Physicochemical characterization of human S-protein and its function in the blood coagulation system

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S-protein, the main inhibitor of the assembly of the membrane attack complex of complement, was isolated from human plasma by a simple purification procedure, which includes barium citrate adsorption, ammonium sulphate precipitation, chromatography on DEAE-Sephacel and Blue Sepharose and gel filtration on Sephacryl S-200. The homogeneous protein (sedimentation coefficient 4.6 S) was obtained in approx. 5% yield relative to its concentration in plasma, which was found to be 0.3–0.5 mg/ml. The final product did not cross-react with antisera against complement proteins or other proteinase inhibitors of human plasma. On polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate, S-protein migrated as a single-chain band with an apparent M_r of 74000 under non-reducing conditions and as a doublet of M_r 78000 and 65000 upon reduction. In plasma or serum S-protein also existed in two forms of corresponding M_r values, as was evidenced by an immunoblot enzyme-linked immunosorbent assay technique. S-protein was found to be an acidic glycoprotein with 10% (w/w) carbohydrate content and several isoelectric points in the range pH 4.75–5.25, and it contained one free thiol group per molecule of protein. The functional properties of S-protein in the complement system were demonstrated by its ability to inhibit complement-dependent cell lysis in a concentration-dependent manner (K_i 0.6 μ M) and by its incorporation into the nascent SC5b-7 complex. A new function for S-protein could be revealed in the blood coagulation system. The slow progressive inhibition of thrombin by antithrombin III was not affected by S-protein, whereas the purified protein interfered with the fast inactivation of thrombin clotting as well as amidolytic activity by antithrombin III–heparin complex. The acceleration of this inhibition reaction by heparin was counteracted by S-protein, indicating the ability of S-protein to neutralize heparin activity.

INTRODUCTION

Activation of complement by the classical or the alternative pathway (Reid & Porter, 1981; Pangburn & Müller-Eberhard, 1984) results in the expression of multiple biological activities, which include clearance of opsonized particles by C3 receptor-bearing cells, production and release of anaphylatoxins and assembly of the membrane-attack complex on the target-cell membrane. The last-mentioned process accounts for the irreversible damage of biological membranes associated with complement-dependent cytolysis (Kolb & Müller-Eberhard, 1975; Bhakdi & Tranum-Jensen, 1978; Mayer *et al.*, 1981; Müller-Eberhard, 1984; Podack & Tschopp, 1984). The formation of the membrane-attack complex is initiated by the self-assembly of the C5b, C6 and C7 subunits. A hydrophilic/amphiphilic transition of C7 leads to the expression of hydrophobic domains on a generated C5b-7 complex, which subsequently becomes inserted into phospholipid bilayer membranes or self-aggregates in their absence (Thompson & Lachmann, 1970; Hammer *et al.*, 1975; Dourmashkin, 1978; Podack *et al.*, 1980; Preissner *et al.*, 1985). Upon addition of C8, membrane bound C5b-8 complexes are formed, which promote insertion of C9 molecules into the hydrocarbon core of the membrane and catalyse C9 polymerization (Podack *et al.*, 1982). In the assembled membrane-attack complex, poly(C9) constitutes the ultrastructural appear-

ance of the characteristic membrane lesions (Tschopp *et al.*, 1982; Podack *et al.*, 1982).

The described reaction sequence is controlled by several serum components, including lipoproteins (Lint *et al.*, 1976; Podack & Müller-Eberhard, 1978), C8 (Nemerow *et al.*, 1979) and S-protein (McLeod *et al.*, 1974b; Kolb & Müller-Eberhard, 1975; Podack & Müller-Eberhard, 1978), which restrict lysis to target membranes and prevent attack of innocent-bystander cells (McLeod *et al.*, 1974a). In particular, S-protein associates with the nascent C5b-7 complex and renders the resulting SC5b-7 complex hydrophilic (Podack *et al.*, 1977). In addition, S-protein prevents C9 polymerization in the subsequently formed cytolytically inactive SC5b-9 complex, which lacks the tubular structure of poly(C9) (Preissner *et al.*, 1983; Podack *et al.*, 1984). Removal of S-protein by limited proteolysis (Bhakdi *et al.*, 1979) or detergent treatment (Podack & Müller-Eberhard, 1980) renders this hydrophilic complex amphiphilic, and as a consequence it may become inserted into phospholipid bilayers.

