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Purification of human vitamin K-dependent protein S and its limited proteolysis by thrombin

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Vitamin K-dependent protein S exists in two forms in human plasma, namely as the free protein and in complex with C4b-binding protein [Dahlbäck & Stenflo (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 2512–2516]. Now reported is a simple purification procedure for human protein S that includes barium citrate adsorption, DEAE-Sephacel chromatography and chromatography on Blue Sepharose. The yield was approx. 30% relative to the concentration of free protein S in plasma, which was found to be approx. 10mg/l. Purified protein S migrated as a single-chain band on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis under non-reducing conditions and as a doublet of M_r approx. 85000 and 75000 on reduction. A third band of M_r 16000 was observed after electrophoresis of ^{125}I-labelled protein S and radioautography of reduced samples. This band appears to be disulphide-linked to the 75000-M_r chain before reduction. Thrombin converted the 85000-M_r chain of protein S into a 75000-M_r chain and an 8000-M_r fragment, the latter again being detectable only by radioautography of reduced samples. The 16000-M_r fragment was not observed, suggesting its degradation by thrombin. Under non-reducing conditions, no change in apparent molecular weight of thrombin-treated protein S was observed, indicating disulphide linkage of the fragments. Thrombin also affected the mobility of protein S on agarose-gel electrophoresis in the presence of Ca^{2+}, suggesting a decreased affinity to Ca^{2+} of the cleaved form of protein S as compared with the undegraded molecule. After activation of the complement system in human serum, protein S was found to be a constituent part of the complex formed by C4b-binding protein and component C4b.

DiScipio *et al.* (1977) reported the isolation and identification of a previously unknown vitamin K-dependent protein in human plasma. This protein was referred to as protein S. Two years later, the bovine counterpart was identified and purified by DiScipio & Davie (1979) and by Stenflo & Jönsson (1979). Human and bovine protein S are both single-chain molecules (M_r 69000 and 64000 respectively), with approx. 10 γ-carboxyglutamic acid residues in the *N*-terminal part of the molecule. The amino acid sequence in this part of protein S is highly homologous to corresponding regions of vitamin K-dependent clotting factors (Jackson & Nemerson, 1980). It has not yet been possible to demonstrate whether or not protein S is a zymogen of a serine proteinase, and no definite function has yet been assigned to it. Walker (1980) has reported that bovine protein S enhances the rate of degradation of phospholipid-bound clotting Factor Va by the vitamin K-dependent protein, protein C_a. This effect was later found to be due to an increased binding of protein C_a to the phospholipid surface in the presence of protein S (Walker, 1981). It has also been demonstrated (Dahlbäck & Stenflo, 1981) that protein S in plasma exists in two forms, namely as free protein and in complex with the complement component C4b-binding protein (C4bp). C4bp is an important cofactor in the degradation of fluid-phase C4b by the enzyme Factor I (previously named C3b inactivator) (Scharfstein *et al.*, 1978; Fujita

The nomenclature used for the complement proteins is that recommended by the World Health Organisation (1968, 1981). Other abbreviations used: C4bp, C4b-binding protein; C4bp(s), C4bp purified from a barium citrate eluate; $\overline{\text{C1s}}$, activated form of C1s; Factor I, C3b inactivator; SDS, sodium dodecyl sulphate.

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et al., 1978; Nagasawa et al., 1980). The function of protein S in the C4b–protein S complex remains to be elucidated.

In the present paper I report an improved purification procedure for human protein S. The effects of blood clotting and complement activation on protein S in plasma were also investigated. Studies of the formation of the complex between protein S and C4bp, and of the influence of protein S on the degradation of C4b, are reported in the following papers (Dahlbäck, 1983; Dahlbäck & Hildebrand, 1983).

Materials and methods

Materials

DEAE-Sephacel, Blue Sepharose and molecular-weight markers for polyacrylamide-gel electrophoresis were from Pharmacia Fine Chemicals, Uppsala, Sweden, and Ultrogel AcA-34 was from LKB Produkter, Bromma, Sweden. The flexible polyester film Gelbond TM and agarose were from Marine Colloid, Rockland, ME, U.S.A.

Trypsin [1-chloro-4-phenyl-3-tosylamidobutan-2-one ('TPCK')-treated], N^{α}-benzoyl-DL-arginine *p*-nitroanilide, bovine serum albumin, soya-bean trypsin inhibitor, phenylmethanesulphonyl fluoride and di-isopropyl phosphorofluoridate were from Sigma Chemical Co., St. Louis, MO, U.S.A.

Poly(ethylene glycol), with a mean molecular weight of 6000, was from Kebo-Grave AB, Malmö, Sweden. The chromogenic substrates compounds S 2238, S 2160 and S 2251 and plasmin were from Kabi, Stockholm, Sweden.

Succinoylalanylalanylalanine *p*-nitroanilide was from Protein Research Foundation, Minoh, Shi, Osaka, Japan. Acrylamide and bisacrylamide were from E. Merck, Darmstadt, Germany. $Na^{125}I$ (carrier-free) and [^{3}H]di-isopropyl phosphorofluoridate were from The Radiochemical Centre, Amersham, Bucks., U.K.

