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# Modulation of Heparin Cofactor II Function by S Protein (Vitronectin) and Formation of a Ternary S Protein-Thrombin-Heparin Cofactor II Complex

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**Key words**

Heparin cofactor II – S protein (vitronectin) – Glycosaminoglycan neutralization – Thrombin inhibition – Ternary complex

**Summary**

The complement inhibitor S protein, which is identical to the adhesive protein vitronectin, functions as heparin-neutralizing factor by protecting thrombin as well as factor Xa against fast inactivation by antithrombin III. The interference of S protein with glycosaminoglycan-catalyzed inhibition of thrombin by heparin cofactor II was investigated in these studies. S protein significantly counteracted the anticoagulant activity of heparin and pentosan polysulfate but not of dermatan sulfate. In the presence of 0.3 μg/ml heparin, 0.5 μg/ml pentosan polysulfate, or 2 μg/ml dermatan sulfate, S protein induced a concentration-dependent reduction of the inhibition rate of thrombin by heparin cofactor II. This resulted in a decrease of the apparent pseudo first-order rate constants by about 17-fold (heparin), or about 7-fold (pentosan polysulfate), whereas no neutralization of dermatan sulfate was demonstrable at a physiological ratio of S protein to heparin cofactor II. Exposure of the glycosaminoglycan-binding region of S protein by reduction and carboxymethylation of the protein increased the neutralizing activity of S protein towards heparin and pentosan polysulfate. The results of these functional experiments correlated well with the demonstration of direct binding of S protein to both polysaccharides but not to dermatan sulfate. While reduced/carboxymethylated S protein remained also ineffective in neutralizing other dermatan sulfate compounds with varying degree of sulfation, a synthetic highly basic tridecapeptide, representing a portion of the glycosaminoglycan-binding domain of S protein, counteracted their anticoagulant activity. Independent on the polysaccharide used, S protein was found incorporated within a ternary complex with thrombin and heparin cofactor II during the inhibition reaction as judged by crossed immunoelectrophoresis, ultracentrifugation as well as ELISA analysis, emphazising the function of S protein as scavenger protein for enzyme-inhibitor complexes of the coagulation system. These findings demonstrate the role of S protein as effective neutralising plasma protein of the anticoagulant activity of various glycosaminoglycans also with respect to heparin cofactor II. Although the glycosaminoglycan-binding domain of S protein readily neutralized different dermatan sulfate compounds, physiological modulation of heparin cofactor-II-dependent inhibition of thrombin by native S protein appears to be restricted to the vascular compartments, where other glycosaminoglycans than dermatan sulfate appear to be operative.

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## Introduction

The multifunctional role of S protein is appreciated by its diverse properties as complement inhibitor (1, 2), as heparin-neutralizing factor (3), and as adhesive protein (4), being identical with the serum spreading factor vitronectin (5–8). As such it is not only found in plasma in high concentration of 4–6 μM, but also in tissues (9) and functionally resembles fibronectin in its ability to bind to cells and promote their spreading (4, 10, 11). S protein is comprised of 458 amino acids and contains an amino-terminal sequence of 44 amino acids that corresponds to the primary structure of somatomedin B (6, 12). Adjacent to this domain is a cell-attachment sequence Arg-Gly-Asp, found also in fibronectin and other adhesive proteins such as fibrinogen and von Willebrand factor (13). In the carboxy-terminal portion of the molecule a sequence of 34 amino acids containing a high proportion of basic residues is thought to mediate the binding of S protein to glycosaminoglycans (6). The involvement of this domain in the neutralization of heparin anticoagulant activity during the inhibition of the coagulation enzymes thrombin as well as factor Xa by antithrombin III has recently been documented by our laboratory (14, 15). In particular, a synthetic tridecapeptide representing part of the glycosaminoglycan-binding region of S protein was effective in exerting the neutralizing properties of the native molecule. Although S protein resembles histidine-rich glycoprotein (16, 17) and platelet factor 4 (18, 19) in its function as heparin-neutralizing protein, differences exist between these three proteins (15): Unlike histidine-rich glycoprotein and platelet factor 4, S protein effectively inhibits the anticoagulant activity of heparan sulfate (20) and associates with the thrombin-antithrombin III complex (21–24) to form a macromolecular ternary complex with apparent $M_r$ of 350,000 (25).

Besides antithrombin III, heparin cofactor II is believed to be operative as an additional protease inhibitor of blood coagulation (26–28), whose activity is solely expressed in the presence of certain glycosaminoglycans such as dermatan sulfate (29) and whose inhibitory function in the blood coagulation system is limited towards thrombin (30). This specificity in function prompted us to investigate the requirements for modulation of heparin cofactor II activity by S protein in the glycosaminoglycan-dependent inhibition of thrombin. Although the glycosaminoglycan-binding domain of S protein was essential in neutralising the anticoagulant activity of various polysaccharides, native S protein was not effective in counteracting the activity of dermatan sulfate. Despite the diverse distribution of S protein in tissues, these results may indicate the restricted role of native S protein as glycosaminoglycan-neutralizing protein in the vascular compartment.