Thrombin may also form a reversible complex with S-protein, as was evidenced by two-dimensional immunoelectrophoresis (Podack *et al.*, 1978a). The activity of thrombin is inhibited by various proteinase inhibitors, in particular by antithrombin III. Heparin accelerates the formation of the covalent complex of thrombin with antithrombin III by 100-fold and more (Rosenberg,

The nomenclature used for the complement proteins is that recommended by the World Health Organization (1968, 1981).

1982). In this respect, the thrombin-S-protein complex was found to be more resistant to inactivation by antithrombin III than was thrombin alone (Podack *et al.*, 1979). The isolation of S-protein in rather low yield from human serum has been described previously (Podack & Müller-Eberhard, 1979). S-protein is a single-chain glycoprotein with an approx. M_r of 80000.

In the present paper we report an improved purification procedure for human S-protein. Investigations were initiated to characterize the protein further and to study its role in the blood coagulation system.

EXPERIMENTAL

Materials

DEAE-Sephacel, DEAE-Sephadex A-50, Blue Sepharose, Sepharose CL-4B, Sephadex G-25, Sephadex G-50, SP- (sulphopropyl)-Sephadex C-50 and Sephacryl S-200 were products from Pharmacia Fine Chemicals, Freiburg, West Germany. Reagents for electrophoresis were from Bio-Rad Laboratories, Munich, West Germany. Nitrocellulose membrane was purchased from Schleicher and Schüll, Dassel, West Germany. Aprotinin was from Bayer, Leverkusen, West Germany. Other chemicals were obtained from Sigma Chemical Co., Munich, West Germany. Human fibrinogen and the chromogenic substrate compound S 2238 were from Kabi AB, Stockholm, Sweden. The flexible polyester film Gelbond and agarose were products of Marine Colloids, Rockland, ME, U.S.A. Iodo[14 C]acetamide and Na 125 I (carrier-free) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Antisera to plasma proteinase inhibitors were obtained from Behringwerke, Marburg, West Germany. Antisera to various complement components were a gift from Dr. H. J. Müller-Eberhard, Scripps Clinic Research Institute, La Jolla, CA, U.S.A. An anti-(human α_1 -antichymotrypsin) IgG fraction and peroxidase-labelled pig anti-rabbit IgG were obtained from Dakopatts, Copenhagen, Denmark. The IgG fractions of various antisera were prepared by using standard procedures. Human C5b-6 complex was isolated as described by Podack *et al.* (1978b) and was a gift from Dr. J. Tschopp, University of Lausanne, Lausanne, Switzerland.

Human complement components C3 and C7 were isolated as described by Hammer *et al.* (1981).

Human antithrombin III was isolated, after barium citrate adsorption of plasma and subsequent precipitation with 6% (w/v) poly(ethylene glycol) from the remaining supernatant, by heparin-Sepharose chromatography and chromatography on DEAE-Sephacel as described by Miller-Anderson *et al.* (1974). It exhibited both progressive antithrombin activity and heparin cofactor activity.

Human prothrombin was isolated from barium citrate eluent by chromatography on DEAE-Sephadex A-50 and chromatography on sulphated Sephadex G-50, which was prepared as described by Miletich *et al.* (1981). Prothrombin was converted into α -thrombin (EC 3.4.21.5) by the method of Morita & Iwanaga (1981) with the use of the prothrombin activator from *Echis carinatus* venom. α -Thrombin was purified as described by Lundblad *et al.* (1975) on SP-Sephadex C-50. It had a specific activity of 2200 units/mg when determined by the method of Fenton & Fasco (1974). The isolated proteins were quantified spectrophotometrically by using

$A_{1\text{cm}}^{280\text{nm}}$ at 280 nm: antithrombin III, 6.5 (Nordenman *et al.*, 1977); thrombin, 18.3 (Fenton *et al.*, 1977).

