Neutralization and Binding of Heparin by S Protein/Vitronectin in the Inhibition of Factor Xa by Antithrombin III

IN Volvement of an Inducible Heparin-Binding Domain of S Protein/Vitronectin*

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The interference of the heparin-neutralizing plasma component S protein (vitronectin) (Mr = 78,000) with heparin-catalyzed inhibition of coagulation factor Xa by antithrombin III was investigated in plasma and in a purified system. In plasma, S protein effectively counteracted the anticoagulant activity of heparin, since factor Xa inhibition was markedly reduced in comparison to heparinized plasma deficient in S protein. Using purified components in the presence of heparin, S protein induced a concentration-dependent reduction of the inhibition rate of factor Xa by antithrombin III. This resulted in a decrease of the apparent pseudo-first order rate constant by more than 10-fold at a physiological ratio of antithrombin III to S protein. S protein not only counteracted the anticoagulant activity of commercial heparin but also of low molecular weight forms of heparin (mean Mr of 4,500). The heparin-neutralizing activity of S protein was found to be mainly expressed in the range 0.2–10 μg/ml of high Mr, as well as low Mr, heparin. S protein and high affinity heparin reacted with apparent 1:1 stoichiometry to form a complex with a dissociation constant Kd = 1 × 10–8 M as determined by a functional assay. As deduced from dot-blot analysis, direct interaction of radiolabeled heparin with S protein revealed a dissociation constant Kd = 4 × 10–7 M. Heparin binding as well as heparin neutralization by S protein increased significantly when reduced/carboxymethylated or guanidine-treated S protein was employed indicating the existence of a partly buried heparin-binding domain in native S protein. Radiolabeled heparin bound to the native protein molecule as well as to a BrCN fragment (Mr = 12,000) containing the heparin-binding domain as demonstrated by direct binding on nitrocellulose replicas of sodium dodecyl sulfate-polyacrylamide gels. Kinetic analysis revealed that the heparin neutralization activity of S protein in the inhibition of factor Xa by antithrombin III could be mimicked by a synthetic tridecapeptide from the amino-terminal portion of the heparin-binding domain. These data provide evidence that the heparin-binding domain of S protein appears to be unique in binding to heparin and thereby neutralizing its anticoagulant activity in the inhibition of coagulation factors by antithrombin III. The induction of heparin binding and neutralization may be considered a possible physiological mechanism initiated by conformational alteration of the S protein molecule. Consequently, a secondary structure model of S protein is proposed in which the β-sheet structure of the heparin-binding domain is localized in a buried position within the protein molecule.

Antithrombin III (ATIII) plays an important role among the proteinase inhibitors in plasma by controlling the activity of various coagulation enzymes, especially thrombin and factor Xa. The rate of enzyme inhibition by ATIII is enhanced by catalytic amounts of heparin (1) or by the presence of heparin-like molecules derived from endothelial cells or mast cells (2, 3). While thrombin expresses high affinity for heparin, and heparin fractions with decreasing Mr, have been shown to exhibit decreasing anticoagulant activity, the inhibition of factor Xa by ATIII is similarly enhanced by heparin as well as by heparin fragments with Mr as small as 2400 (4, 5). Consequently, low Mr, heparin fragments are ineffective in the inhibition of thrombin, and a minimal octadecasaccharide sequence is required for acceleration of thrombin inhibition by ATIII (6). These differences in the kinetics of inhibition can be best described in a random order bireactant model for thrombin or an ordered bireactant model for factor Xa inhibition by heparin-ATIII (7).

The expression of the biological activity of endogenous or exogeneous heparin-like compounds in vivo may be regulated by the action of various binding and neutralizing proteins. The platelet release product platelet factor 4 (8, 9) as well as the plasma protein histidine-rich glycoprotein (10, 11) bind to heparin and protect thrombin as well as factor Xa against heparin-catalyzed inhibition by ATIII. Both neutralizing proteins require interaction with saccharide sequences in addition to the ATIII-binding sequence of heparin in order to effectively express their heparin-neutralizing activity (6, 12). Recently, the complement inhibitor S protein has been described by our laboratory to bind to heparin and to exert a heparin-neutralizing activity in the heparin-catalyzed inhibition of thrombin by ATIII (13, 14), a finding confirmed by others (15). S protein has been shown to be identical with the serum-spreading factor vitronectin (16–19) and structural studies have revealed the existence of a heparin-binding domain in the carboxyl-terminal portion of the S protein/vitronectin molecule (20). In contrast to platelet factor 4 and histidine-rich glycoprotein, S protein/vitronectin becomes incorporated into a high Mr, ternary complex with thrombin and ATIII (21–24).