Human thrombin was purified by the method of Lundblad *et al.* (1975). It had a specific activity of 2300 units/mg when determined by the method of Fenton & Fasco (1974). Bovine Factor Xa and protein C_a were purified and activated as described by Jesty & Nemerson (1976) and Kisiel *et al.* (1977). The Factor X activator from Russell's-viper venom was purified by the procedure of Kisiel *et al.* (1976). $\overline{C1s}$ was purified as described by Gigli *et al.* (1976), and Factor I by using the method described by Crossley & Porter (1980). The thrombin inhibitor dansylarginine *N*-(3-ethylpentane-1,5-diyl)amide was synthesized as described by Nesheim *et al.* (1979).

Purification of protein S

Human protein S was purified by a procedure similar to that described, for bovine protein S (Stenflo & Jönsson, 1979). Chromatography and centrifugation were run at 4°C, all manipulations being performed on an ice bath. Freshly frozen platelet-poor plasma (−70°C) in citrate/phosphate/dextrose/adenine (Travenol) was obtained from the blood bank. After thawing at 37°C, the following proteinase inhibitors were added to the plasma: benzamidine hydrochloride (10mM), di-isopropyl phosphorofluoridate (1mM), phenylmethanesulphonyl fluoride (1mM) and soya-bean trypsin inhibitor (50mg/l). Then 1M-$BaCl_2$ was added dropwise (80ml/l of plasma). After stirring of the mixture for 1h, the barium citrate precipitate was collected by centrifugation at 5000**g** for 10min. Protein S and most of the C4bp in plasma were adsorbed on the barium citrate. This C4bp is referred to below as C4bp(s). The barium citrate precipitate was suspended in 0.9% NaCl containing 5mM-benzamidine hydrochloride (80ml/l of plasma) and centrifuged again. The washing procedure was repeated once. The barium citrate precipitate was dissolved in 0.2M-EDTA, pH7.4, containing 10mM-benzamidine hydrochloride, 0.5mM-di-isopropyl phosphorofluoridate and 0.1mM-phenylmethanesulphonyl fluoride (150ml/l of plasma). The suspension was stirred for 20min and then dialysed against 0.1M-sodium phosphate buffer, pH6.0, containing 10mM-benzamidine hydrochloride. After dialysis a small precipitate was removed by centrifugation at 10000**g** for 20min. After the addition of di-isopropyl phosphorofluoridate (1mM) and phenylmethanesulphonyl fluoride (1mM) to the dialysed barium citrate eluent, it was applied to a column (2.5cm × 39cm) of DEAE-Sephacel in 0.1M-sodium phosphate buffer, pH6.0, containing 1mM-benzamidine hydrochloride, 0.1mM-di-isopropyl phosphorofluoridate and 0.1mM-phenylmethanesulphonyl fluoride. The chromatographic details are given in the legend to Fig. 1(*a*). The positions of protein S and C4bp(s) in the column effluent were monitored immunochemically. The fractions containing C4bp(s) were pooled as indicated in Fig. 1(*a*). The further purification and characterization of C4bp(s) is reported in the following paper (Dahlbäck, 1983). The protein S pool from the DEAE-Sephacel was dialysed against 50mM-Tris/HCl buffer, pH7.5, containing 0.1M-NaCl and 1mM-EDTA and applied to a column (2.5cm × 38.5cm) packed with Blue Sepharose in the same buffer, also containing 0.1mM-di-isopropyl phosphorofluoridate and 0.1mM-phenylmethanesulphonyl fluoride. Before application the protein S pool was made 1mM with respect to both di-isopropyl phosphorofluoridate and phenylmethanesulphonyl fluoride. Details of the column chromatography are given in the legend to Fig. 1(*b*). Protein S was pooled as indicated in Fig. 1(*b*), concentrated by ultrafiltration on an Amicon YM10 filter

and stored at −70°C. The isolated protein S was quantified spectrophotometrically by using $A_{1\,cm}^{1\%}$ at 280 nm = 9.5 (DiScipio & Davie, 1979).

Electrophoretic and immunochemical methods

Agarose-gel electrophoresis was run at pH 8.6 in 75 mM-sodium barbital buffer containing either 2 mM-calcium lactate or 2 mM-EDTA on the flexible polyester film Gelbond TM as described by Jeppsson *et al.* (1979). Crossed immunoelectrophoresis and electroimmunoassay were performed as described by Ganrot (1972) and Laurell (1972) respectively. SDS/polyacrylamide-slab-gel electrophoresis was performed as described by Blobel & Dobberstein (1975) with 5–15%-gradient gels. The buffer system of Maziel (1971) was used. After the gels had been fixed, dried and stained with Coomassie Brilliant Blue R-250, the distribution of ^{125}I-labelled protein S was detected by radioautography. Molecular weights were calculated from comparison with those of the following standard proteins: phosphorylase *b* (M_r 94000), bovine serum albumin (M_r 67000), ovalbumin (M_r 73000), carbonic anhydrase (M_r 3000), soya-bean trypsin inhibitor (M_r 20100) and α-lactalbumin (M_r 14400). Antisera against purified protein S and C4bp were raised in rabbits. The C3 and C4 rabbit antisera were available at the laboratory.