Purification of S-protein

All manipulations, including chromatography and centrifugation, were performed at 4 °C. After thawing of freshly frozen platelet-poor plasma at 37 °C, the following proteinase inhibitors were added: benzamidine hydrochloride (20 mM), ϵ -aminohexanoic acid (30 mM), phenylmethanesulphonyl fluoride (1 mM), di-isopropyl phosphorfluoridate (1 mM), soyabean trypsin inhibitor (50 mg/l) and aprotinin (10^6 units/l). Barium citrate adsorption was performed by using a standard procedure (Stenflo, 1976). The supernatant from this precipitation step was substituted with EDTA (10 mM) and glutathione (0.5 mM), and $(\text{NH}_4)_2\text{SO}_4$ fractionation was performed between 40 and 85% saturation at pH 6.9. The dialysate was chromatographed on a DEAE-Sephacel column (4.5 cm $\times$ 25 cm) with a flow rate of 100 ml/h, 11 ml fractions being collected. Proteins were eluted with a 2 $\times$ 1800 ml linear NaCl concentration gradient (conductivity 4–25 mS/cm). Fractions containing S-protein were pooled, concentrated and dialysed against the starting buffer for Blue Sepharose chromatography. Proteins were eluted from this column (2.5 cm $\times$ 40 cm) at a flow rate of 40 ml/h by a linear NaCl concentration gradient of 2 $\times$ 1000 ml (conductivity 2–25 mS/cm), 6 ml fractions being collected. S-protein-containing fractions were pooled, concentrated and after dialysis applied to a column (2.5 cm $\times$ 135 cm) packed with Sephacryl S-200 in 40 mM-Tris/HCl buffer, pH 8.0, containing 1 M-NaCl and 5 mM-benzamidine hydrochloride. The column was eluted at 20 ml/h, and eight fractions/h were collected. Fractions containing S-protein were pooled and passed over affinity columns to which rabbit anti-(human C3), anti-(human serum albumin) or anti-(human α_1 -antichymotrypsin) IgG fraction was coupled. The final pool was dialysed against 40 mM-Tris/HCl buffer, pH 7.4, containing 0.12 M-NaCl, and stored at –70 °C.

Protein concentrations were measured by the dye-binding method of Bradford (1976), with bovine serum albumin as a standard. The absorption coefficient $A_{1\text{cm}}^{280\text{nm}}$ at 280 nm of 9.0 for S-protein was determined from the absorbance at 280 nm of solutions with known protein concentration. Radiolabelling of proteins was carried out with the iodogen procedure (Fraker & Speck, 1980) to reach specific radioactivities of 2×10^5 – 8×10^5 c.p.m./ μ g of protein. Functional properties of the proteins were not affected by the labelling procedure.

Antibodies against purified S-protein were raised in rabbits, and the IgG fraction of the antiserum was prepared by using standard methods. The purified S-protein reacted with an antiserum, kindly provided by Dr. E. R. Podack (Scripps Clinic Research Institute, La Jolla, CA, U.S.A.) and Dr. B. Dahlbäck (Malmö General Hospital, Malmö, Sweden).

Electrophoretic and immunochemical methods

Two-dimensional immunoelectrophoresis was carried out by the procedure of Ganrot (1972) on the flexible polyester film Gelbond. Single radial immunodiffusion was performed as described by Mancini *et al.* (1965) with 1% agarose gel containing 2.5% anti-S-protein serum.

Sodium dodecylsulphate/polyacrylamide-slab-gel electrophoresis was performed by the procedure of Laemmli (1970) with 10% gels. Portions of pooled plasma and

Table 1. Purification of human S-protein

The protein concentration was measured from the absorbance at 280 nm, assuming $A_{1\text{cm}}^{1\%} = 10$. S-protein was determined by radial immunodiffusion. A typical preparation is depicted.

Fraction	Volume (ml)	Protein (mg)	S-protein (mg)	Recovery (%)	Purification (fold)
Plasma	1000	69000	450	100	1
Barium citrate supernatant	940	60000	440	98	1.1
(NH ₄) ₂ SO ₄ pellet (40–85% satn.)	780	34100	317	70	1.4
DEAE-Sephacel	420	1320	79	18	9
Blue Sepharose	260	210	54	12	38.5
Sephacryl S-200	34	33	27	6	122
Immunoabsorption	23	24	23	5	143

serum were incubated with Sepharose-coupled anti-(human serum albumin) IgG for 1 h at 4 °C. After electrophoresis, proteins were transferred from polyacrylamide gels to nitrocellulose by electroblotting, with the buffer system of Towbin *et al.* (1979). The nitrocellulose was treated with several washings with 0.15 M-NaCl/10 mM-sodium phosphate buffer, pH 7.5, containing 0.5% (w/v) gelatin and 0.1% (w/v) Tween 20 (Batteiger *et al.*, 1982). Subsequently, the membrane was treated with an IgG fraction of anti-S-protein serum followed by an incubation with peroxidase-labelled anti-rabbit IgG and 4-chloro-1-naphthol as substrate.