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In the present report, the heparin-neutralizing activity of S protein/vitronectin in the inhibition of factor Xa by ATIII catalyzed by high and low Mf, heparin components was examined in purified systems and in plasma. The direct interaction of heparin with S protein/vitronectin was characterized by functional assay as well as by direct binding studies. Based on the present findings, a structural model for the heparin-binding domain of S protein/vitronectin is proposed.

EXPERIMENTAL PROCEDURES

Materials

DEAE-Sephadex, Sephadex G-50, and BrCN-activated Sepharose 4B were from Pharmacia and nitrocellulose was from Hoefer Instruments. Heparin sodium salt from porcine intestinal mucosa (mean Mf = 15,000, 169 units of anti-factor Xa/mg), bovine and human albumin, Polybrene and Russell’s viper venom were obtained from Sigma. High affinity heparin was obtained from commercial heparin by affinity chromatography on immobilized ATIII, as described (25). The synthetic substrate N-benzoyl-L-Glu-Gly-Gly-p-nitroanilide (S-2222) and heparin sodium salt which had been treated with nitrous acid (26) to obtain fragments with mean Mf of 4,500 (160 units of anti-factor Xa/mg) were purchased from Kabi Diagnostics; hirudin was from Pentapharm and 3-(p-hydroxyphenyl)propionic acid from Behring Diagnostics. All chemicals used were analytical grade.

S protein and ATIII were purified and assayed as previously described (13), and affinity-purified rabbit IgG against S protein was prepared as described elsewhere (24). The synthetic tridecapeptide Lys-Lys-Gln-Arg-Phe-Arg-His-Arg-Lys-Gly-Tyr representing positions 347–359 of the sequence of S protein/vitronectin (16, 17) was kindly provided by Dr. J. Tschopp, University of Lausanne (Switzerland). Bovine factor Xa was purchased from Diagnostic Reagents (United Kingdom). Human factor Xa was isolated from the barium citrate eluate which was obtained after barium citrate adsorption of normal human plasma. Following chromatography on DEAE-Sephadex and sulfated Sephadex G-50 as described for prothrombin (13), factor Xa was pooled and activated by Russell’s viper venom immobilized on Sepharose 4B according to (27). All proteins were homogeneous as judged by 10% polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) (28). Following modification with 3-(p-hydroxyphenyl)propionic acid essentially as described elsewhere (29), heparin was labeled with 35Cl (carrier free) (Du Pont-New England Nuclear) using the iodogen procedure (30), reaching a specific activity of 4 mCi/μg. The anticoagulant activity of heparin was not affected by the labeling procedure.

Kinetics of Heparin-induced Factor Xa Inhibition by ATIII and Neutralization of Heparin Activity by S Protein and S Protein-treated Proteins

A—The effect of various concentrations of S protein on heparin-induced fast inactivation of factor Xa by ATIII was tested in an amidoxydase assay system similar to that previously described for thrombin inhibition (14). ATIII (0.44 μM final concentration), various concentrations of S protein (0–1.46 μM), and the synthetic substrate S-2222 (0.4 mM final concentration) were preincubated for 1 min at 37 °C in a total volume of 7.5 μl 0.05 M TRIS HCl, 0.14 M NaCl, pH 7.4 (TBS), containing 0.5 mM CaCl2 and 0.5% (w/v) human serum albumin. 75 μl of a mixture of bovine factor Xa (5 nM final concentration) and heparin (0.3 μg/ml final concentration) or factor Xa alone was then added to initiate the reaction, and the disappearance rate of the amidoxydase activity was continuously recorded. The Michaelis constant for S-2222 (0.3 mM).