## Materials and Methods

*Proteins*

S protein was isolated from fresh-frozen human plasma as previously detailed (3). Heparin cofactor II was purified from Australia-Ag negative human plasma as recently described (31) and was essentially antithrombin-III-free as judged by immunological and gel electrophoretic criteria. Human α-thrombin was purified as outlined elsewhere (3) and had a fibrinogen clotting activity of 2,200–2,500 NIH units/mg as determined by the method of Fenton and Fasco (32). All proteins were homogeneous as judged by gel electrophoresis in the presence of sodium dodecyl sulfate using the buffer system of Laemmli (33): S protein showed the characteristic doublet with $M_r$ of 78,000 and 65,000 under reducing conditions while heparin cofactor II ($M_r$ 65,000) and α-thrombin ($M_r$ 37,000) appeared as a single band under non-reducing conditions. S protein was reduced and alkylated as described elsewhere (15). A synthetic basic tridecapeptide, representing part of the glycosaminoglycan-binding domain of S protein (positions 347–359), was kindly provided by Dr. J. Tschopp (University Lausanne, Switzerland). S protein, thrombin and heparin cofactor II were labelled with $^{125}I$ (carrier-free) (New England Nuclear, Dreieich, West-Germany) using the iodogen procedure (34) reaching specific activities of 1–2 μCi/μg. The labelling procedure did not affect the functional activities of the proteins. Antibodies against S protein and heparin cofactor were raised in rabbits, and monospecific antibodies against thrombin were kindly donated by Dr. H. Pelzer, Behring Research Laboratories (Marburg, West-Germany).

*Glycosaminoglycans*

Heparin sodium salt from porcine intestinal mucosa, polybrene and protamine chloride from Salmon were obtained from Sigma Chemical Co. (Munich, West-Germany). Natural dermatan sulfate (S/C ratio of 1.05, essential heparin-free) as well as oversulfated dermatan sulfate compounds (S/C ratio of 2.0 and 3.7, respectively) were a kind gift of Dr. M. Petitou (Choay Institute, Paris, France). The heparinoid pentosan polysulfate was kindly provided by Dr. T. Halse (Benechemie, Munich, West-Germany).

*Assay Procedures*

The influence of various concentrations of S protein on glycosaminoglycan-induced inactivation of thrombin by heparin cofactor II was investigated in an amidolytic assay system, similar to that previously described for thrombin inhibition by antithrombin III (14). Briefly, thrombin (30 nM final concentration) was incubated with heparin cofactor II (0.5 μM final concentration) and various concentrations of S protein (0.3–3 μM final concentration) in the presence of heparin (0.3 μg/ml), pentosan polysulfate (0.5 μg/ml), or dermatan sulfate (2 μg/ml) in a total volume of 150 μl in 20 mM Tris/HCl, 0.15 M NaCl, pH 7.4, containing 3 mM $CaCl_2$ and 0.5% (w/v) human serum albumin (Sigma). At various time intervals, 25 μl portions were removed from the reaction mixture and transferred to a cuvette with 0.2 mM chromogenic substrate compound S-2238 (Kabi AB, Stockholm, Sweden) dissolved in 875 μl 40 mM Tris/HCl, 0.12 M NaCl, pH 8.0, containing 0.1% (w/v) bovine serum albumin (Sigma). Residual thrombin amidolytic activity was recorded at 405 nm on an Uvikon 810 spectrophotometer (Kontron, Offenbach, West-Germany). Under these conditions, pseudo-first-order reaction conditions apply and the apparent rate constants $k_1$ were calculated from the slopes of the linear plots of the logarithm of residual thrombin concentration versus time.

In another experimental set-up the ability of S protein and its reduced/carboxymethylated derivative to neutralize the anticoagulant activity of various concentrations of glycosaminoglycans in the inhibition of thrombin by heparin cofactor II was tested at 37° C. Heparin cofactor II (85 nM final concentration), various final concentrations of heparin (0–18 μg/ml), pentosan polysulfate (0–300 μg/ml), or dermatan sulfate (0–30 μg/ml) were preincubated for 1 min at 37° C in the absence or presence of 340 nM S protein (final concentration). Thrombin (4 nM final concentration) was added to initiate the reaction in a total volume of 150 μl 20 mM Tris/HCl, 0.15 M NaCl, pH 7.4, containing 0.5% (w/v) human serum albumin and 3 mM $CaCl_2$. After exactly 30 s at 37° C, prewarmed 500 μl Tris-buffer, pH 8.0, containing 0.1 mg/ml polybrene, 0.1 (w/v) bovine serum albumin and 0.2 mM (final concentration) synthetic substrate S-2238 was added to terminate the inhibition reaction and to quantitate the amount of residual thrombin amidolytic activity. After additional 5 min at 37° C, 150 μl 50% (v/v) acetic acid was added and the concentration of liberated p-nitroaniline was measured at 405 nm.

Similarly, the effect of various dermatan sulfate compounds (at final concentrations of 0.5 μg/ml) on the thrombin-heparin cofactor II reaction and the neutralisation of their anticoagulant activity by reduced/carboxymethylated S protein (S protein: heparin cofactor II = 5, mole/mole) and the synthetic tridecapeptide (0.17 mg/ml final concentration) was analyzed. Results were expressed as percent neutralisation, 100% neutralisation defined in the absence of glycosaminoglycans whereas no neutralisation occurred in the absence of S protein or synthetic peptide.

*Binding of S Protein to Glycosaminoglycans*

Binding of S protein to heparin, dermatan sulfate as well as pentosan polysulfate was analyzed simultaneously by crossed immunoelectrophoresis as recently described (14). 10 μl portions of pooled normal human plasma as well as of purified S protein (0.2 mg/ml) were preincubated in the absence or presence of 0.5 mg/ml glycosaminoglycan for 15 min at 37° C and electrophorized in the first dimension with 4 V/cm for 2.5 hr at 22° C through agarose-gel, which either contained no additive or each of the respective glycosaminoglycans at a concentration of 0.5 mg/ml. The agarose-gel in the second dimension contained 0.8% (v/v) antiserum against S protein.

