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Characterization of Protein S, a γ-Carboxyglutamic Acid Containing Protein from Bovine and Human Plasma†

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ABSTRACT: Protein S is a vitamin K dependent protein of unknown function, which is present in mammalian plasma. It was isolated from bovine plasma by barium citrate adsorption and elution, ammonium sulfate fractionation, and column chromatography on DEAE-Sephadex, heparin-agarose, and polyhomoarginine-Sepharose. Bovine Protein S (M_r 64 200) is a single-chain glycoprotein with an amino-terminal sequence of Ala-Asn-Thr-Leu-Leu-. It contains 7.0% carbohydrate and 10 residues of γ-carboxyglutamic acid per mol of protein. Human Protein S (M_r 69 000) is also a single-chain glycoprotein with an amino-terminal sequence of Ala-Asn-Ser-Leu-Leu-. It contains 7.8% carbohydrate and 10 residues of γ-carboxyglutamic acid per mol of protein. These results indicate that Protein S from bovine or human plasma shows many similarities to the other vitamin K dependent proteins present in plasma.

Three of the four vitamin K dependent proteins in plasma which are involved in blood coagulation have been shown to contain γ-carboxyglutamic acid (Stenflo et al., 1974; Magnusson et al., 1974; Nelsestuen et al., 1974; Bucher et al., 1976). These proteins include prothrombin, factor IX (Christmas factor),[1] and factor X (Stuart factor). γ-Carboxyglutamic acid is formed by the carboxylation of specific glutamic acid residues in the amino-terminal region of these proteins in a series of reactions requiring vitamin K. Furthermore, a cell-free system that carries out these carboxylation reactions has been described (Esmon et al., 1975; Suttie et al., 1975; Mack et al., 1976; Esmon & Suttie, 1976; Jones et al., 1977).

In 1976, Stenflo identified and characterized another vitamin K dependent protein from bovine plasma which was called Protein C. This protein is converted to activated Protein C by thrombin and may play a regulatory role in blood coagulation (Kisiel et al., 1976; Esmon et al., 1976; Kisiel et al., 1977). Activated Protein C is probably identical with an inhibitor which interferes with the intrinsic pathway of blood coagulation (Marciniak, 1970, 1972; Seegers et al., 1976). It differs, however, from a bovine plasma protein, called Protein Z, which has been described in a preliminary report (Prowse & Esnouf, 1977). Protein Z contains γ-carboxyglutamic acid, but its amino-terminal sequence is not homologous with either Protein C or the other vitamin K dependent proteins of plasma.

More recently, we have described another protein from human plasma which was homologous to the other known vitamin K dependent proteins (DiScipio et al., 1977). This protein, called Protein S, was only partially characterized since rather small amounts were available. Its biological function was not established. In the present paper, we present a more thorough characterization of Protein S from human plasma, as well as its isolation and characterization from bovine plasma. The γ-carboxyglutamic acid content of human and bovine Protein S was quantitated along with that of prothrombin, factor VII, factor IX, factor X, and Protein C.

Experimental Section

Materials. 4-Morpholineethanesulfonic acid (Mes),[2] 4-morpholinepropanesulfonic acid (Mops), thiobarbituric acid, galactose, *N*-acetylneuraminic acid, and poly(L-lysine) (type I-B) were obtained from the Sigma Chemical Co., St. Louis, MO. Cyclohexanone, *O*-methylisourea, and benzamidine hydrochloride were purchased from Aldrich Chemical Co., Milwaukee, WI. Barium chloride and cyanogen bromide were obtained from Baker Chemical Co., Phillipsburg, NJ. DEAE-Sephadex A-50 and Sepharose 4B were products of

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

[2] Abbreviations used: Mes, 4-morpholineethanesulfonic acid; Mops, 4-morpholinepropanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane; $NaDodSO_4$, sodium dodecyl sulfate; Gla, γ-carboxyglutamic acid.