B—Under identical conditions, reduced/carboxymethylated S protein (10), S protein treated with BrCN dissolved in 70% formic acid for 1 h at 22 °C, or S protein data treated with 5% sodium hydrochloride for 1 h at 37 °C were included in the inhibition assays. Prior to analysis, modified S protein samples were extensively dialyzed against TBS. Similarly, various concentrations (0–20 μg/ml) of the protein-tridecapeptide instead of S protein were used, and the kinetics of factor Xa inhibition followed exactly as outlined in the present paragraph in the presence of three different ATIII concentrations (0.22, 0.44, and 0.98 μM).

Influence of S protein on the inhibition of factor Xa by ATIII in the presence of heparin was quantitated in a similar experiment. ATIII (220 nM final concentration) was preincubated with 55 nM (final concentration) high affinity heparin and various concentrations of S protein (6–90 nM) in a total volume of 275 μl of TBS containing 0.5% (w/v) human serum albumin and 3 mM CaCl2 for 1 min at 37 °C. 25 μl of a solution of bovine factor Xa (3.5 mM final concentration) was added to initiate the reaction, and after 30 s at 37 °C the whole mixture was transferred into a prewarmed cuvette with 0.27 mM S-2222 dissolved in 450 μl of TBS, pH 8.0, containing 0.1% (w/v) bovine serum albumin. Residual factor Xa amidolytic activity was recorded at 405 nm and was used to determine residual free heparin concentration. For this purpose a calibration curve was constructed using different concentrations of high affinity heparin (0.8–67 nM final concentration) in the absence of S protein. Residual factor Xa activity was plotted versus heparin concentration on a semilogarithmic scale. The residual free heparin concentration determined by this apparent heparin concentration, which was estimated from residual factor Xa activity of the calibration curve and expressed in percent of the total heparin concentration, was plotted against residual free S protein concentration. The concentration of free S protein itself was determined from the total amount minus that bound to heparin assuming a 1:1 interaction between S protein and heparin.

D—The ability of S protein to neutralize the anticoagulant activity of various heparin preparations in the inhibition of factor Xa by ATIII was tested at 37 °C. ATIII (0.5 μM final concentration), various concentrations (0–100 μg/ml) of commercial heparin, 3rd International Heparin Standard (National Institute for Biological Standards and Control, London, U.K.), or nitrous acid-treated heparin were preincubated for 1 min at 37 °C in the absence or presence of 1.5 μM S protein (final concentration). 38 nM bovine factor Xa (final concentration) was added to initiate the reaction in a total volume of 50 μl of TBS, containing 0.5% (w/v) human serum albumin and 2 mM CaCl2. After 30 s at 37 °C, 750 μl of prewarmed TBS, pH 8.0, containing 0.1 mg/ml polyethylene, 0.1% (w/v) bovine serum albumin, and 0.3 mM (final concentration) S-2222 was added to terminate the inhibition reaction and to quantify the amount of residual factor Xa amidolytic activity. After an additional 5 min at 37 °C, 200 μl of 50% (v/v) acetic acid was added and the concentration of liberated p-nitroanilide was measured at 405 nm.

Neutralization of Heparin Activity by S Protein in Plasma

Pooled normal human plasma was supplemented with 5 IU/ml hirudin and one sample was incubated with affinity-purified rabbit IgG against human S protein, the other sample was mixed with the same amount of nonimmune rabbit IgG for 1 h at 4 °C, followed by centrifugation. S protein activity was measured by rate chromotography according to Laurier (31), using anti-S protein at a concentration of 2% (v/v) in the agarose gel. Human factor Xa (130 nm final concentration) was added to plasma samples which contained either 0.05 units/ml or 0.1 units/ml heparin in a total volume of 300 μl. After incubation for exactly 30 s at 37 °C, 700 μl of TBS, pH 8.0, containing 0.1% (w/v) bovine serum albumin, 0.1 mg/ml Polyethylene and 0.6 mM S-2222 was added. Residual factor Xa amidolytic activity was recorded at 405 nm and was expressed as percentage of the factor Xa activity measured under identical conditions in the absence of heparin.