*Ternary Complex Formation*

The formation of a ternary complex between thrombin, heparin cofactor II and S protein was studied in defibrinated normal human plasma, which was obtained by incubation of normal human plasma with 10 U/ml batroxobin (Pentapharm, Basel, Switzerland) for 5–10 min at 37° C followed by centrifugation at 11,000 × g for 10 min to remove the fibrin clot. In 20 μl of this plasma supplemented either with 5 μl $^{125}I$-labelled heparin cofactor II or 5 μl $^{125}I$-labelled S protein, complex formation was induced by the addition of 1 μg thrombin either in the presence of 75 μg/ml heparin or 120 μg/ml dermatan sulfate. Reaction was continued for 10 min at 37° C and samples were simultaneously analyzed by crossed immunoelectrophoresis on the flexible polyester film Gelbond (Marine Colloids, Rockland, ME) (35) as outlined elsewhere (25). The agarose-gel in the second dimension either contained 0.8% (v/v) rabbit anti-(human S protein) serum or 0.5% (v/v) rabbit anti-(human heparin cofactor II) serum. After the agarose-containing films had been washed, dried and stained, the distribution of radiolabel was detected by autoradiography.

Ultracentrifugation of preformed complexes through linear sucrose density gradients was carried out essentially as outlined elsewhere (25). Briefly, 5 μg radiolabelled thrombin was incubated with 10 μg heparin cofactor II or with 10 μg heparin cofactor II and 20 μg S protein, both in the presence of 0.5 μg/ml dermatan sulfate for 30 min at 37° C in a final volume of 100 μl Tris-buffer. Thereafter, samples were layered on top of linear 10–35% (w/v) sucrose density gradients and centrifugation was carried out for 16 hr at 4° C in a SW 60 rotor (Beckman Instruments, Munich, West-Germany) with 45,000 rpm. As markers for sedimentation coefficients, IgM (19 S) and human serum albumin (4.1 S) were run in parallel gradients. After fractionation, the gradient fractions containing the high molecular weight ternary complex were pooled and immunoprecipitated with antibodies against heparin cofactor II as well as S protein utilizing goat-anti(rabbit IgG) coupled to *Staphylococcus aureus* cell walls ("Tachysorb", Calbiochem, Frankfurt, West-Germany) as solid phase reactant. Unspecific adsorption of radiolabelled thrombin alone by these antibodies was about 5% of radioactivity totally added; results were corrected for unspecific adsorption.

Complex formation between S protein and thrombin-heparin cofactor II was also documented by ELISA analysis: 96-well microtiter plates (Flow Laboratories, Meckenheim, West-Germany) were coated with 50 μl portions of S protein (20 μg/ml) dissolved in 15 mM $Na_2CO_3$, 35 mM $NaHCO_3$ (pH 9.6) for 16 hr at 4° C. Control wells were coated with 0.1% (w/v) human serum albumin. After saturation of wells with 200 μl portions of 3% (w/v) milk proteins for 1 hr at 22° C and washing with phosphate-buffered saline containing 0.1% (w/v) Tween 20 (PBS-Tween), the wells were incubated with 50 μl portions of either heparin cofactor II alone

(10 μg/ml) or thrombin-heparin cofactor II complex (5 μg/ml and 10 μg/ml, respectively). These components were preincubated for 30 min at 37° C at a ten-fold concentration in the presence of 5 μg/ml dermatan sulfate or 15 μg/ml heparin, respectively, prior to reaction with solid phase-bound S protein. Following incubation for 2 hr at 22° C and washing with PBS-Tween, dilutions of rabbit-anti(human heparin cofactor II)serum as well as rabbit-anti(human thrombin)IgG were incubated with wells for 2 hr at 22° C. Following washing and incubation with horse radish peroxidase-conjugated IgG against rabbit immunoglobulins (Dako, Hamburg, West-Germany), the extent of complex formation was quantitated by absorption at 414 nm utilizing 2,2'-azino-di-[3-ethyl-benzthiazolinsulfonat (6)] (Boehringer, Mannheim, West-Germany) as substrate. The absorption in control wells which were treated identically were used to correct values for unspecific binding.

## Results

*Interference of S Protein with the Activity of Glycosaminoglycans in the Thrombin-Heparin Cofactor II Reaction*

The glycosaminoglycan-induced inhibition of thrombin by heparin cofactor II and the neutralisation of the anticoagulant activity of polysaccharides by S protein was studied in a purified system. In the presence of 0.3 μg/ml heparin, or 2 μg/ml dermatan sulfate, or 0.5 μg/ml pentosan polysulfate, respectively, thrombin activity was inhibited by heparin cofactor II with apparent rate constants $k_1$ of 8, 4 and 2.1 $min^{-1}$, respectively (Fig. 1). The addition of S protein induced a concentration-dependent reduction of inhibition rates as reflected by a decrease of the apparent rate constants by about 17-fold (heparin), or about 7-fold (pentosan polysulfate), at a molar ratio of S protein to heparin cofactor II of 6. No effect of S protein was noted in the case of dermatan sulfate. While the S protein-dependent reduction of $k_1$ was linear for heparin on a semi-logarithmic plot, a biphasic characteristic was noted for pentosan polysulfate (Fig. 1) such that a slightly more effective neutralisation for the latter polysaccharide occurred at low S protein concentrations. Control experiments in the same system revealed neither an influence of S protein and glycosaminoglycans on thrombin alone nor an effect of S protein on thrombin activity in the presence of heparin cofactor II.