The determination of isoelectric points of S-protein was carried out on a Desaphor isoelectric-focusing chamber (Desaga, Heidelberg, West Germany) with ampholytes (Serva, Heidelberg, West Germany). The pI values in the presence of 8 M-urea were determined by the procedure of Altland *et al.* (1981).

Physicochemical analysis of the membrane-attack-complex inhibitor

Sedimentation-velocity runs to determine the sedimentation coefficient of S-protein were performed by the method of Vinograd *et al.* (1963), as modified by Kranz & Schmidt (1981), with a Beckman model E analytical ultracentrifuge. The Stokes radius of S-protein was determined by the procedure of Ackers (1964). Amino acid analysis was carried out by the method of Moore & Stein (1963) and Spackman *et al.* (1958) with a Durrum D 500 amino acid analyser, with standard procedures for hydrolysis. Tryptophan content was quantified by the procedure of Edelhoch (1967). Half-cystine was determined as carboxymethylcysteine by the procedure of Crestfield *et al.* (1963). Neutral sugar was determined by the method of Dubois *et al.* (1956), with a 1:1 mixture of mannose and galactose as a standard. Neuraminic acid was analysed by the thiobarbituric acid method of Warren (1959), with *N*-acetylneuraminic acid as a standard. The partial specific volume of S-protein was calculated from published partial specific volumes of amino acid and sugar residues (Cohn & Edsall, 1943; Gibbons, 1966).

The content of free thiol groups in S-protein was determined either by amino acid analysis of carboxymethylated S-protein or by measuring the incorporation of iodo[¹⁴C]acetamide into the protein (Crestfield *et al.*, 1963) in the presence and in the absence of 6 M-guanidinium chloride.

The inhibition of C5b-6-initiated lysis of unsensitized

sheep erythrocytes in the presence of terminal complement components by S-protein was measured as described by Podack *et al.* (1978b) in a modified version. The formation of the SC5b-7 complex was analysed by ultracentrifugation in 10–35% (w/v) linear sucrose density gradients as described (Podack *et al.*, 1977; Preissner *et al.*, 1983).

Functional properties of S-protein in the coagulation system

The effect of purified S-protein on heparin-induced fast inactivation of α -thrombin by antithrombin III was tested in a clotting as well as an amidolytic assay system. After preincubation of antithrombin III (0.6 μ M) without or together with S-protein (2.4 μ M) in the absence and in the presence of heparin (0.02 unit/ml) in 40 mM-Tris/HCl buffer, pH 7.4, containing 120 mM-NaCl, 3 mM-CaCl₂ and 0.1% (w/v) human serum albumin for 2 min at 37 °C, α -thrombin (70 nM) was added to the reaction mixture. After various time periods, 75 μ l portions were removed and transferred to a rotating cuvette at 37 °C with 150 μ l of human fibrinogen (3 mg/ml) in 20 mM-Tris/HCl buffer, pH 7.4, containing 120 mM-NaCl. Clotting times were measured in a KC 10 automatic coagulometer (Amelung, Lemgo, West Germany) and are converted into percentage thrombin-clotting activity by using a calibration curve.

In a second experimental setting, human antithrombin III (0.6 μ M) and human α -thrombin (50 nM) were incubated at 37 °C in a total volume of 150 μ l of 20 mM-Tris/HCl buffer, pH 7.4, containing 150 mM-NaCl and 0.1% (w/v) human serum albumin in the absence and in the presence of purified S-protein (2.4 μ M) and heparin (0.01 unit/ml). At various time intervals, 20 μ l portions were removed from the reaction mixture and transferred to a cuvette with 0.2 mM-compound S 2238 dissolved in 880 μ l of 40 mM-Tris/HCl buffer, pH 8.0, containing 120 mM-NaCl and 0.1% (w/v) bovine serum albumin. Residual thrombin amidolytic activity was recorded at 405 nm in a Uvikon 810 spectrophotometer (Kontron, Munich, West Germany).