Binding of 122I-Heparin to S Protein

Direct binding of radiolabeled heparin was studied using a dot-blot chamber (Bio-Rad), following the instructions of the manufacturer. After adsorption of 100-μl portions of purified S protein (12, 20, 25 μg/ml) to nitrocellulose and saturation with 0.5% (w/v) bovine albumin solution, various concentrations of 122I-heparin, dissolved in TBS containing 0.05% (v/v) bovine albumin, were allowed to react with the precoated nitrocellulose for 2 h at 22 °C. After extensive washing with TBS containing 0.05% (w/v) bovine serum albumin, the nitrocellulose sheet was removed from the chamber and individual dots were cut out and counted for radioactivity. Measurements were performed at least in triplicate. As data were corrected for nonspecific binding by subtracting the radioactivity of dots which had not been precoated with S protein but otherwise treated identically. Likewise,
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reduced/carboxymethylated S protein, S protein treated with BrCN, or guanidine-treated S protein was included in the binding assay.

After electrophoresis of purified S protein and BrCN-treated S protein on a 15% SDS-polyacrylamide gel, proteins were transferred onto nitrocellulose using the buffer system of Towbin et al. (32). The nitrocellulose was rinsed three times with TBS and incubated with [3H]heparin (2 μCi/ml) for 2 h at 22 °C, followed by several washes with TBS and drying. Distribution of radiolabel was detected by autoradiography. Finally, the nitrocellulose was stained and destained as previously described (29).

RESULTS

Neutralization of Heparin by S Protein in Plasma—Human plasma which was made deficient in S protein by immunoprecipitation (containing 10–15% of the initial S protein concentration) showed loss of its potential to neutralize heparin anticoagulant activity. Depending on the heparin concentration, factor Xa inhibition was prolonged 3- to 4-fold in normal plasma in the presence of S protein (Table I).

Neutralization of Heparin Activity by S Protein and Determination of the Dissociation Constant of the Complex between S Protein and Heparin—The heparin-neutralizing effect of S protein on factor Xa inhibition by ATIII-heparin was also demonstrated in a purified system. While in the absence of S protein, 50% factor Xa activity was inhibited by ATIII and 0.03 μg/ml heparin in less than 3 s, the addition of S protein induced a concentration-dependent reduction of the inhibition rate as reflected by a more than 10-fold increase of the half-life of factor Xa activity. Thus, the apparent first-order rate constant decreased from k_{app} = 730 × 10^{-5} s^{-1} in the absence to k_{app} = 63 × 10^{-5} s^{-1} in the presence of a 1.5-fold molar excess of S protein over ATIII (Table II). Control experiments in the same system revealed neither an influence of S protein and heparin on factor Xa nor an effect of S protein on the inhibition of factor Xa by ATIII alone.

Since S protein may compete with ATIII for heparin in the inhibition reaction thereby neutralizing the heparin activity, a determination of the dissociation constant between S protein and heparin was attempted. In a factor Xa inhibition assay in the absence of S protein, residual factor Xa activity could be correlated with the apparent heparin concentration with establishment of a calibration curve. For each S protein concentration, the percentage of neutralized heparin could thus be determined. A plot of apparent heparin concentration (as percentage of total heparin) versus the concentration of free S protein (total S protein minus the amount bound to heparin) revealed a sigmoidal-shaped curve which is consistent with a single association reaction between heparin and S protein (Fig. 1). The dissociation constant of this interaction, determined as the concentration of S protein that decreases the apparent heparin concentration to 50% of its initial value, was found to be K_{D} = 1 × 10^{-8} M.

Neutralization of High and Low Molecular Weight Heparin by S Protein—Commercial heparin as well as low M, heparin were able to accelerate the inhibition of factor Xa by ATIII in a concentration-dependent manner (Fig. 2). As with 3rd International Heparin Standard, 50% inhibition of factor Xa was induced at 0.1–0.2 μg/ml high M, as well as low M, heparin. Addition of a 3-fold molar excess of S protein over ATIII resulted in the neutralization of the activity of high and low M, heparin. The point of 50% inhibition of factor Xa at 30 s, which was equivalent to a second-order rate constant k_{2} = 2.8 × 10^{9} M^{-1} min^{-1}, was shifted from 0.1–0.2 μg/ml heparin in the absence to 8–15 μg/ml heparin in the presence of S protein. Heparin concentrations higher than 30 μg/ml were no longer neutralized by S protein.

