partial structure of bovine protein C. Arrow indicates the point of cleavage in the heavy chain of protein C by either RVV-X or trypsin. Amino acid sequence of the light chain was taken from Stenflo (1976) and confirmed in part by this study.

Acknowledgments

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[3] A recent report by Esmon et al. (1976) demonstrates that protein C can be converted by trypsin to a species which reacts with diisopropyl phosphorofluoridate. Presumably, this species is equivalent to the activated protein C observed in this study.

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