RESULTS

Isolation of S-protein and physicochemical characterization

S-protein was isolated from human plasma by a six-step procedure, including barium citrate adsorption, (NH₄)₂SO₄ fractionation of the supernatant, chromato-

Fig. 1. Chromatography used in the purification of S-protein

(a) DEAE-Sephacel chromatography after $(\text{NH}_4)_2\text{SO}_4$ fractionation. After application of the sample and washing of the column (4.5 cm $\times$ 25 cm) with 5 vol. of starting buffer (20 mM-sodium phosphate buffer, pH 6.2, containing 5 mM-EDTA, 5 mM-benzamidine hydrochloride, 20 mM- ϵ -aminohexanoic acid, 0.5 mM-glutathione and 0.5 mM-phenylmethanesulphonyl fluoride), proteins were eluted with a linear gradient of NaCl. The column effluent was monitored immunochemically with antisera against S-protein, C3, caeruloplasmin (Caerul.) and C9. Fractions were pooled as indicated by the horizontal bar. ●, A_{280} ; ○, conductivity (mS/cm). (b) Blue Sepharose chromatography of the S-protein pool from the DEAE-Sephacel chromatography. After application of the sample, the column (2.5 cm $\times$ 40 cm) was washed with 2 column volumes of equilibration buffer (10 mM-Tris/HCl buffer, pH 7.4, containing 1 mM-glutathione and 0.5 mM-phenylmethanesulphonyl fluoride). The arrow indicates the start of a linear gradient of NaCl. The effluent was monitored immunochemically with an antiserum against S-protein. Fractions were pooled as indicated by the horizontal bar. ●, A_{280} ; ○, conductivity (mS/cm).

graphy on DEAE-Sephacel and Blue Sepharose, gel filtration and immunoabsorption (Table 1). Unlike the vitamin K-dependent proteins, which all contained γ -carboxyglutamic acid (Stenflo, 1976; DiScipio *et al.*, 1977), S-protein was not retained to the barium citrate. On DEAE-Sephacel (Fig. 1a), S-protein was eluted between complement component C3 and the prominent caeruloplasmin peak and trailed further into the C9 and C4 peaks, as deduced from immunochemical analysis.

Pooling of S-protein in a narrow range proved to be essential for the purity of the final product, however, and loss of material had to be tolerated. After chromatography on Blue Sepharose (Fig. 1b) (Gianazza & Arnaud, 1982) and gel filtration in the presence of 1 M salt to separate S-protein from IgM, C3, caeruloplasmin, α_1 -antichymotrypsin and albumin, traces of contaminants were removed by immunoabsorption. The overall recovery of S-protein was dependent on the early precipitation step and amounted to 5%. The purification was approx. 150-fold, which is as expected considering the physiological concentration of S-protein, which was determined to be the range 0.3–0.5 mg/ml in plasma of healthy donors by radial immunodiffusion analysis. Immunologically, S-protein did not cross-react with antisera against complement proteins and α_1 -antitrypsin, α_1 -antichymotrypsin, inter- α -trypsin inhibitor, α_2 -plasmin inhibitor, α_2 -macroglobulin, antithrombin III, C1-inactivator or histidine-rich glycoprotein.

Human S-protein migrated as a single-chain polypeptide with M_r 74000 on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis under non-reducing conditions (Fig. 2, lane 1). Despite the use of proteinase inhibitors, the purified protein appeared as a doublet (apparent M_r 78000 and 65000) when analysed after reduction of disulphide bridges (lane 2). In different preparations both protein bands showed variable intensity. A peptide with an apparent M_r 12000, which poorly stained with Coomassie Blue, was occasionally observed. After electrophoresis under reducing conditions, an immunoreplication procedure was utilized to analyse plasma or serum for the presence of S-protein. Lane 5 (Fig. 2) demonstrates the detection of two bands in purified S-protein by the anti-S-protein IgG fraction, essentially identical with the occurrence of Coomassie Blue-stained bands in Fig. 2, lane 2. In lanes 3 and 4 on the immunoreplicas, containing serum and plasma samples respectively, virtually the same band pattern was detectable. In addition, a low- M_r component (M_r 12000) was recognized by the antibody. The used antiserum to S-protein proved to be monospecific, as deduced from crossed immunoelectrophoresis, since it formed one precipitin arc against plasma at a position with inter- α -mobility.