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Pharmacia Fine Chemicals, Piscataway, NJ. *N,N'*-methylenebis(acrylamide), 2-mercaptoethanol, and *N,N,N',N'*-tetramethylethylenediamine were purchased from Eastman Kodak Co., Rochester, NY. Acrylamide was a product of Bio-Rad Laboratories, Richmond, CA. Guanidine hydrochloride (ultrapure) was purchased from Schwarz/Mann, Van Nuys, CA. All guanidine solutions were filtered before use. The sequenator reagents were of Sequanol grade (Pierce Chemical Co., Rockford, IL). PAG plates containing ampholine carriers in the pH range 3.5–9.5 were purchased from LKB-Produkter AB, Bromma, Sweden. All other chemicals were of the highest quality available.

Methods. Polyhomoarginine-Sepharose and heparin-Sepharose were prepared as previously described (DiScipio et al., 1977). Human prothrombin, human factor X, human factor IX, and human Protein S were purified by the method of DiScipio et al. (1977) with the minor alteration that Protein S was renatured after the polyhomoarginine-Sepharose column chromatography step by dialyzing overnight against 10 volumes of 0.04 M Tris-HCl buffer (pH 7.8) containing 2.0 M NaCl, 10 mM benzamidine, and 10% glycerol and then two more times against 0.02 M Tris-HCl buffer (pH 7.8) containing 0.2 M NaCl, 2 mM benzamidine, and 10% glycerol.

Bovine factor VII was purified by the method of Kisiel & Davie (1975). Bovine factor IX and bovine factors X_1 and X_2 were purified by the methods of Fujikawa et al. (1973) and Fujikawa et al. (1972), respectively. Bovine Protein C was purified by the method of Stenflo (1976) as modified by Kisiel et al. (1976). Human Protein C was purified by the method of Kisiel (1978). These purified plasma proteins all migrated as single bands when subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

The preparation of bovine Protein S was as follows. Five liters of plasma was fractionated by the procedure of Stenflo (1976) for the purification of bovine Protein C up to and including the DEAE-Sephadex column chromatography step. The first half of the major protein peak from the DEAE-Sephadex column, which contained the major portion of the factor IX activity, was pooled and dialyzed against 0.05 M imidazole hydrochloride buffer (pH 6.0). This fraction was then applied to a heparin-agarose column as previously described by Fujikawa et al. (1973) for the purification of bovine factor IX. The first protein peak from the heparin-agarose column chromatography step contained about 95% prothrombin and about 2–3% Protein S. It was pooled, dialyzed against 10 volumes of 0.02 M Mes–Tris buffer (pH 5.9) containing 0.01 M EDTA, 2 mM benzamidine, and 0.02% azide, and then applied to a polyhomoarginine-Sepharose column (1.7 × 45 cm) which had previously been equilibrated with buffer A (0.02 M Mes–Tris buffer, pH 5.9, containing 0.02% sodium azide). The column was washed with 50 mL of buffer A, followed by 350 mL of the same buffer containing 3.0 M NaCl. The proteins were eluted with 350 mL of 2.7 M guanidine hydrochloride and 2.4 M NaCl in buffer A containing 10^{-3} M $PhCH_2SO_2F$. The flow rate was 0.1 mL/min, and 4.2-mL fractions were collected. The second peak from this column, which contained the bovine Protein S, was pooled and dialyzed once against 10 volumes of 0.04 M Tris-HCl buffer (pH 7.8) containing 10 mM benzamidine, 2.0 M NaCl, and 10% glycerol. This sample was subsequently dialyzed twice more against 0.02 M Tris-HCl buffer (pH 7.8) containing 0.2 M NaCl, 2 mM benzamidine, and 10% glycerol. When desired, the bovine prothrombin peak was also pooled and the sample was renatured in a fashion similar to what has been described for Protein S. This preparation of bovine prothrombin was devoid of Protein S.

Extinction coefficients were determined in an analytical ultracentrifuge by the method of Babul & Stellwagen (1969) in 0.02 M 4-morpholinepropanesulfonic acid buffer (pH 7.2) containing 0.1 M NaCl. These determinations were performed at least twice.