In order to study the neutralization capacity of S protein in this reaction, the anticoagulant function of glycosaminoglycans over the range of 0.01–20 μg/ml was examined in the inhibition of thrombin by heparin cofactor II. The concentrations of glycosaminoglycans at the point of 50% inhibition of thrombin at 30 s, which was equivalent to a second-order rate constant $k_2 = 1.6 \times 10^7\ M^{-1}min^{-1}$, were different and amounted to 0.05 μg/ml for heparin, 3.0 μg/ml for dermatansulfate and 0.3 μg/ml for pentosan polysulfate (Fig. 2). As expected from the previous experiment, at a four-fold molar excess of S protein over heparin cofactor II, the point of 50% inhibition of thrombin was significantly shifted towards higher concentrations of heparin and pentosan polysulfate, while there was virtually no neutralizing

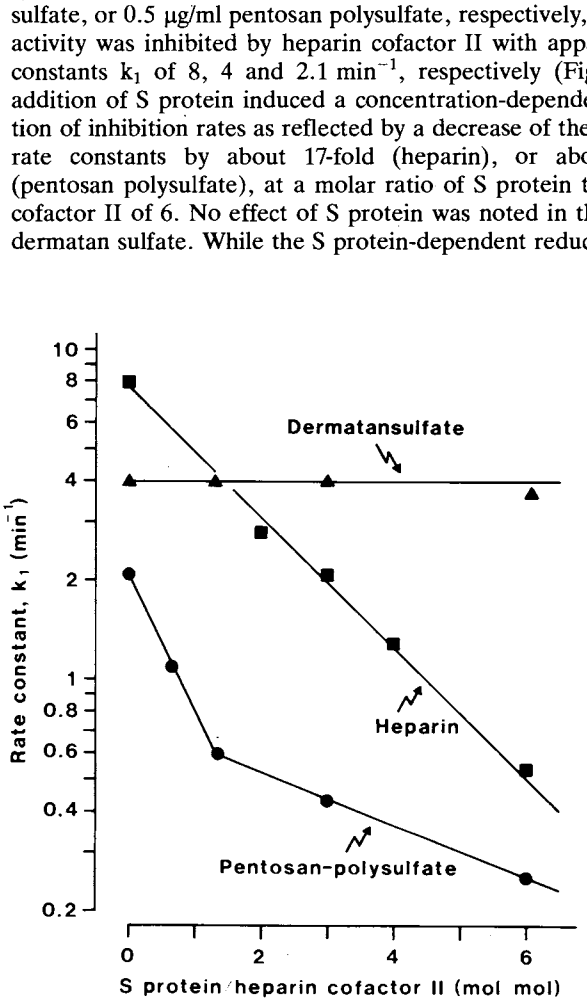


*Fig. 1* Neutralisation of various glycosaminoglycans by S protein in the inhibition of thrombin by heparin cofactor II. The pseudo first-order rate constants $k_1$ (which correlate directly with the rate of inhibition) were determined by kinetic analysis of the reaction at various ratios of S protein/heparin cofactor II in the presence of 0.3 μg/ml heparin, 2 μg/ml dermatan sulfate, or 0.5 μg/ml pentosan polysulfate, respectively

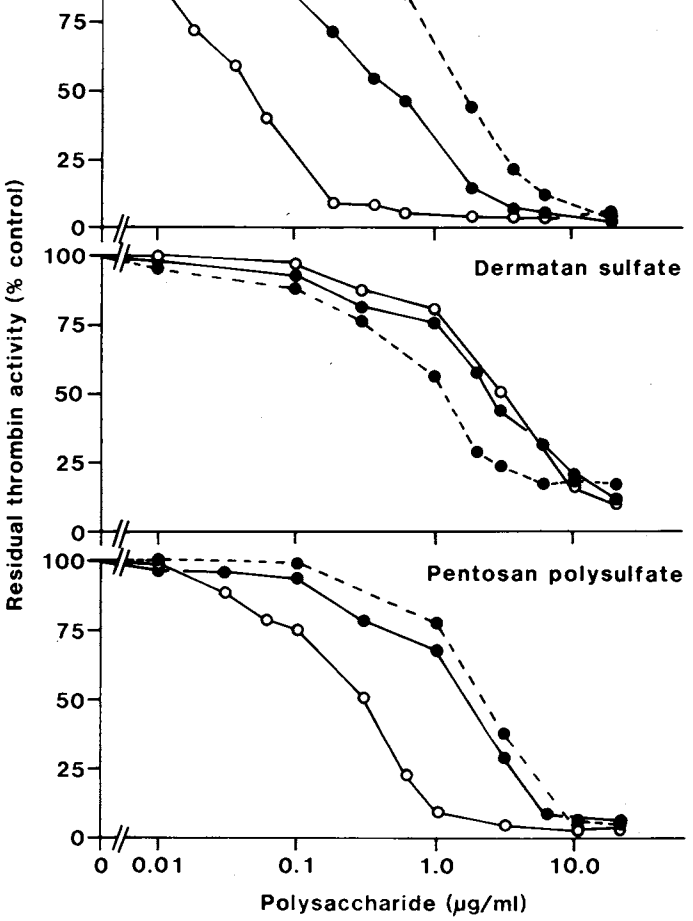


*Fig. 2* Neutralisation of glycosaminoglycans by S protein in the inhibition of thrombin by heparin cofactor II as a function of polysaccharide concentration. Four nM thrombin was incubated with various concentrations of glycosaminoglycans and 85 nM heparin cofactor II in the presence of 340 nM S protein (●, solid lines), 340 nM reduced/carboxymethylated S protein (●, broken lines) or in its absence (○). Residual thrombin amidolytic activity was measured by a chromogenic assay after exactly 30 s reaction

effect of S protein on the activity of dermatan sulfate. The ability of S protein to counteract the anticoagulant activity of heparin and pentosan polysulfate even increased after modification of the protein by reduction/carboxymethylation (Fig. 2, broken lines).