Characterization of Heparin Binding by S Protein—Direct interaction of S protein with radiolabeled heparin was studied by dot-blot analysis and was used to determine the dissociation constant between both components. Increasing concentrations of [3H]-heparin were allowed to react with two different concentrations of adsorbed S protein and were both found to bind in a saturable manner. The interaction was specific, since unlabeled heparin in 100-fold molar excess over labeled heparin was no longer bound by S protein.

| TABLE I |
| Influence of S protein on inhibition of factor Xa in heparinized human plasma |
| Human factor Xa (130 nM) was added to either normal human plasma or plasma partially deficient in S protein in the absence and presence of heparin as indicated. After reaction for 30 s, residual factor Xa activity (mean of three determinations) was determined in a chromogenic assay and expressed as percentage of the control. |
| Residual factor Xa activity |
| Normal plasma | S-Deficient plasma |
| % control | IU/ml |
| 100 | 100 | 0 |
| 79 | 28 | 0.05 |
| 44 | 9 | 0.1 |

| TABLE II |
| Influence of S protein on heparin-catalyzed inhibition of factor Xa by ATIII |
| Apparent first-order rate constants (k_{app}) for the factor Xa-ATIII reaction (5 nM factor Xa, 440 nM ATIII) in the presence of 0.3 μg/ml heparin and various concentrations of S protein. |
| S protein | S/ATIII | First-order rate constant |
| μM | mol/mol | k_{app} × 10^{9} s^{-1} |
| 0 | 0 | 730 |
| 1.25 | 0.55 | 275 |
| 2.5 | 1.10 | 102 |
| 3.3 | 1.46 | 63 |

FIG. 1. Direct interaction of S protein with heparin. Inhibition of 3.5 nM factor Xa in the presence of 0.22 μM ATIII, 55 nM high affinity heparin (mean Mᵢ = 15,000), and different concentrations of S protein was measured in an amidolytic assay system. From a calibration curve constructed with various concentrations of heparin in the absence of S protein, the apparent heparin concentration which was available for factor Xa inhibition by ATIII was determined. A plot of the apparent heparin concentration (percentage of the initial value) versus the concentration of free S protein (total S protein minus that bound to heparin) is shown.
ligand inhibited binding by more than 90%, while albumin was ineffective in displacing labeled heparin. When binding values for two different S protein concentrations were expressed in a double-reciprocal plot (Fig. 3A), a dissociation constant $K_D = 3.8 \times 10^{-8}$ M (mean of three determinations) was found. Likewise, binding at two different concentrations of $^{125}$I-heparin to preadsorbed S protein was measured in the absence and presence of various concentrations of unlabeled heparin which displaced the radiolabeled ligand in a concentration-dependent fashion. Expression of binding data in a Dixon-plot (Fig. 3B) revealed competitive inhibition with a dissociation constant $K_D = 4 \times 10^{-8}$ M (mean of three determinations).

Following modification of S protein by reduction and carboxymethylation or by treatment with guanidine hydrochloride, direct binding of $^{125}$I-heparin increased more than 2-fold as compared with the native protein. Fragmentation of S protein by BrCN also increased heparin binding significantly (Fig. 4, gray bars). Concomitantly, an increase in functional heparin-neutralizing activity of modified S protein was observed when tested in the factor Xa inhibition assay, and values were expressed as relative neutralization activity utilizing the reciprocal apparent first-order reaction constants (Fig. 4, closed bars). While native S protein showed moderate neutralization activity, modified samples including BrCN-treated S protein expressed nearly maximal heparin-neutralizing capacity. In addition, modified S protein has been noted to be more effective than unmodified S protein in functioning as complement inhibitor in vitro.