Amino acid analyses and preparation of samples were carried out by the methods of Moore & Stein (1963) and Spackman et al. (1958), employing a Durrum Model D500 amino acid analyzer. The samples were hydrolyzed in 6 N HCl at 110 °C for 24, 48, 72, and 96 h in evacuated tubes. The values of threonine and serine were determined by extrapolation to zero hydrolysis time. Tryptophan content was ascertained by the method of Edelhoch (1967), and half-cystine was determined as cysteic acid by the method of Hirs (1967).

N-Acetylneuraminic acid was determined by the thiobarbituric acid method of Warren (1959) by use of *N*-acetylneuraminic acid as a standard. The content of neutral sugar was evaluated by the anthrone procedure (Spiro, 1966) by use of a 1:1 mixture of mannose and galactose as a standard. Glucosamine and galactosamine were determined in a Durrum Model D500 amino acid analyzer after the samples were hydrolyzed in 2.0 N HCl for 24 h at 110 °C (Spackman et al., 1958).

Ultracentrifugation was carried out in a Beckman Model E analytical ultracentrifuge which was equipped with an electronic speed control. Sedimentation equilibrium experiments were performed by the method of Yphantis (1964) and analyzed by the method of Teller et al. (1969). Sedimentation equilibrium experiments of human and bovine Protein S were performed at three different concentrations (1.2, 0.8, and 0.6 mg/mL) in 6.0 M guanidine hydrochloride in 0.1 M sodium acetate buffer (pH 5.5). The analyses were carried out with a rotor speed of 22 000 rpm at 20 °C.

Automated Edman degradations were performed in a Beckman Model 890C sequenator. The method employed was an adaptation (Hermodson et al., 1972) of the technique of Edman & Begg (1967). For the amino-terminal analyses, 6 mg of human and bovine Protein S was used and each analysis was performed at least twice. Phenylthiohydantoin amino acids were identified and quantitated by high-pressure liquid chromatography (Bridgen et al., 1976). Amino-terminal residues were quantitated after determination of protein concentration by amino acid analysis. Norleucine was used as an internal standard to calculate protein recovery.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed by the method of Weber & Osborn (1969) as modified by Kisiel et al. (1976). Samples were subjected to electrophoresis at room temperature for 3–4 h in 8.5–9.0% polyacrylamide gels at 4 mA/gel. The gels were stained for protein with a solution of 0.2% Coomassie brilliant blue R. The molecular weights were estimated by interpolation from a linear semilogarithmic plot of molecular weight vs. distance of migration by use of the following protein standards: phosphorylase *a* (97 000), bovine serum albumin (68 000), aldolase (40 000), bovine carbonic anhydrase (29 000), soybean trypsin inhibitor (21 000), and cytochrome *c* (12 000).

Partial specific volumes were calculated for human Protein S and bovine Protein S by the method of Lee & Timasheff (1974) and corrected for carbohydrate content (Gibbons, 1966).

γ-Carboxyglutamic acid content of the various plasma proteins was determined after alkaline hydrolyses by the method of Tabor & Tabor (1977). Analyses were performed in duplicate for each protein. The data were quantitated by

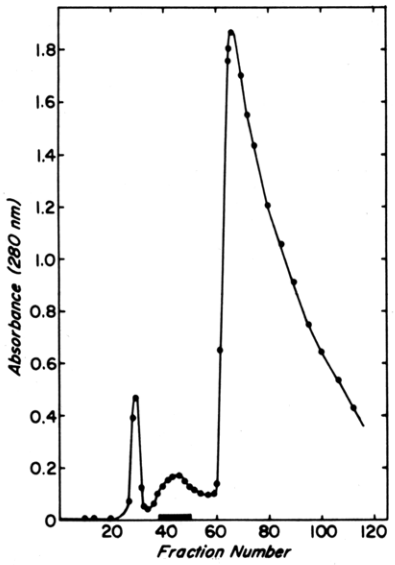

FIGURE 1: Elution pattern of bovine Protein S from polyhomoarginine-Sepharose. Protein was eluted from the column (1.7 × 45 cm) with 0.02 M Mes–Tris buffer, pH 5.9, containing 2.7 M guanidine hydrochloride, 2.4 M NaCl, 0.02% sodium azide, 10^{-3} M $PhCH_2SO_2F$. Fractions (4.2 mL) were collected at a flow rate of 0.1 mL/min. The fraction that was pooled for Protein S is indicated by the bar. (●) Absorbance at 280 nm.

comparison to a series of bovine prothrombin standards. Bovine prothrombin has been shown to contain 10 mol of γ-carboxyglutamic acid/mol of protein (Magnusson et al., 1975).