Binding studies with glycosaminoglycans by crossed-immunoelectrophoresis revealed a direct interaction of heparin as well as of pentosan polysulfate with purified S protein and resulted in the shift of the mobility of the protein towards the anode (Fig. 3). The presence of dermatan sulfate did not influence the migration of S protein. Similar results were obtained when plasma instead of isolated S protein was analyzed (data not shown).

Since a high affinity interaction between S protein and standard dermatan sulfate was not evident from these experiments, two different dermatan sulfate fractions with a higher degree of sulfation were included. In addition, a synthetic, basic tridecapeptide (positions 347–359 of S protein) which appeared to express heparin-neutralizing activity (15), was utilized to further investigate the interaction of S protein with dermatan sulfate compounds. Although reduced/carboxymethylated S protein also failed to counteract the anticoagulant activity of both oversulfated dermatan sulfate compounds, the basic peptide showed appreciable neutralisation for all dermatan sulfate compounds, and it was found similar in its effect compared with protamine chloride (Fig. 4), which neutralized more than 90% anticoagulant activity of the glycosaminoglycans in each case.

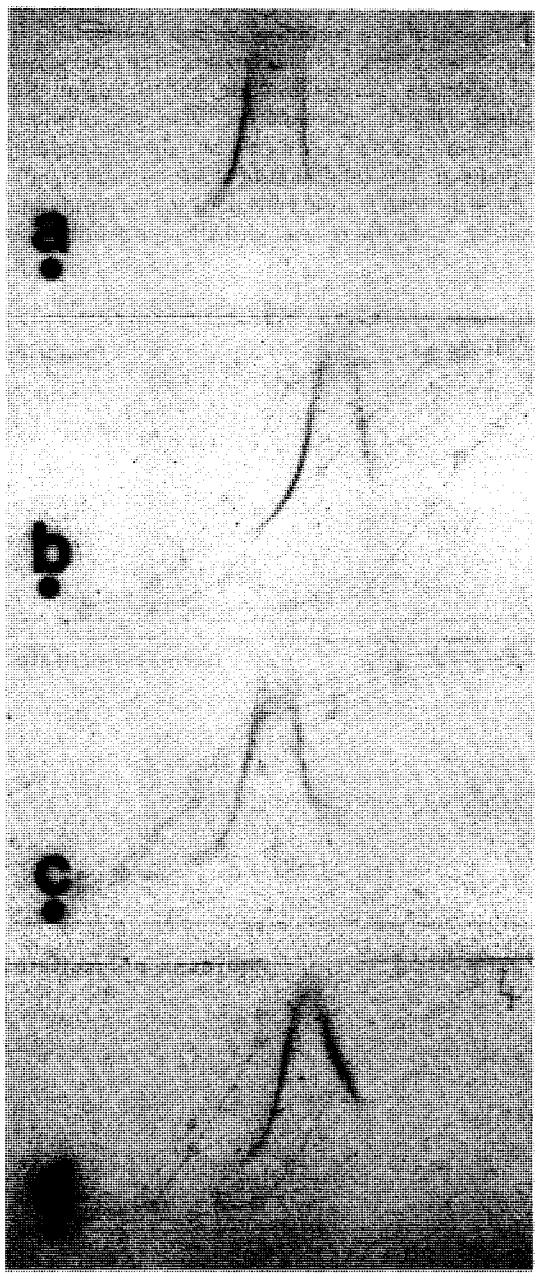


*Fig. 3* Binding of various glycosaminoglycans to S protein, analyzed by immunoelectrophoresis. Two μg of purified S protein were preincubated with various glycosaminoglycans as indicated below and subjected to electrophoresis in the first dimension (anode to the right): (a) without additive; in the presence of 0.5 mg/ml (in the agarose gel and the sample) each of (b) heparin; (c) dermatan sulfate; (d) pentosan polysulfate. The second dimension (anode to the top) contained 0.8% (v/v) anti-(human S protein) serum

### *Formation of a Ternary Complex Consisting of Thrombin, Heparin Cofactor II and S Protein*

The appearance of an additional cathodal shoulder in the immunoprecipitate of S protein in serum has constantly been attributed to association with the thrombin-antithrombin III complex during the clotting process. By analogy with the formation of this ternary S protein-thrombin-antithrombin III complex, it was tested whether S protein may also become incorporated into a macromolecular complex together with thrombin and heparin cofactor II. Using the immuno-electrophoretic methodology, in defibrinated plasma supplemented with radiolabelled components, glycosaminoglycans and thrombin, ternary complex formation was evidenced by precipitation of radioactivity originating from S protein by anti-(heparin cofactor II) serum and vice versa