Localization of the heparin-binding site in S protein was investigated by SDS-polyacrylamide gel electrophoresis of native and BrCN-treated S protein, followed by transfer onto nitrocellulose and the determination of direct binding of radiolabeled heparin. While $^{125}$I-heparin bound to both the 78,000-dalton as well as the 65,000-dalton band of the intact S protein, only a 12,000-dalton BrCN fragment was consistently found associated with heparin (Fig. 5). This fragment has recently been shown to bind to heparin-Sepharose (20) and resides in the carboxyl-terminal portion of the S protein molecule (16, 17). A synthetic tridecapeptide representing the amino-terminal part of the heparin-binding domain (positions 347-359 of S protein) was studied for its ability to function as a heparin-neutralizing component in the factor Xa inhibition assay. As with the native molecule, the tridecapeptide mimicked the functional activity of intact S protein in a concentration-dependent fashion at three different ATIII concentrations (Fig. 6). The interaction constant of the peptide in this neutralization reaction was $K = 2 \mu$M, which is about 3-fold higher than was found for intact S protein in the thrombin-ATIII-heparin reaction (14).

**DISCUSSION**

The generation of activated factor X (factor Xa) by both the intrinsic as well as the extrinsic pathway of blood coagulation points to its central position in the blood-clotting cascade. While in the absence of heparin, factor Xa is mainly controlled by the two plasma protease inhibitors ATIII and $\alpha_1$-proteinase inhibitor (33, 34), ATIII becomes the major inhibitor of factor Xa in the presence of catalytic amounts of heparin (1). In this respect, no factor Xa-heparin interaction

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Fig. 2. Neutralization of high and low $M_r$ heparin by S protein. The residual amidolytic activity of 38 nM factor Xa was measured in the presence of 0.5 $\mu$M ATIII (C); 0.5 $\mu$M ATIII and 1.5 $\mu$M S protein (○); or without additions (△) at different concentrations of: (A), 3rd International Heparin Standard; (B), commercial heparin; (C), low $M_r$ heparin.

Fig. 3. Direct binding of $^{125}$I-heparin to S protein as measured by dot-blot analysis. A, 0.8 $\mu$g (△) and 2 $\mu$g (○) S protein was adsorbed to nitrocellulose in a 96-multi-well chamber and binding of various amounts (38–300 ng) $^{125}$I-heparin was measured. A double-reciprocal plot of bound versus free heparin is shown. B, 2 $\mu$g of S protein was adsorbed to nitrocellulose in a 96-multi-well chamber and binding of 125 ng (○) and 33 ng (C) of $^{125}$I-heparin in the presence of various concentrations of unlabeled heparin was measured. Data were expressed in a Dixon-plot.

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is required for expression of anticoagulant activity of heparin (4–6, 35) and the rate of inhibition of factor Xa is proportional to the concentration of heparin-ATIII complex (5).

Since previous studies have shown that S protein/vitronectin neutralizes the anticoagulant activity of heparin in the inhibition of thrombin by ATIII (14), S protein/vitronectin was further examined for its neutralizing ability in the heparin-catalyzed inhibition of factor Xa by ATIII. The rate of factor Xa inhibition was already decreased by S protein/vitronectin at concentrations lower than those of ATIII, indicating a competition between S protein/vitronectin and ATIII for binding to heparin. Indeed, the dissociation constant for the interaction of S protein/vitronectin with heparin determined by functional assay is $K_D = 1 \times 10^{-8}$ M and when determined by direct binding experiments is $K_D = 4 \times 10^{-8}$ M. Both values appeared to be comparable to the dissociation constant $K_D = 5.7 \times 10^{-3}$ M for the ATIII-heparin interaction (36).

Likewise, the dissociation constant for the interaction between histidine-rich glycoprotein and heparin has been reported to be $K_D = 0.7 \times 10^{-8}$ M (11), suggesting that both S protein/vitronectin and histidine-rich glycoprotein may exert a potent and similar anti-heparin effect. In addition, S protein/vitronectin and histidine-rich glycoprotein express a comparable heparin-neutralizing activity in plasma. Like histidine-rich glycoprotein and platelet factor 4 (12), S protein/vitronectin was equally effective in neutralizing the anticoagulant activity of high $M_r$, as well as low $M_r$ heparin. However, differences between S protein/vitronectin, histidine-rich glycoprotein, and platelet factor 4 do exist in the interaction with heparin as described in the following paragraphs.

(a) While the neutralizing properties of histidine-rich glycoprotein are dependent on divalent cations and are abolished by EDTA (11), those of platelet factor 4 (37) and S protein/vitronectin (13, 14) are not.