Radioimmunoassays of human Protein S were performed by the following procedure. Rabbit antibodies were developed against human Protein S and purified by the method previously described for bovine factor IX (Fujikawa et al., 1973). Human Protein S was radiolabeled with ^{125}I to a specific activity of 1.8×10^8 cpm/mg by the lactoperoxidase-Sepharose method of David (1972). A standard competition curve was established by adding 1.0 μg of radioiodinated human Protein S (1.8×10^5 cpm) to each siliconized glass tube along with various amounts of unlabeled human Protein S. Test samples were analyzed in a similar manner. Anti-Protein S antibody (15 μg) was added and the reactions were allowed to proceed at 4 °C for 12 h. Subsequently, 100 μL of a 1:1 suspension of anti-rabbit goat antibody-Sepharose (10 mg of protein/mL of gel) in 0.02 M Mops–Tris buffer (pH 7.2) containing 0.15 M NaCl was added. The samples were mixed intermittently for 3 h at room temperature and then centrifuged. The pellets were washed twice with 0.02 M Mops–Tris buffer (pH 7.2) containing 0.15 M NaCl, and the samples were counted in a Beckman Model 4000 γ counter.

Isoelectric points for the various vitamin K dependent proteins were determined in an LKB 2117 multiphor apparatus equipped with an LKB 2103 power supply. The procedure followed was that suggested by the manufacturer in its methodology bulletin.

Results

Isolation of Bovine Protein S. Protein S was purified by a series of steps that included adsorption and elution on barium citrate, ammonium sulfate fractionation, DEAE-Sephadex column chromatograhy, heparin-agarose column chromatography, and polyhomoarginine-Sepharose column chromatography (see Methods). The elution profile from the polyhomoarginine-Sepharose column is shown in Figure 1. Three protein peaks were observed. The first peak contained a single-chain protein of 28 000 daltons. The second peak was identified as Protein S, and the third peak was shown to be prothrombin.

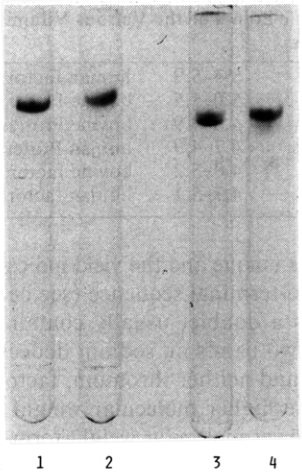

FIGURE 2: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis patterns of human and bovine Protein S. Gel electrophoresis was carried out in 8.5% polyacrylamide gels for 3.5 h, and the gels were stained with 0.2% Coomassie brilliant blue R as described under Methods. All of the gels contain 10 μg of protein. Gel 1 is unreduced human Protein S; gel 2 is reduced human Protein S; gel 3 is unreduced bovine Protein S; gel 4 is reduced bovine Protein S. The anode was at the bottom of the gel.

Table I: Physical Properties of Human and Bovine Protein S

	human	bovine
molecular weight	69 000 ± 2700	64 000 ± 2300
sedimentation equilibrium (M_1)[a]		
$NaDodSO_4$ gel electrophoresis		
unreduced	69 000 ± 1000	65 000 ± 1000
reduced	72 000 ± 2000	70 000 ± 3000
partial specific volume ($\bar{v}$)	0.721 mL/mg	0.717 mL/mg
extinction coefficient ($E_{280}^{1\%}$)	9.5	10.0

[a] M_1 refers to the smallest molecular weight species calculated by the method of Teller et al. (1969).

