Specific Glycosaminoglycans Support the Inhibition of Thrombin by Plasminogen Activator Inhibitor 1

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Received April 30, 1992; Revised Manuscript Received December 4, 1992

ABSTRACT: In the absence of accessory components, plasminogen activator inhibitor 1 (PAI-1) rapidly forms equimolar, inactive complexes both with tissue-type (t-PA) and with urokinase-type (u-PA) plamsinogen activator. In the presence of either the glycoprotein vitronectin or the glycosaminoglycan heparin, PAI-1 is endowed with additional, efficient thrombin-inhibitory properties (Ehrlich et al., 1990, 1991a). Here, we have investigated the interaction between PAI-1, thrombin, and glycosaminoglycans in more detail. Inhibition of thrombin by PAI-1 was quantitatively analyzed in the presence of a wide range of concentrations of heparin, heparan sulfate, dermatan sulfate, chondroitin 4-sulfate, chrondoitin 6-sulfate, keratan sulfate, and hyaluronic acid by measuring residual amidolytic activity. In addition, a qualitative analysis was performed by determining the formation of SDS-stable, equimolar complexes between thrombin and PAI-1 in the presence of various glycosaminoglycans. Heparin, at concentrations between 0.1 and 1 μg/mL, significantly promoted thrombin inhibition by PAI-1 as well as SDS-stable complex formation. Suboptimal inhibition was observed with dermatan sulfate, chondroitin 4-sulfate, and heparan sulfate at concentrations that are at least 1 order of magnitude higher than that required for optimal inhibition in the presence of heparin. Virtually no inhibition of thrombin and SDS-stable complex formation was detected with any of the other glycosaminoglycans at concentrations between 0.1 and 1 μg/mL. Competition experiments between solid-phase heparin and soluble glycosaminoglycans for binding to metabolically labeled ^{35}S-PAI-1 revealed a half-maximal inhibition value (IC_{50}) of 4 μg/mL for heparin and approximately 200 μg/mL for dermatan sulfate and chondroitin 4-sulfate, whereas values larger than 200 μg/mL were found for the other glycosaminoglycans, supporting the notion that the ability to sustain thrombin inhibition is due to binding of the inhibitor. Partial depolymerizaton of heparin, followed by chromatographic size fractionation of different heparin species, allowed the determination of the number of monosaccharide units required for efficient promotion of both thrombin inhibition and SDS–stable complex formation between thrombin and PAI-1. Species below about 14 monosaccharide units did not promote thrombin inhibition and the formation of SDS-stable complexes. Optimal inhibition of thrombin by PAI-1 and promotion of SDS-stable complex formation were achieved with fractionated high molecular weight heparin. Finally, a dye displacement assay was used to establish difference spectra of heparin/proflavine vs proflavine in the presence of either thrombin or PAI-1. The displacement of the dye by thrombin, but not by PAI-1, indicated that the protease and the inhibitor occupy different binding sites on heparin. Our results are consistent with a template mechanism for the assembly of thrombin and PAI-1 on high molecular weight heparin.

Plasminogen activator inhibitor 1 (PAI-1) is a key regulatory protein of the fibrinolytic system [reviewed by Sprengers and Kluft (1987), Schleef and Loskutoff (1988), and Collen and Lijnen (1991)]. In vitro, PAI-1 rapidly forms equimolar, inactive complexes with the serine proteases tissue-type (t-PA) and urokinase-type (u-PA) plasminogen activator, thus preventing the generation of plasmin. Evidence for the significance of PAI-1 in the control of this process, and consequently during thrombolysis in vivo, has been recently documented in an experimental animal model where, upon inclusion of an anti-PAI-1 monoclonal antibody, increased thrombolysis and a limitation of thrombus extension were observed (Levi et al., 1992). The molecular cloning and DNA sequencing of full-length PAI-1 cDNA (Ny et al., 1986; Pannekoek et al., 1986; Ginsberg et al., 1986; Andreasen et al., 1986) have revealed a significant homology between its amino acid sequence and that of other members of the serine protease inhibitor ("serpin") family. On the basis of this observation, it has been assumed that PAI-1 has a similar structure as α_1-antitrypsin, the prototype of this family (Loebermann et al., 1984), as well as an equivalent mechanism of action. Indeed the elucidation of the three-dimensional structure of PAI-1 (Mottonen et al., 1992), which matches those of other serpins (Loebermann et al., 1984; Stein et al., 1990; Wright et al., 1990), has substantiated this assumption and provides considerable support for the inhibitory mechanism. The reactive center P1 residue (R346) is the main constituent of a serpin and ultimately forms a tight bond with the active-site serine residue of the "target" protease, resulting in an SDS-stable complex between the serpin and the protease (Travis & Salvesen, 1983; Huber & Carrell, 1989).