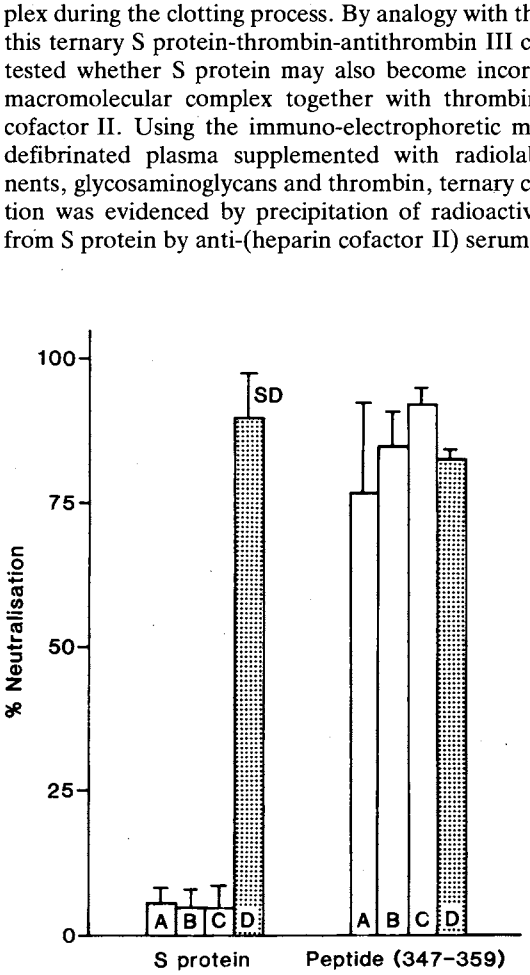


*Fig. 4* Neutralisation of various dermatan sulfate compounds by S protein and S protein-derived synthetic peptide. The neutralisation activity of reduced/carboxymethylated S protein or the synthetic peptide (residues 347–359) in the inhibition of thrombin by heparin cofactor II was measured in the presence of: (A) standard dermatan sulfate; (B) dermatan sulfate with a ratio S/O = 2.0; (C) dermatan sulfate with a ratio S/O = 3.7; (D) heparin. Results are expressed as percent of total neutralisation which is measured in the absence of glycosaminoglycans (mean ± S.D., n = 3)

(Fig. 5). Moreover, there was a corresponding mobility in all cases of the radioactive part of the precipitin line, indicating that the ternary complex was positioned cathodal to the original uncomplexed S protein and anodal to the original uncomplexed heparin cofactor II peak. The immunoelectrophoretic patterns of the complexes were virtually identical regardless whether heparin or dermatan sulfate was used to initiate inhibition of thrombin.

Like the ternary S protein-thrombin-antithrombin III complex (25), the S protein-thrombin-heparin cofactor II complex, which had been preformed by reaction of radiolabelled thrombin with heparin cofactor II and S protein in the presence of dermatan sulfate, sedimented as high molecular weight product with an apparent sedimentation coefficient larger than 10 S in sucrose density gradients (Fig. 6). From the pooled heavy fractions of the sucrose density gradient (Fig. 6A, bar), 68% or 70% of thrombin-originating radioactivity could be immunoprecipitated by monospecific antibodies against heparin cofactor II or S protein, respectively. In contrast to the ternary complex, the binary thrombin-heparin cofactor II complex sedimented only slightly faster than uncomplexed thrombin (Fig. 6B).

Complex formation between S protein and thrombin-heparin cofactor II was also evidenced utilizing the ELISA methodology. Solid phase-adsorbed S protein bound the thrombin-heparin cofactor II complex regardless whether it was preformed in the presence of heparin or dermatan sulfate (Fig. 7), as documented by the binding of antibodies against thrombin as well as heparin cofactor II to a similar degree. Although to a much lesser extent, we repeatedly observed a reproducible binding of heparin cofactor II to S protein in the absence of thrombin (Fig. 7).