The overall recovery and extent of purification of bovine Protein S was not established since a simple assay procedure for this protein was not available. The total yield, however, was 5–8 mg from 5 L of starting plasma. Bovine Protein S was free of prothrombin, factor VII, factor IX, and factor X as measured by specific clotting assays. It showed some cross-reactivity to antibodies to human Protein S by employment of a specific radioimmunoassay. In these experiments, bovine Protein S was 6% as effective as the human protein in its ability to compete with the latter protein in the radioimmunoassay. In contrast, bovine prothrombin or bovine factor X_1 had virtually no ability to compete with human Protein S in the radioimmunoassay.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis Experiments. Figure 2 shows sodium dodecyl sulfate–polyacrylamide gel electrophoresis experiments of human and bovine Protein S. Human Protein S migrated as a single, sharp band before and after reduction (gels 1 and 2), indicating that it is composed of a single polypeptide chain. The apparent molecular weight of the protein before reduction was found to be 69 000 by this method (Table I). Bovine Protein S was also observed to consist of a single polypeptide chain (gels 3 and 4, Figure 2), and the apparent molecular weight of this protein before reduction was 65 000. The unreduced gel of bovine Protein S sometimes appeared as a doublet, and the molecular weight difference between the two bands was less than 1000. It appears highly probable that both of these bands represent bovine Protein S since the amino-terminal sequence

Table II: Isoelectric Points of the Various Vitamin K Dependent Proteins

bovine S	5.4–5.9	human factor X	4.9–5.2
human S	5.0–5.5	bovine factor VII	4.8–5.1
bovine prothrombin	4.4–4.9	bovine Protein C	4.2–4.5
human prothrombin	4.7–4.9	human Protein C	4.4–4.8
bovine factor X_1	4.8–5.2	bovine factor IX	4.0–4.5
bovine factor X_2	4.7–5.1	human factor IX	4.0–4.5

of this protein was unique and the yield indicated the presence of only one amino-terminal sequence (see below). Protein S that appeared as a doublet usually contained about equal amounts of the two bands on sodium dodecyl sulfate–polyacrylamide gels, and neither thrombin, factor X_a, nor factor IX_a converted the higher molecular weight form of bovine Protein S to the lower molecular weight form. Whether these variants in bovine Protein S result from minor degradation, carbohydrate heterogeneity, or genetic polymorphism is not known.

Sedimentation Equilibrium Studies. Sedimentation equilibrium studies on human and bovine Protein S were carried out in the presence of 6 M guanidine hydrochloride. The smallest molecular weight species calculated by the method of Teller et al. (1969) was 69 000 ± 2700 and 64 000 ± 2300 for human and bovine Protein S, respectively (Table I). These values are in good agreement with those obtained by sodium dodecyl sulfate–polyacrylamide gel electrophoresis with the unreduced proteins. These data indicate that human Protein S is slightly larger than bovine Protein S. Both proteins, however, are slightly smaller than bovine prothrombin (71 550; Magnusson et al., 1975) or human prothrombin (72 000; Kisiel & Hanahan, 1973).

Isoelectric Points. The isoelectric points of Protein S and various other vitamin K dependent proteins from bovine and human plasma are shown in Table II. All of these proteins displayed microheterogeneity upon isoelectric focusing, and thus the range of isoelectric values has been tabulated. These plasma proteins are acidic, and bovine and human Protein S have the highest isoelectric point ranges in this group of proteins.

Amino Acid and Carbohydrate Composition. The amino acid and carbohydrate compositions of human and bovine Protein S are shown in Table III. Some similarity in their compositions is noted when these two proteins are compared, although substantial differences in their content of serine and alanine are apparent. Protein S from human and bovine plasma is particularly rich in aspartic acid, glutamic acid, and leucine. The similarity in composition between human and bovine Protein S is greater when these proteins are compared to each other than when either of these is compared to any of the other plasma proteins which contain γ-carboxyglutamic acid (see DiScipio et al., 1977). It is also noteworthy that the carbohydrate content of human and bovine Protein S is the lowest of any of the members of this family of plasma proteins.