Concentration of free protein S in human plasma

A trace amount of ^{125}I-labelled protein S (less than 0.2 μg/ml) was added to pooled human citrated plasma. After incubation for 2 h at 37°C to equilibrate the labelled protein S between the different forms of protein S in plasma (see the following paper, Dahlbäck, 1983), samples of the plasma were treated with poly(ethylene glycol) (0–6%, w/v) for 1 h at 4°C. The precipitates that formed were removed by centrifugation at 5000 **g** for 10 min, and the supernatants were analysed by electroimmunoassay in the presence of 2 mM-EDTA. A mixture of a high concentration of antiserum against C4pb and a lower concentration of antiserum against protein S was used. This was to immunoprecipitate, and thereby suppress, the C4bp–protein S complexes early during electrophoresis, i.e. well before the immunoprecipitates between free protein S and anti-(protein S) antibodies were formed. Pure protein S, with a tracer amount of ^{125}I-labelled protein S, was used as standard. The protein S was quantified spectrometrically. The dilution buffer for the standard was either 50 mM-Tris/HCl buffer, pH 7.5, containing 0.15 M-NaCl, 40 mg of bovine serum albumin/ml and 10 mg of human polyclonal immunoglobulin G/ml, or barium citrate-adsorbed bovine plasma diluted 1 : 5 in distilled water. The precipitates were located by radioautography and, after each immunoprecipitate had been cut out, the amount of radioactivity was measured in a γ-radiation counter.

Effects of clotting and complement activation on protein S in plasma

^{125}I-labelled protein S (100 μl) was added to 1 ml of human citrated platelet-rich or platelet-poor plasma. After incubation for 2 h at 37°C the plasmas were transferred to glass tubes; clotting was initiated by the addition of $CaCl_2$ (10 mM) and $MgCl_2$ (10 mM), and, after a further 30 min at 37°C, the fibrin clots were removed. The complement system was activated by the addition of heat-aggregated human immunoglobulin G (incubated at 63°C for 30 min in 50 mM-Tris/HCl buffer, pH 7.5, at a concentration of 10 mg/ml) to a final concentration of 1 mg/ml, and the reaction mixtures were then incubated at 37°C for 1 h. Before and after the clotting and complement activation, samples were rapidly frozen at −70°C and later analysed on crossed immunoelectrophoresis with antisera against protein S, C4 and C3. Samples (5 μl) were also analysed by 5–15%-gradient SDS/polyacrylamide-slab-gel electrophoresis, both before and after reduction of disulphide bonds with 5% (v/v) 2-mercaptoethanol for 2 min at 95°C.

Effects of thrombin and other proteinases on purified protein S

Both in the presence of $CaCl_2$ (2.5 mM) and C4bp(s) (180 μg/ml) and in their absence, purified protein S (0.2–0.4 mg/ml) in 50 mM-Tris/HCl buffer, pH 7.5, containing 0.15 M-NaCl, with a trace amount of ^{125}I-labelled protein S, was incubated at 37°C for 1–2 h separately with each of the following: thrombin (0.4–50 μg/ml), trypsin (0.2–40 μg/ml), plasmin (2–40 μg/ml), Russell's-viper-venom Factor X activator (10 μg/ml), protein C_a (10–40 μg/ml), Factor Xa (5–40 μg/ml), Factor I (7–37 μg/ml) and $\overline{C1s}$ (4 μg/ml). Samples of the incubation mixture were analysed by 5–15%-gradient SDS/polyacrylamide-slab gel electrophoresis before and after treatment with 5% 2-mercaptoethanol for 2 min at 95°C.

Before and after incubation with thrombin, tests were made to check for any esterolytic activity of protein S. Protein S (0.4 mg/ml) in 50 mM-Tris/HCl buffer, pH 7.5, containing 0.15 M-NaCl was incubated with thrombin (0.4–40 μg/ml) for 1 h at 37°C. The highly specific thrombin inhibitor dansylarginine *N*-(3-ethylpentane-1,5-diyl)amide was then added (final concentration 80 μM) to inhibit the esterolytic activity of thrombin. Samples (10–20 μl) containing 4–8 μg of protein S were then tested for esterolytic activity, with each of the following: compounds S2160 (benzoylphenylalanylvalylargining *p*-nitroanilide hydrochloride), S2238 (D-phenylalanylpipecolylarginine *p*-nitroanilide hydro-

chloride) and S2251 (D-valyl-leucyl-lysine *p*-nitroanilide hydrochloride) (Svendsen *et al.*, 1972), N^{α}-benzoylarginine *p*-nitroanilide (Erlanger *et al.*, 1961) and succinoylalanylalanylalanine *p*-nitroanilide (Bieth *et al.*, 1974).