When the number of amino acid residues of purified S-protein were calculated on the basis of M_r 69900 for the carbohydrate-free protein, the following composition was obtained (residues/molecule of protein): Lys (26), His (23), Arg (45), Asp (58), Thr (25), Ser (44), Glu (84), Pro (44), Gly (50), Ala (40), Cys (15), Val (28), Met (9), Ile (19), Leu (40), Tyr (28), Phe (30) and Trp (8), resulting in 616 total residues/molecule of protein, representing 90.3% (w/w) of the glycoprotein. Carbohydrate analysis revealed hexose (20), *N*-acetylhexosamine (9) and *N*-acetylneuraminic acid (6), resulting in 35 total residues/molecule of protein, representing 9.7% (w/w) of the glycoprotein. The carbohydrate content may account for the appearance of a diffused protein band on electrophoresis on non-reduced sodium dodecyl sulphate/polyacrylamide gels. Upon isoelectric focusing, S-protein exhibited microheterogeneity (Fig. 2, lane 6). Several isoelectric points of the protein exist between pH 4.75 and 5.25 in the presence of 8 M-urea. Other physicochemical parameters that are based on amino acid composition and sedimentation coefficient are listed in Table 2.

Fig. 2. Electrophoretic analysis of purified S-protein

Lanes 1 and 2, sodium dodecyl sulphate/polyacrylamide-slab-gel electrophoresis of purified S-protein under non-reducing (lane 1) or reducing conditions (lane 2). Lanes 3–5, immunoblotting of serum (lane 3), plasma (lane 4) and purified S-protein (lane 5) after sodium dodecyl sulphate/polyacrylamide-gel electrophoresis under reducing conditions. S-protein was detected by rabbit anti-SIgG followed by a peroxidase-conjugated pig anti-rabbit IgG and substrate. Lane 6, isoelectric focusing of purified S-protein in the presence of 8 M-urea. The pH values of slices from a parallel gel were determined after equilibration with water. Anodal positions are at the bottom.

Table 2. Physical parameters of S-protein

Abbreviation: SDS, sodium dodecyl sulphate.

Sedimentation coefficient, $s_{20,w}$	4.6 S
Partial specific volume, $\bar{v}$	0.711 cm ³ /g
M_r (from SDS/polyacrylamide-gel electrophoresis)	78 000
Absorption coefficient, $A_{280nm, 1cm}^{1\%}$	9.0
Frictional ratio, f/f_0	1.37
Stokes radius, r_H	3.7 nm
Isoelectric point (in 8 M-urea), pI	4.75–5.25

Upon incubation with iodo[¹⁴C]acetamide in the presence of 6 M-guanidinium chloride, 0.5 ± 0.02 mol (mean $\pm$ s.d., $n = 3$) of the reagent was incorporated per mol of S-protein. Amino acid analysis of carboxymethylated S-protein revealed an additional peak preceding aspartic acid at the position of carboxymethylcysteine, with a concentration of 1.5 residues/molecule of protein.

The functional property of S-protein as the main inhibitor of assembly of the membrane-attack complex was verified by its ability to interfere with C5b-6-initiated lysis of unsensitized sheep erythrocytes in the presence of terminal complement components with an apparent inhibition constant K_i 0.55 μ M and its incorporation into the nascent SC5b-7 complex.

Biological properties of S-protein in the blood coagulation system

Purified S-protein was tested in a clotting as well as an amidolytic assay system for its interference with the thrombin-antithrombin III inhibition reaction, which was studied under pseudo-first-order kinetic conditions. There was no difference in the Michaelis constant (K_m 7.2 μ M) for the reaction with the chromogenic substrate whether thrombin was tested alone or in the presence of S-protein. The ratio of antithrombin III to S-protein in the system *in vitro* was 1:4 (mol/mol). S-protein did not

affect the slow progressive inhibition of thrombin clotting or amidolytic activity by antithrombin III in the absence of heparin. However, the accelerative effect of heparin in this inhibition reaction was counteracted by S-protein in both assay systems (Fig. 3).

Thrombin clotting activity was inhibited by 50% within 140 s (apparent rate constant k' 0.3 min⁻¹) upon incubation with antithrombin III. The inhibitory effect of antithrombin III was accelerated in the presence of 0.02 unit of heparin/ml (k' 4.3 min⁻¹). However, the presence of S-protein partly prevented the heparin effect (k' 0.9 min⁻¹) (Fig. 3a) and thereby prolonged the life-time of enzymic activity. Similarly, thrombin amidolytic activity was progressively inhibited by antithrombin III (k' 0.35 min⁻¹) and accelerated in the presence of 0.01 unit of heparin/ml (k' 2.5 min⁻¹). S-protein was able to slow down this fast inactivation (k' 0.65 min⁻¹) and thereby protected thrombin against inhibition by antithrombin III-heparin (Fig. 3b).