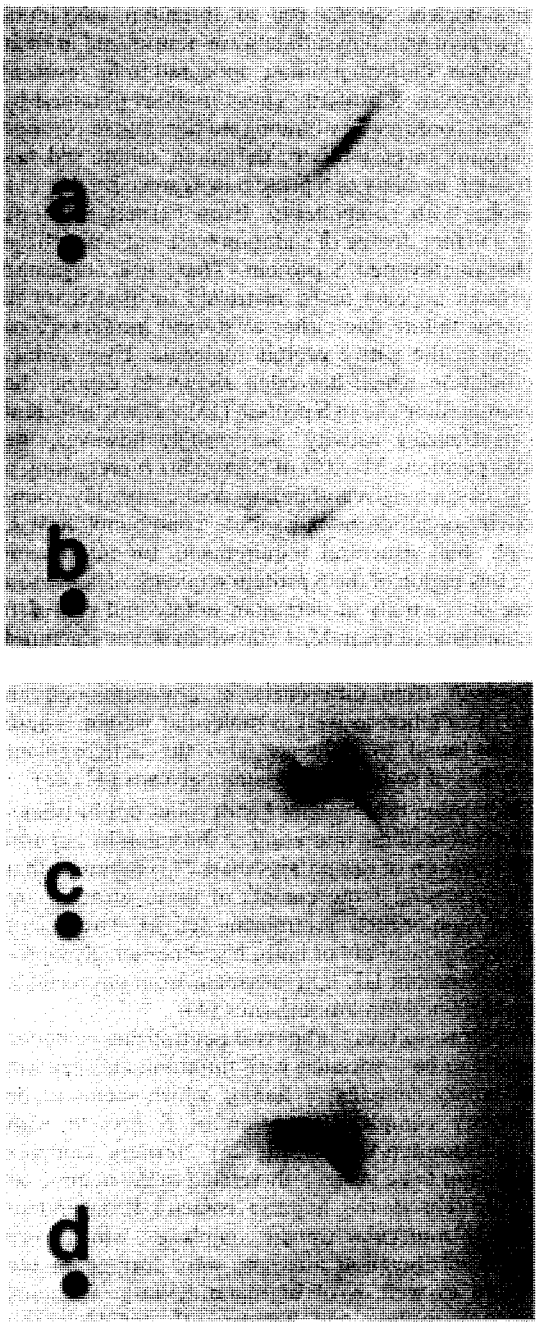


*Fig. 5* Demonstration of ternary complex formation between thrombin, heparin cofactor II and S protein by crossed immuno-electrophoresis. Thrombin was added to de-fibrinated normal human plasma which was either supplemented with ratiolabelled S protein (a, b) or radiolabelled heparin cofactor II (c, d) either in the presence of heparin (a, c) or dermatan sulfate (b, d) and subjected to electrophoresis in the first dimension (anode to the right). Samples containing radiolabelled S protein were analyzed in the second dimension (anode at the top) against anti-(heparin cofactor II) serum (a, b) and samples containing radiolabelled heparin cofactor II against anti-(S protein) serum (c, d). Autoradiographs of the respective gels are shown. Note the appearance of radioactivity which constitutes part of the entire precipitine line at corresponding positions in the gels

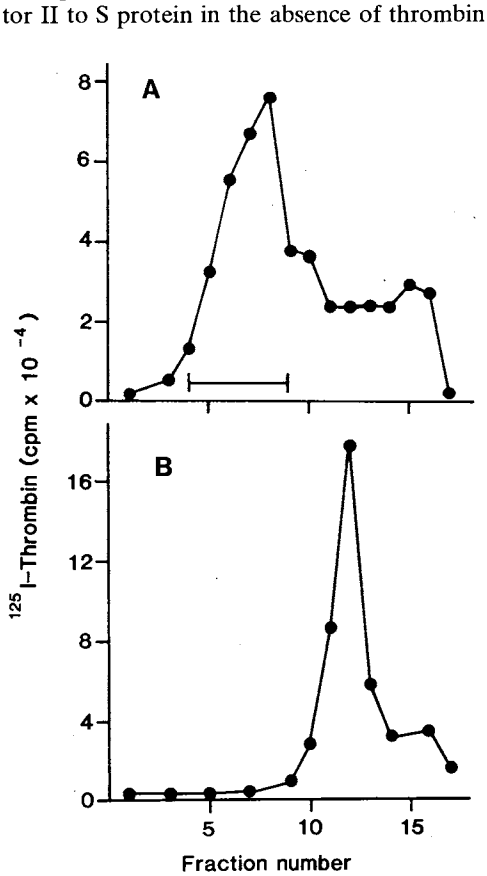


*Fig. 6* Formation of a high molecular weight ternary S protein-thrombin-heparin cofactor II complex as demonstrated by ultracentrifugation. Radiolabelled thrombin was incubated with heparin cofactor II (B) or heparin cofactor II and S protein (A) in a molar ratio of 1:1.1:1.8 and centrifuged through 10–35% (w/v) linear sucrose density gradients (direction of centrifugation is to the left). The high molecular weight ternary complex in the heavy fractions of the gradient A was pooled as indicated by the bar. IgM (19 S) sedimented in fraction 5, albumin (4.1 S) in fraction 13 and uncomplexed radiolabelled thrombin in fraction 14 in simultaneously analyzed gradients

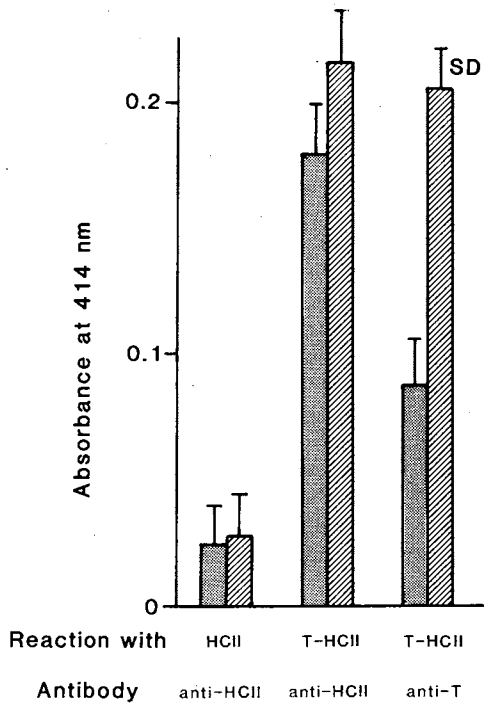


*Fig. 7* Demonstration of ternary complex formation by ELISA analysis. Microtiter wells coated with S protein were reacted with heparin cofactor II (HC II) or thrombin-heparin cofactor II complex (T-HC II) in the presence of heparin (grey columns) or dermatan sulfate (hatched columns). Proteins bound to S protein were detected by monospecific antibodies as indicated followed by peroxidase-conjugated IgG against rabbit immunoglobulins and substrate. Control wells were coated with albumin instead of S protein but otherwise treated identically. The results are given as means ± S.D. (n = 4) and are corrected for unspecific binding