The γ-carboxyglutamic acid content of human and bovine Protein S is shown in Table IV along with that of the other vitamin K dependent proteins of plasma. Protein S from both human and bovine plasma contains about 10 residues of γ-carboxyglutamic acid per mol of protein. The data also indicate the presence of 9–12 residues of γ-carboxyglutamic acid in the other vitamin K dependent proteins from human and bovine plasma.

Amino-Terminal Sequence Analyses. Table V shows the first 13 amino-terminal residues in human and bovine Protein S. The sequence homology between these two proteins is clearly evident in that 10 residues are identical and two

Table III: Amino Acid and Carbohydrate Compositions of Human and Bovine Protein S[a]

component	human (69 000)	bovine (64 200)
amino acid		
Lys	43.1	35.0
His	11.8	8.8
Arg	20.2	17.2
Asp[b]	66.7	59.9
Thr	29.7	25.7
Ser	43.8	31.3
Glu[c]	62.8	68.1
Pro	26.8	24.7
Gly	39.4	36.4
Ala	39.5	30.0
half-cystine	21.3	25.9
Val	36.0	35.3
Met	5.1	8.9
Ile	32.4	27.5
Leu	51.7	44.5
Tyr	16.1	19.0
Phe	19.3	24.1
Trp	9.5	9.6
carbohydrate		
hexose	12.3 (3.2%)	13.8 (3.8%)
N-acetylglucosamine	9.3 (2.8%)	4.8 (1.6%)
N-acetylneuraminic acid	4.0 (1.8%)	3.3 (1.6%)
protein (%)	92.2	93.0
carbohydrate (%)	7.8	7.0

[a] Expressed in residues per molecule of glycoprotein. [b] The aspartic acid includes asparagine. [c] The glutamic acid content includes glutamine and 10 residues of γ-carboxyglutamic acid (see Table IV).

Table IV: γ-Carboxyglutamic Acid Content of the Vitamin K Dependent Plasma Proteins

	γ-carboxyglutamic acid[a]	molecular weight
bovine Protein S	10.0	64 200
human Protein S	10.3	69 000
bovine prothrombin	10.0	71 600[b]
human prothrombin	10.1	72 000[c]
bovine factor X_1	12.3	55 100[d]
bovine factor X_2	11.8	55 100[d]
human factor X	11.6	58 900[e]
bovine factor VII	9.4	45 500[f]
bovine Protein C	10.8	57 000[g]
human Protein C	10.4	57 000
bovine factor IX	12.2	55 400[h]
human factor IX	12.4	57 100[e]

[a] Expressed in residues per molecule of glycoprotein. [b] From Magnusson et al. (1975). [c] From Kisiel & Hanahan (1973). [d] From Fujikawa et al. (1972) and Titani et al. (1975). [e] From DiScipio et al. (1977). [f] From Kisiel & Davie (1975). [g] From Kisiel et al. (1976). [h] From Fujikawa et al. (1973).

conservative substitutions are present in positions 3 and 10. Residue 8 of human Protein S was not identified. Comparison of these sequences with those of several other γ-carboxyglutamic acid containing plasma proteins reveals considerable homology among the various members of this family of proteins (Fujikawa et al., 1974; DiScipio et al., 1977). Human and bovine Protein S show the greatest degree of amino-terminal sequence homology with the light chains of human and bovine factor X.

Discussion

The present paper indicates that Protein S from human and bovine plasma is a single-chain glycoprotein containing γ-carboxyglutamic acid. The physiological role of this protein has not been established. Thus, the present data do not definitely prove that the two proteins we have isolated from

Table V: Amino-Terminal Sequences of Human and Bovine Protein S

	residue												
	1	2	3	4	5	6	7	8	9	10	11	12	13
human	Ala	Asn	Ser	Leu	Leu	(Gla)[b]	(Gla)[b]	?[c]	Lys	Gln	Gly	Asn	Leu
equivalents	0.7	0.3	0.3	0.8	0.9	NQ[a]	NQ		0.3	0.1	0.3	0.1	0.3
bovine	Ala	Asn	Thr	Leu	Leu	(Gla)[b]	(Gla)[b]	Thr	Lys	Lys	Gly	Asn	Leu
equivalents	0.8	0.3	NQ	0.8	1.0	NQ	NQ	NQ	0.6	0.8	0.4	0.2	0.3