DISCUSSION

In the present study, an improved method has been described for purification of S-protein from human plasma, which was obtained with 10-fold higher recovery than previously reported (Podack & Müller-Eberhard,

Fig. 3. Interference of S-protein with the inhibition of thrombin activity by antithrombin III-heparin

(a) The inhibition of clotting activity of thrombin (70 nM) by antithrombin III (0.6 μM)/heparin (0.02 unit/ml) was analysed in the absence and in the presence of S-protein at 37 °C. ○, Slow progressive inhibition of thrombin by antithrombin III in the absence of heparin; ●, fast inactivation of thrombin by antithrombin III in the presence of heparin; ■, inhibition of thrombin by antithrombin III in the presence of heparin and S-protein (2.4 μM); ▲, thrombin activity in the absence or the presence of heparin and S-protein. (b) The inhibition of amidolytic activity of human thrombin (50 nM) by antithrombin III (0.6 μM) in the presence of heparin (0.01 unit/ml) was monitored in a time-course reaction at 37 °C. ○, Slow progressive inhibition of thrombin by antithrombin III in the absence of heparin; ●, fast inactivation of thrombin by antithrombin III in the presence of heparin; ■, inhibition of thrombin by antithrombin III in the presence of heparin and S-protein (2.4 μM); ▲, thrombin activity in the absence or the presence of heparin and S-protein.

1979). Our isolation procedure consisted of fewer chromatographic steps than the earlier published method, and could be accomplished within 10 days. It yielded a product that was homogeneous as judged by several independent means, and free of other proteinase inhibitors as deduced from immunochemical analysis. The appearance of two forms of S-protein in plasma or serum was not due to the isolation procedure. The native

molecule (M_r 78000) and a proteolysed form (M_r 65000 and 12000) could unequivocally be demonstrated by an immunoblot enzyme-linked immunosorbent assay technique with monospecific anti-S-protein antibody. The lack of detection of the small peptide on the immunoreplica in purified S-protein could be due to the removal of this fragment during purification.

In addition to the amino acid composition, which was in good agreement with the previously reported data (Podack & Müller-Eberhard, 1979), we analysed the carbohydrate content of S-protein, which amounted to 10% of the mass of total glycoprotein. A heterogeneity of S-protein in its carbohydrate moiety, or a microheterogeneity in the primary structure, may account for the appearance of several isoelectric points around pH 5. Although these values have to be corrected (0.2–0.4 pH unit) for protein denaturation and solvent effects (Gelsema *et al.*, 1979), they differ from the earlier reported isoelectric point of 3.9 by more than 1 pH unit. The presence of one free thiol group in S-protein was evidenced by two independent methods in the presence of guanidinium chloride, indicating a presumably buried thiol group in the native molecule. Before reaction with iodoacetamide, a portion of the purified protein may have undergone oxidation, resulting in incorporation of less than 1 mol of reagent per mol of protein. An additional proof for the identity of the isolated S-protein was its ability to inhibit C5b-6-initiated cell lysis and its incorporation into the nascent SC5b-7 complex (Kolb & Müller-Eberhard, 1975; Podack *et al.*, 1977; Preissner *et al.*, 1983). Whether the free thiol group in S-protein is involved in the complement inhibition mechanism and may thereby prevent dimerization of C9 (Yamamoto *et al.*, 1982) or C9 polymerization (Podack *et al.*, 1984) remains to be established.

A new function for S-protein as a heparin-neutralizing protein is described in the present study. In contrast with previously reported data by Podack *et al.* (1978a, 1979), we could not detect any direct effect of purified S-protein on the slow progressive inhibitory activity of antithrombin III in the absence of heparin. Neither in an amidolytic nor in a clotting assay system was a protective effect of S-protein on thrombin evident. However, the fast inhibition of thrombin by antithrombin III in the presence of heparin was counteracted by S-protein. This activity of S-protein was dependent on the initial concentration of heparin and S-protein in the assay systems *in vitro*. These data suggest either a competition of S-protein and antithrombin III for binding to heparin or an interference of S-protein with antithrombin III-heparin complex for the binding to thrombin. Thus, besides histidine-rich glycoprotein (Lijnen *et al.*, 1983) and platelet factor 4 (Jordan *et al.*, 1982), S-protein may act as a component in plasma that counteracts heparin effects in blood coagulation.

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