Reaction of [^{3}H]di-isopropyl phosphorofluoridate with protein S both before and after modification by trypsin and thrombin was investigated. Protein S (0.2mg/ml) or C4bp(s) (0.1mg/ml) in 50mM-Tris/HCl buffer, pH 7.5, was incubated for 2h at 37°C with thrombin (1 and 10μg/ml) or for 1h at 37°C with trypsin (0.2 and 2μg/ml). A mixture of protein S (0.2mg/ml) and C4p(s) (0.1mg/ml) was likewise incubated with trypsin or with thrombin. The volumes of incubation mixtures were 0.2ml. At the end of the incubation period, 5μl of [^{3}H]di-isopropyl phosphorofluoridate was added to each mixture. After 1h at room temperature, 40μl of the reaction mixture was adsorbed on Whatman 3MM filter paper (1cm diam.). The filter papers were washed in ice-cold 10% (w/v) trichloroacetic acid for 30min and then in 5% trichloroacetic acid at 100°C for 10min. The filter papers were washed twice in ethanol and finally in diethyl ether. After being dried, the filter papers were put in 10ml of Lumagel in counting vials, and the amount of ^{3}H associated with the filter papers was measured in a liquid-scintillation counter. Trypsin (10 and 100μg/ml) and thrombin (10μg/ml) served as positive controls for the assay.

Other methods

The amino acid composition of protein S was determined in acid hydrolysates (24 and 72h in 6M-HCl at 110°C *in vacuo*) by standard procedures by using a single-column programme on a Beckman model 119CL amino acid analyser. Half-cysteine was determined as *S*-carboxymethylcysteine. γ-Carboxyglutamic acid was determined after alkaline hydrolysis as described by Fernlund *et al.* (1975). The isolated protein was [^{125}I]iodinated by a lactoperoxidase method (Thorell & Johansson, 1971). The molar ratio of iodide to protein varied from 0.1 to 0.5.

Results

Comments on the purification of protein S

Protein S was purified from freshly frozen human plasma by barium citrate adsorption, elution and two chromatographic steps (Table 1). An abundance of inhibitors of proteolytic enzymes were used, especially in early purification steps, to minimize proteolytic degradation or activation during handling. Most purification procedures for vitamin K-dependent proteins include a $(NH_4)_2SO_4$ fractionation of the barium citrate eluate. This step, however, is unnecessary for achieving purity in the final product; furthermore, the protein S yield is greater when it is excluded. The first large protein peak in the DEAE-Sephacel chromatography (Fig. 1*a*) contained mainly C4bp in complex with protein S, confirming that this material reacts with monospecific antisera against both protein S and C4bp, as reported previously (Dahlbäck & Stenflo, 1981). Free protein S was eluted just in front of the prominent prothrombin peak. The protein S pool from the DEAE-Sephacel was then chromatographed on Blue Sepharose (Fig. 1*b*). Protein S was not retained on the column and appeared pure in the first protein peak. Factor X and protein C were retarded and appeared in the second protein peak. Prothrombin and C4bp(s) were adsorbed on the column and were eluted together during the gradient in the third major peak. The purification procedure was repeated several times and consistently yielded a high-quality product.

The amino acid composition of the purified protein S was very similar to that previously published (DiScipio & Davie, 1979). The γ-carboxyglutamic acid content was measured in two different protein S preparations. It was 8.4 and 14.2 residues/molecule of protein S when calculated relative to aspartic acid in the hydrolysates, or 8 and 9.5 residues/molecule of protein S when calculated from the amount of analysed material (weight). Within acceptable experimental error, these results agree with the reported value of 10 γ-carboxyglutamic acid residues per molecule of protein S (DiScipio & Davie, 1979).

Table 1. *Purification of human protein S*

For full experimental details see the text. The protein concentration was measured from the absorbance at 280nm, assuming $A_{1cm}^{1\%} = 10$. Protein S was determined by electroimmunoassay as described in the Materials and methods section. The recovery was calculated from the concentration of free protein S in plasma.

Fraction	Volume (ml)	Protein (mg)	Protein S (arbitrary units)	Recovery (%)	Purification (fold)
Plasma	5000	330000	520	100	1
Barium citrate eluate	930	7440	440	85	39
DEAE-Sephacel	85	189	260	50	943
Blue Sepharose	82	14.6	154	29	6600