[a] NQ, not quantitated. [b] Tentative identification shown in parentheses. [c] Question mark indicates not identified.

human and bovine plasma are functionally homologous. It appears highly probable that they are counterparts, however, since (a) both proteins can be purified by nearly identical procedures and each elutes prior to prothrombin on poly-homoarginine-Sepharose columns, (b) both proteins have similar amino acid and carbohydrate compositions, physical properties, and amino-terminal sequences, and (c) some immunological cross-reactivity between the two proteins was observed in a specific radioimmunoassay.

The presence of γ-carboxyglutamic acid in Protein S indicates that it is another vitamin K dependent protein containing 10 residues per mol of protein. We also observed the presence of about 12 residues of γ-carboxyglutamic acid in human factor X and bovine factors X_1 and X_2. These data are similar to that of Lindhout et al. (1978) who reported 12 and 13 residues of γ-carboxyglutamic acid in bovine factors X_1 and X_2, respectively. Our data, however, differ from that of Neal et al. (1976) who reported the presence of 6.8–8.1 residues in bovine factor X_1 and 12.8–13.3 in bovine factor X_2. Our results on the content of γ-carboxyglutamic acid in human factor IX also differ somewhat from those of Østerud et al. (1978) who have reported 8–10 residues of γ-carboxyglutamic acid per mol of protein. We have consistently found 12 γ-carboxyglutamic acid residues in both bovine and human factor IX. Furthermore, our data are in accord with the content of γ-carboxyglutamic acid as determined from the primary sequence of factor IX (K. Titani, personal communication). The present experiments also clearly establish the presence of γ-carboxyglutamic acid in factor VII. The presence of γ-carboxyglutamic acid in prothrombin, Protein C, factor IX, and factor X has been shown previously in many different laboratories (Stenflo et al., 1974; Magnusson et al., 1974; Nelsestuen et al., 1974; Bucher et al., 1976; Stenflo, 1976; Fryklund et al., 1976). In summary, the plasma proteins that have been shown to date to contain γ-carboxyglutamic acid and have been characterized in detail include prothrombin, factor X, factor IX, factor VII, Protein C, and Protein S. The existence of γ-carboxyglutamic acid containing proteins, however, has been established to be a more general phenomenon, as such proteins have been found in bone (Hauschka et al., 1975; Price et al., 1976), kidney (Hauschka et al., 1976), renal stones (Lian et al., 1977), and certain pathological fluids and tissues (Lian et al., 1976).

Bovine and human Protein S have the highest isoelectric point ranges of the various plasma proteins which contain γ-carboxyglutamic acid. Our data for the isoelectric values of bovine and human prothrombin and factor IX are in agreement with the results of Pechet & Smith (1970), Chandra & Pechet (1973), and Suomela (1976).

The physiological role of Protein S remains to be established. It shows many characteristics which are similar to the other vitamin K dependent proteins in plasma, including its binding to barium citrate (DiScipio et al., 1977) and phospholipid membranes in the presence of calcium ions (Nelsestuen et al., 1978), as well as its composition and amino-terminal sequence. Thus, it seems probable that Protein S is also a precursor to a serine protease and participates in blood as a protease with limited specificity toward one or more protein or peptide substrates. Protein S does not appear to play a role associated with the activation of prothrombin since its complete removal from the latter protein had no influence on the biological activity of prothrombin in a specific two-step assay, including factor X_a, factor V, prothrombin, and fibrinogen (R. DiScipio, unpublished results).

Studies on the function of human Protein S have proven to be difficult because of the small amount of this protein that can be isolated (maximum yield was 2 mg of human Protein S from 5 L of plasma). The rationale for developing an isolation procedure for bovine Protein S has been that bovine plasma was readily available in large quantities; furthermore, the yield of Protein S from bovine plasma was 5–8 mg from 5 L of plasma, which was substantially higher than that obtained from human plasma. Activation studies on Protein S are currently under investigation in our laboratory.

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