(b) In contrast to the tetrameric structure of platelet factor 4 (38) which permits interaction with heparin via lysine residues (8) by forming multiple complexes (39), and the apparently monomeric histidine-rich glycoprotein, a free thiol group in S protein/vitronectin (13, 40) renders this molecule susceptible to dimer formation (40). Although S protein/vitronectin and heparin appear to react with a 1:1 stoichiometry, the exact distribution of monomeric and dimeric S protein/vitronectin in the interaction with heparin is not known.

(c) Unlike platelet factor 4 and histidine-rich glycoprotein, S protein/vitronectin forms a ternary complex with thrombin and ATIII (15, 21–24) which is detectable in serum. Although the formation of a ternary complex seems to be a secondary event not necessarily dependent on binding of S protein/vitronectin to heparin (24), the reversible binding to thrombin (15) may at least in part be an indication for the interference of S protein/vitronectin with enzyme-inhibitor complex formation.

(d) In contrast to ATIII, platelet factor 4, histidine-rich glycoprotein, or the adhesive protein fibronectin (41) in which the heparin-binding domains are less distinct, the heparin-binding site of S protein/vitronectin appears to be a well-defined domain (17, 20) located in the carboxyl-terminal portion of the protein molecule. The involvement of this domain in direct binding of heparin and neutralization of
the cell attachment domain. Partial proteolysis, heparin-binding of S protein may increase as enzymes described in the text.

The position of analysis of the secondary structure of S protein deduced from the protein/vitronectin molecule. Moreover, the anti-heparin activity was unequivocally demonstrated in the present study. Interaction of radiolabeled heparin with the 12,000-dalton BrCN-fragment substantiated the heparin-binding site in this portion of the S protein/vitronectin molecule. Treatment with denaturing agents, reduction and carboxymethylation or fragmentation of S protein by BrCN resulted in significant increases of both activities, indicating the existence of a (partly) buried heparin-binding domain within the molecule. This result confirms the recent finding that S protein/vitronectin binds to heparin-Sepharose but only in the presence of high concentrations of urea (42, 43).

Yet another observation suggests the existence of a buried and thus inducible heparin-binding site. Among other epitopes, the relatively large heparin-binding domain and, in particular, the sequence Arg-His-Arg-Asn-Arg-Lys (positions 352–357) contained within this domain exhibit appreciable hydropathy (17) and thereby might show relatively high antigenicity. However, the observed weak immunogenicity of S protein itself, purified under nondenaturing conditions (13, 21, 40), may partly be explained by the shielded location of the heparin-binding site in the native molecule. This proposal only holds true if the high positive charge density of the heparin-binding domain in its buried location is neutralized by adequate negative charge. Interestingly, the amino terminus of S protein/vitronectin, known as the “somatomedin” portion of the molecule (16, 17), may provide such a high negatively charged domain. Furthermore, the somatomedin domain appears to be of high rigidity, since it contains four disulfide bridges and is rather resistant to proteolysis (44). Moreover, both the heparin-binding domain as well as the somatomedin portion contain mostly β-sheet secondary structure as deduced from their amino acid sequence (45), allowing them to interact intimately within the protein. A hypothetical model for S protein/vitronectin is shown in Fig. 7. The exposure of the heparin-binding domain under in vivo conditions may either be facilitated by interaction of S protein/vitronectin with other protein components, with surfaces, or by partial proteolysis, whereby the most sensitive peptide bond toward trypsin-like enzymes is actually located proximal to the heparin-binding site (Fig. 7) (40). Despite the fact that such a conformational change in S protein/vitronectin is required for its originally described function as a complement inhibitor (46), it is not known whether the heparin-binding site of S protein/vitronectin is involved in this inhibition of the assembly of the membrane attack complex of complement and thus in the inhibition of C9 polymerization (47). Since complement component C9 buries highly charged domains in its native conformation which become exposed upon polymerization (48), it is, however, easy to envisage that S protein/vitronectin may inhibit this process by interacting with these sites. Thus, the heparin-binding domain in S protein/vitronectin may be a candidate for this physiological mechanism and thereby exhibits multifunctional properties in addition to its anti-heparin effect.

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