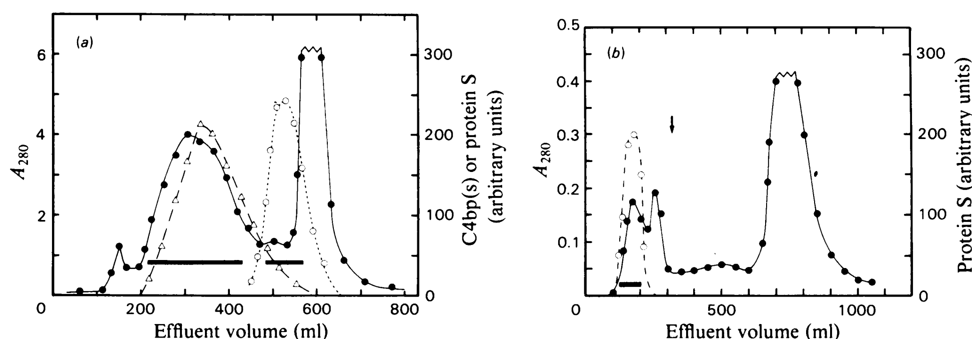

Fig. 1. *Chromatography used in the purification of protein S*
(*a*) DEAE-Sephacel chromatography of the barium citrate eluate. The column (2.5cm × 39cm) was equilibrated in 0.1M-sodium phosphate buffer, pH 6.0, containing 1mM-benzamidine hydrochloride, 0.1mM-di-isopropyl phosphorofluoridate and 0.1mM-phenylmethanesulphonyl fluoride. The proteins were eluted with a linear gradient of NaCl (0.1–0.7M), 600ml in each vessel. The chromatography was run at 47ml/h, and 5.5ml fractions were collected. The effluent was monitored immunochemically with antisera against C4bp and protein S. Fractions were pooled as indicated by the horizontal bars. ●, A_{280}; △, C4bp(s); ○, free protein S. (*b*) Blue Sepharose chromatography of the protein S pool from the DEAE-Sephacel chromatography. The column (2.5cm × 38.5cm) was equilibrated in 50mM-Tris/HCl buffer, pH 7.5, containing 0.1M-NaCl, 1mM-EDTA, 0.1mM-di-isopropyl phosphorofluoridate and 0.1mM-phenylmethanesulphonyl fluoride. The column was eluted at 80ml/h, and 5.3ml fractions were collected. The arrow indicates the start of a linear gradient of NaCl (0.1–1M), 600ml in each vessel. The effluent was monitored immunochemically with an antiserum against protein S. Fractions were pooled as indicated by the horizontal bar. ●, A_{280}; ○, protein S.

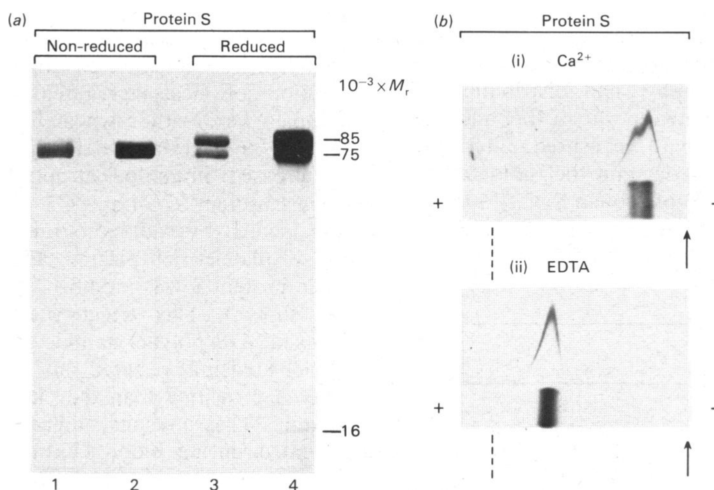

Fig. 2. *Purified protein S analysed by SDS/polyacrylamide-gel electrophoresis and by crossed immunoelectrophoresis*
(*a*) SDS/polyacrylamide-slab-gel electrophoresis of purified protein S, with a ^{125}I-labelled protein S tracer. Approx. 10μg of protein was applied per track. Lanes 1 and 3 show the Coomassie-Blue-stained gels, and lanes 2 and 4 the radioautography of the same gels. The sample shown in lane 1 and 2 was non-reduced, and the sample in lanes 3 and 4 was reduced. (*b*) Crossed immunoelectrophoresis of purified protein S with the first dimension either in the presence of Ca^{2+} (i) or in the presence of EDTA (ii). Arrows indicate the application slot and the broken line the position of albumin in the first dimension.

Characterization of protein S by electrophoresis

Despite the use of proteinase inhibitors, the purified protein S appeared as a closely spaced doublet (apparent M_r 85000 and 75000) on SDS/polyacrylamide-slab-gel electrophoresis after reduction of disulphide bridges (Fig. 2*a*). Staining with Coomassie Blue failed to detect any lower-molecular-weight protein bands, though a peptide with apparent M_r 16000 was observed when

^{125}I-labelled protein S was analysed by radioautography. No lower-molecular-weight material was observed on non-reduced gels, and the protein S band appeared to be homogeneous both on the stained gels and on the radioautography. The ratio of the two bands in the doublet varied from preparation to preparation. The purified protein S was also analysed by agarose-gel electrophoresis and crossed immunoelectrophoresis (Fig. 2*b*). When the agarose-gel electrophoresis was run in the presence of 2mM-EDTA, protein S appeared to be homogeneous. When analysed in the presence of 2mM-$CaCl_2$, protein S was greatly retarded, indicating a strong Ca^{2+}-binding by the protein. On comparison of different preparations of protein S with different ratios of the two forms, it was observed that the protein S corresponding to the 75000-M_r band was slightly less retarded than was the single-chain 85000-M_r protein S. Both forms reacted well with a monospecific antibody against protein S, and gave a precipitation line of identity.