## Discussion

The expression of the biological activity of endogenous or exogeneous heparin-like compounds in vivo may be restricted by the action of various binding and neutralizing proteins. Besides the platelet release product factor 4 (18, 19) and the plasma component histidine-rich glycoprotein (16, 17) which both bind to heparin and thereby protect thrombin as well as factor Xa against heparin-catalyzed inhibition by antithrombin III, S protein (vitronectin) exerts a potent function as heparin-neutralizing factor as well (3, 14, 15). While all three proteins appear to require interaction with saccaride sequences in addition to the antithrombin III-binding sequence of heparin in order to effectively express their activity (20, 36, 37), a unique heparin-binding domain in S protein (6) enables it to express a similar affinity in heparin-binding compared to antithrombin III (15). In contrast to platelet factor 4 and histidine-rich glycoprotein, S protein readily neutralizes the anticoagulant activity of polysaccharides with minimal size for the inhibition of thrombin (octadecasaccharide) as well as factor Xa (pentasaccharide) by antithrombin III (20). Thus, it appears that S protein exerts its primary effect by competing with antithrombin III for binding to heparin rather than to prevent formation of a ternary complex between antithrombin III, heparin and the protease (38), although weak interactions between S protein and thrombin have been proposed (24). While heparin interacts with both, antithrombin III and heparin cofactor II, dermatan sulfate as well as pentosan polysulfate do not bind to antithrombin III and thus, have no appreciable effect on the rate of inhibition of thrombin by antithrombin III (29, 39, 40). In addition, the diverse reactivities of antithrombin III and S protein towards dermatan sulfate and pentosan polysulfate clearly documented different structural (and functional) requirements for interaction.

The different modes of interaction between the various polysaccharides and S protein, as investigated in the present study, may allow to approach the molecular mechanisms operative in the S protein-induced modulation of heparin cofactor II function and its possible physiological significance in thrombin inhibition. While heparin and pentosan polysulfate were neutralized in their anticoagulant function, dermatan sulfate appeared to be resistent towards neutralisation by S protein. These findings obtained by kinetic analysis were confirmed by binding studies, and in contrast to heparin and pentosan polysulfate, dermatan sulfate did not significantly affect the mobility of S protein in agarose gel due to a low affinity among both components. Even dermatan sulfate compounds with a higher degree of sulfation were not neutralized by reduced/carboxymethylated S protein. Thus, it is suggested that native S protein appears to be restricted in its activity to neutralize dermatan sulfate due either to sterical hinderance or charge distribution in the native protein molecule. Even reduction/carboxymethylation which has been shown to expose the glycosaminoglycan-binding site in S protein (15) was not sufficient to create any neutralizing activity. Surprisingly however, the synthetic peptide (positions 347–359 of S protein) representing a portion of the glycosaminoglycan-binding domain readily counteracted the activity of all dermatan sulfate compounds tested, indicating that minimal structural requirements for interaction were fulfilled for this region of the S protein sequence. The reason for the difference in reactivity of dermatan sulfate and pentosan polysulfate (a heparinoid of mean $M_r$ 4,500 which is manufactured by sulfation of a carbohydrate extract from tree bark) with native S protein may result from differences in glycosaminoglycan structure, degree of sulfation as well as molecular weight. These criteria have been found to influence also the anticoagulant activity of these types of sulfated glycosaminoglycans (41, 42).

The recent observation that platelet factor 4 but not histidine-rich glycoprotein prevented the inhibition of thrombin by heparin cofactor II in the presence of dermatan sulfate (43) indicates that indeed particular structural requirements of these proteins are necessary for expression of their neutralizing activity. The lack of interaction with dermatan sulfate of both, histidine-rich glycoprotein as well as antithrombin III, may originate from appreciable homology within their heparin binding sites (44).

Moreover, in contrast to these other two neutralizing proteins, S protein was found to be associated with thrombin and heparin cofactor II in a ternary complex, a finding which seems to be analogous to ternary complex formation of S protein with thrombin and antithrombin III (21–23, 25). Ternary complex formation appeared not to be necessarily related to the neutralisation effect of S protein in the heparin cofactor II-dependent inhibition, since high molecular weight complexes, which were evidenced by ultracentrifugation analysis, were formed in the presence of heparin as well as of dermatan sulfate. Complex formation between S protein and heparin cofactor II in the absence of thrombin was also noted, an interaction which may depend on sulfate residues recently identified in the disulfide loop-free amino-terminal portion of the heparin cofactor II molecule (45, 46). Based on our results, heparin cofactor II in its native conformation may directly interact to a certain extent with S protein, whereas antithrombin III has to be modified by radioiodination or adsorption to surfaces in order to express direct binding to S protein (23). Upon reaction with thrombin, however, both inhibitors appear to be in the "correct" conformation to interact with S protein.

Taken together, our findings may also implicate obvious differences for S protein-related modulation of glycosaminogly-

can-dependent inhibition reactions of thrombin by antithrombin III and heparin cofactor II occuring in intra- and extravascular compartments:

(a) The observed affinity of S protein for different polysaccharides possibly exposed at the vessel wall surface (20), suggests an interference of this neutralizing protein with thrombin inhibition by antithrombin III at this site. Heparin cofactor II, however, appears to be secondary or even ineffective in this respect. This conclusion is also in accordance with clinical data from patients, deficient in either one of both inhibitors. While hereditary antithrombin III-deficiency predisposes for the development of thrombosis (47), isolated hereditary heparin cofactor II-deficiency (48, 49) appears not to be a risk factor for development of thrombophilia (50). Thus, the physiological importance of heparin cofactor II in the circulation remains unclear.

(b) In extravascular compartments, however, heparin cofactor II may fully express its inhibitory capacity, since dermatan sulfate is a major component found in extravascular sites (51). Additionally, despite the presence of S protein (vitronectin) in extracellular matrices (9) the native protein appears to be ineffective in neutralizing dermatan sulfate, such that heparin cofactor II function in the extracellular space may be modulated only by possible proteolysis products of S protein (e.g. glycosaminoglycan-binding domain) or by platelet factor 4 (43).

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