Concentration of free protein S in plasma

The concentrations of free protein S in plasma was measured by electroimmunoassay, by using a combination of antisera against C4bp and protein S (Fig. 3). Because of the low concentration of free protein S, a trace amount of ^{125}I-labelled protein S was added to the plasma. Immunoprecipitates were detected by radioautography, and distribution of ^{125}I-labelled protein S between the C4bp and the protein S precipitates was measured after the precipitates had been cut out and the radioactivity counted. Approx. 60–65% of protein S was found in the C4bp immunoprecipitate and 35–40% in the protein S immunoprecipitate. The C4bp–protein S complex was precipitated quantitatively by approx. 5% poly(ethylene glycol), whereas at least 60% of the free protein S (measured both as 'rocket' height and amount of radioactivity) was recovered in the supernatant. The concentration of free protein S in a mixed human citrated plasma pool was estimated to be approx. 10mg/l. Added concentrations of poly(ethylene glycol) to the plasma resulted in a corresponding decrease in height of 'rockets' representing free protein S and amount of radioactivity recovered in corresponding immunoprecipitates. This indicates that quantitative determination of free protein S in untreated plasma samples is not affected by the presence of the C4bp–protein S complexes. Furthermore, as reported in the following paper (Dahlbäck, 1983), dissociation of the complex during electrophoresis did not influence the results significantly.

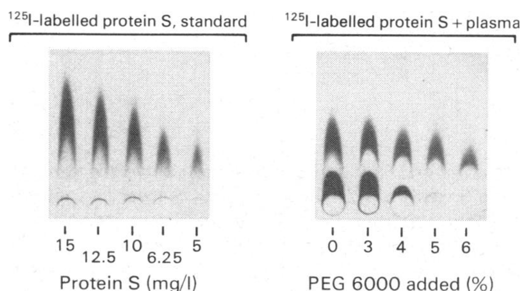

Fig. 3. *Electroimmunoassay of protein S in plasma*
A ^{125}I-labelled protein S tracer was added to plasma. Before application, samples of the plasma were incubated with increasing poly(ethylene glycol) (PEG 6000) concentrations, the precipitates formed being removed by centrifugation. The immunoprecipitates were detected by radioautography. Purified protein S was used as standard (left part of the Figure).

Effects on protein S during clotting and activation of the complement system

Efforts were made to investigate whether protein S was structurally modified during clotting or activation of the complement system. A trace amount of ^{125}I-labelled protein S was added to human citrated platelet-rich or platelet-poor plasma. The clotting process was initiated by the addition of $CaCl_2$. The complement system was then activated by the addition of heat-aggregated immunoglobulin G. Samples were withdrawn at intervals and analysed by SDS/polyacrylamide-slab-gel electrophoresis and by crossed immunoelectrophoresis with antisera against protein S, C4 and C3. ^{125}I-labelled protein S was located by radioautography. During the clotting of platelet-rich plasma, approx. 50% of single-chain protein S was modified by proteolysis (results not shown). This effect was only observed on reduced SDS/polyacrylamide gels. Clotting of platelet-rich plasma resulted in a higher amount of cleaved protein S than did clotting of platelet-poor plasma. These results indicate that an enzyme, activated during blood clotting, cleaves a peptide bond near one of the ends of protein S, resulting in a disulphide-linked two-polypeptide-chain structure. The pattern on crossed immunoelectrophoresis (first dimension in 2mM-EDTA) with an antiserum against protein S remained unchanged after clotting (Fig. 4*a*). Free protein S migrated rapidly in the first dimension, whereas the C4bp–protein S complexes were just anodal to the application slots. After activation of the complement system, the migration rate of the C4bp–protein S complex was enhanced, and the ^{125}I-labelled protein S in the complex was precipitated on crossed immunoelectrophoresis with an anti-C4 serum (Fig. 4*b*), indicating that protein S is part of the C4b–C4bp complex form during complement activation.

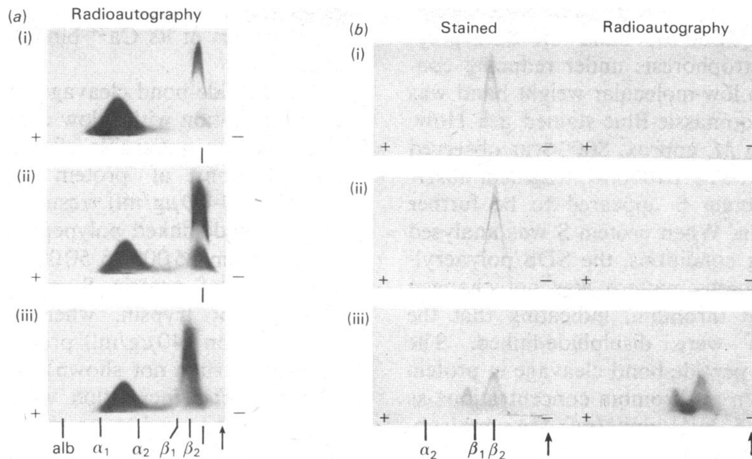

Fig. 4. *Effects of clotting and of activation of the complement system on protein S in plasma, as judged by crossed immunoelectrophoresis*

A ^{125}I-labelled protein S tracer was added to platelet-rich plasma. After equilibrium of the tracer between free and complexed protein S, clotting was initiated by the addition of $CaCl_2$, and the complement system was then activated by aggregated immunoglobulin G. The amount of applied material was equivalent to 10μl of plasma. Arrows indicate the application slots. (*a*) Crossed immunoelectrophoresis with an antiserum against protein S. The picture represents the radioautography. (i) Plasma, (ii) serum and (iii) serum, after complement activation. Both dimensions were run in the presence of 2mM-EDTA. (*b*) Crossed immunoelectrophoresis with an antiserum against C4. The stained gels are shown to the left and the corresponding radioautography to the right. (i) Plasma, (ii) serum, and (iii) serum, after complement activation. Both dimensions were run in the presence of 2mM-EDTA.

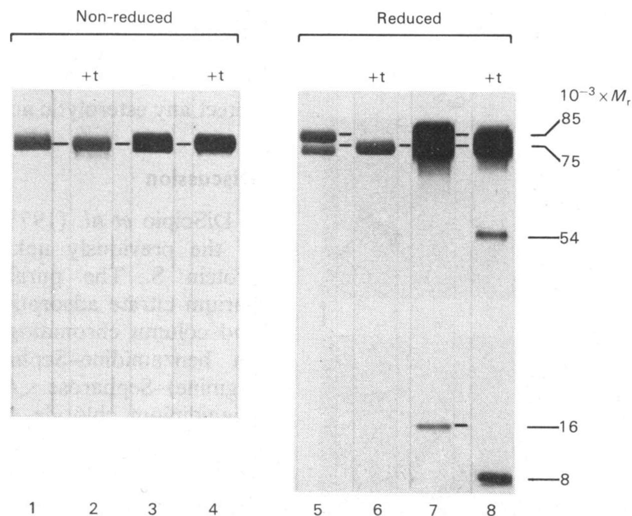

Fig. 5. *SDS/polyacrylamide-slab-gel electrophoresis of protein S before and after incubation with thrombin*

Protein S (0.3mg/ml), with a ^{125}I-labelled protein S tracer, was incubated with thrombin (+t) (20NIH units/ml) at 37°C for 2h. Lanes 1, 2, 5 and 6 show the Coomassie-Blue-stained gels, and lanes 3, 4, 7 and 8 are the radioautographs of the same gels. The gels to the left (1–4) are non-reduced and the gels to the right (5–8) are reduced. Samples incubated with thrombin are denoted by t (lanes 2, 4, 6, 8). Approx. 10–15μg of protein was applied per gel.

Effects of thrombin on purified protein S

The observed modification of protein S by clotting is presumably the result of limited proteolysis by a proteinase involved in clotting. Therefore the effects on protein S by thrombin and by some other proteinases were investigated. Thrombin was found to cleave a peptide bond in single-chain protein S,

giving rise to a 75000-M_r band on SDS/polyacrylamide-gel electrophoresis under reducing conditions (Fig. 5). No low-molecular-weight band was observed on the Coomassie-Blue-stained gel. However, a peptide with M_r approx. 8000 was observed on radioautography. The 16000-M_r fragment observed in purified protein S appeared to be further cleaved by thrombin. When protein S was analysed under non-reducing conditions, the SDS/polyacrylamide-gel-electrophoretic pattern was not changed by incubation with thrombin, indicating that the cleavage products were disulphide-linked. The thrombin-mediated peptide-bond cleavage in protein S was observed even at thrombin concentrations as low as 1 μg/ml (2.5 NIH units/ml) (enzyme/substrate ratio 1:200, w/w), indicating that protein S is very sensitive to the effect of thrombin. At higher thrombin concentrations (30–100 NIH units/ml), additional peptide bonds were cleaved, giving rise to a dominant band, with apparent M_r 54000, and a band that was barely visible either on radioautography or on stained gels, with M_r 41000. On unreduced gels, the protein S band was unchanged after incubation with thrombin. The effect of thrombin on protein S was also analysed by crossed immunoelectrophoresis (Fig. 6). The retarded migration rate of protein S in the presence of Ca^{2+} ions, discussed above was much less pronounced after modification of protein S by thrombin, indicating that part of its Ca^{2+}-binding capacity had been lost.

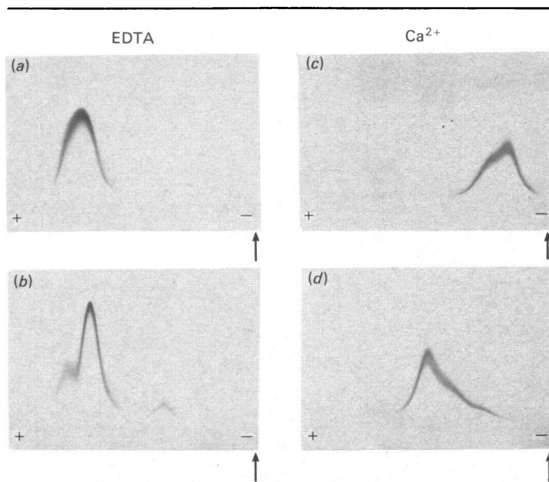

Fig. 6. *Crossed immunoelectrophoresis of protein S before and after modification by thrombin*
Protein S (0.4 mg/ml) was incubated with thrombin (10 NIH units/ml) at 37°C for 1 h and then analysed by crossed immunoelectrophoresis with the first dimension either in the presence of 2 mM-EDTA (*a* and *b*) or in the presence of 2 mM-$CaCl_2$ (*c* and *d*); (*a*) and (*c*) illustrate protein S before incubation with thrombin, and (*b*) and (*d*) the patterns obtained after incubation with thrombin. Arrows indicate the application of each sample.

The peptide-bond cleavage in protein S, observed after incubation with a low concentration of thrombin, was also noticeable after incubation with high concentrations of protein C_a and Factor Xa (approx. 30–40 μg/ml) (results not shown). Five or six disulphide-linked polypeptide chains (M_r values ranging from 15000 to 50000) were observed after incubation of protein S with low concentrations (2 μg/ml) of trypsin, whereas at higher trypsin concentration (40 μg/ml) protein S was extensively digested (results not shown). A similar pattern was observed after incubation with plasmin. However, protein S appeared to be more resistant to proteolysis by plasmin than by trypsin. Proteolysis of protein S by thrombin, trypsin and plasmin was unaffected by the presence of C4bp(s). In the presence of $CaCl_2$, however, the proteolytic effects on protein S of the different proteinases were much less pronounced than in the presence of EDTA. No proteolysis of protein S by $\overline{C1s}$, Factor I or Russell's-viper-venom Factor X activator was observed at the concentrations used. No reaction of [^{3}H]di-isopropyl phosphorofluoridate with protein S could be demonstrated, either before or after incubation with thrombin or trypsin. The assay system worked well, and significant reaction of [^{3}H]di-isopropyl phosphorofluoridate was found with control proteinases. Furthermore, a check made, after modification by thrombin, failed to detect any esterolytic activity of protein S.

Discussion

DiScipio *et al.* (1977) first reported the isolation of the previously unknown vitamin K-dependent protein S. The purification procedure included barium citrate adsorption, $(NH_4)_2SO_4$ fractionation and column chromatography on DEAE-Sephadex, on benzamidine–Sepharose and on poly(homoarginine)–Sepharose. A buffer containing 2.9 M-guanidinium chloride and 2.4 M-NaCl was used to elute protein S from the poly(homoarginine)–Sepharose column, isolated protein S then being renaturated by dialysis. The protein S yield was 0.5 mg from 5 litres of plasma. The purification procedure reported in the present paper is simple and yielded approx. 15 mg of protein S from 5 litres of plasma. Despite the use of an abundance of inhibitors of proteolytic enzymes, the purified material appeared as a doublet on SDS/polyacrylamide-slab-gel electrophoresis under reducing conditions. Stenflo & Jönsson (1979) reported that bovine protein S, purified by a technique similar to that described in the present paper, appeared as a closely spaced doublet on SDS/polyacrylamide-disc-gel electrophoresis under reducing conditions.

Presumably the modified protein S observed in both species was the result of proteolysis during the handling. Human protein S was found to be sensitive to proteolysis by thrombin. Even very low concentrations of thrombin cleaved a peptide bond near one of the ends of single-chain protein S. The 16000-M_r chain of the two-chain form of protein S was also cleaved by thrombin, whereas the 75000-M_r chain remained unaltered. This indicates that the thrombin-sensitive bond and the 16000-M_r chain are located at the same end of the protein S molecule. With the use of bovine material we have localized the thrombin cleavage to the *N*-terminal part of the molecule (B. Dahlbäck & J. Stenflo, unpublished work). The thrombin-modified human protein S did not react with [^{3}H]di-isopropyl phosphorofluoridate, and had no esterolytic activity against synthetic substrates, indicating that protein S is presumably not converted into a serine proteinase by thrombin. Like the other vitamin K-dependent plasma proteins, protein S has a strong affinity for negatively charged phospholipids in the presence of Ca^{2+} ions (Nelsestuen *et al.*, 1978). As in the other vitamin K-dependent proteins (Jackson & Nemerson, 1980), the Ca^{2+}-binding sites are presumably located at the *N*-terminal part of the molecule containing the γ-carboxyglutamic acid residues. Of possible physiological significance is that thrombin-modified protein S appears to have a lower affinity for Ca^{2+} than has intact protein S, judged by its mobility on agarose-gel electrophoresis in the presence of Ca^{2+}, and that thrombin modification of protein S also presumably influences the affinity of protein S for phospholipids. Walker (1980, 1981), using bovine material, has reported that protein S enhances the rate of degradation of Factor Va by the vitamin K-dependent serine proteinase protein Ca, this being due to an increased binding of protein Ca to the phospholipid surface by the presence of protein S. In this respect the modification of protein S by thrombin, described in the present paper, is of interest since thrombin is the physiological activator of both protein C and Factor V (Jackson & Nemerson, 1980). The effects of modification of protein S by thrombin and of the formation of C4bp–protein S complex on the degradation of Factor Va remain to be elucidated.

Owing to the complex-formation between protein S and C4bp, it has been difficult to measure the concentration of free protein S in human plasma. That the C4bp–protein S complexes remain stabile during agarose-gel electrophoresis, reported in the following paper (Dahlbäck, 1983), made it possible to devise a method for the quantitative determination of free protein S in plasma samples. This method should be a useful instrument for the elucidation of variations in protein S concentrations in various disorders.

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