Inhibitory serpins can be classified into two groups of proteins, notably those that interact rapidly with their target protease(s) without additional components and those that require a cofactor, usually a glycosaminoglycan, for efficient

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0006-2960/93/0432-1675$04.00/0

inhibition. The latter group includes antithrombin III, heparin cofactor II, protease nexin I, and protein C inhibitor (Huber & Carrell, 1989). According to this arrangement, PAI-1 belongs to both groups of serpins for the following reasons. Recently, we have demonstrated that defined cofactors can endow PAI-1 with additional inhibitory properties. Specifically, in the presence of either the glycoprotein vitronectin or the glycosaminoglycan heparin, the rate of association between PAI-1 and thrombin increases approximately 200- and 100-fold, respectively (Ehrlich et al., 1990, 1991a; Keijer et al., 1991). The effect of these cofactors is restricted to the interaction between thrombin and PAI-1, since in the presence of these cofactors virtually no alteration is detected on the rate of association with other serine proteases, including the target proteases t-PA and u-PA (Keijer et al., 1991). These observations may indicate that PAI-1, in addition to its regulatory role in the fibrinolytic system, in the presence of heparin or vitronectin is involved in regulation of the coagulation cascade or, alternatively, in other processes that depend on thrombin activity such as degradation of subcellular matrices (Ehrlich et al., 1991b).

The interaction between PAI-1, thrombin, and heparin has now been studied in some detail. First, we demonstrated that PAI-1 specifically binds to heparin and that the effect of heparin on thrombin inhibition by PAI-1 is optimal at about 0.3 unit/mL (approximately 1 μg/mL), whereas at higher concentrations suboptimal inhibition is found (Ehrlich et al., 1991a). These data suggest that heparin acts as a template for the assembly of both thrombin and PAI-1, a mechanism originally proposed for the interaction between thrombin, antithrombin III, and heparin (Pomerantz & Owen, 1978; Griffith, 1982; Nesheim, 1983; Danielsson et al., 1986) and for the interaction between thrombin, heparin cofactor II, and heparin (Maimone & Tollefson, 1986). Second, we recently established the localization and composition of the high-affinity heparin binding site on PAI-1, using site-directed mutagenesis of PAI-1 cDNA and expression of functional PAI-1 mutants in transformed *Escherichia coli* cells (Ehrlich et al., 1992). The basic residues that constitute the heparin binding site, i.e., Lys^{65}, Lys^{69}, Arg^{76}, Lys^{80}, and Lys^{88}, are present in the helix D subdomain and align with the heparin binding residues that have been identified on antithrombin III (Chang, 1989) and on heparin cofactor II (Blinder & Tollefsen, 1990; Whinna et al., 1991). In addition, it can be noted that alignment of the amino acid sequence of protease nexin I also reveals a similar clustering of basic residues (Huber & Carrell, 1989). Hence, it is conceivable that an analogous mechanism is applicable for the heparin-dependent serpins antithrombin III, heparin cofactor II, protease nexin I, and PAI-1. In contrast, the mechanism of action of protein C inhibitor may be quite different, since yet another region (helix A′ subdomain) has been implicated in heparin binding (Kuhn et al., 1990).

In this paper, we investigated the structural requirements of glycosaminoglycans to efficiently sustain the inhibition of thrombin by PAI-1. Various glycosaminoglycans as well as size-fractionated heparin were assessed for their ability to support thrombin inhibition by PAI-1 and to promote the generation of SDS-stable thrombin–PAI-1 complexes. From the data, it is deduced that, at concentrations used for anticoagulant therapy, in essence only high molecular weight heparin, containing at least 14 monosaccharide units, acts as an efficient cofactor for thrombin inhibition by PAI-1. It is argued that high molecular weight heparin accommodates separate binding sites for thrombin and PAI-1. This view is further supported by the results of a dye displacement assay, measuring difference spectra between haprin/proflavine vs proflavine. Displacement of the dye from heparin is observed upon addition of thrombin, but not in the presence of PAI-1.

EXPERIMENTAL PROCEDURES

Materials. The chromogenic substrates H-D-isoleucylprolylarginyl-*p*-nitroanilide (S-2288) and H-D-phenylalanylpipecolylarginyl-*p*-nitroanilide (S-2238) were obtained from KabiVitrum (Stockholm, Sweden). Unfractionated high molecular weight heparin from porcine intestinal mucosa (H-3125; specific activity 178 units/mg; average molecular weight 15 000–18 000), dermatan sulfate from porcine skin, chondroitin 4-sulfate from bovine trachea, chondroitin 6-sulfate from shark cartillage, keratan sulfate from bovine cornea, hyaluronic acid from human umbilical cord, and proflavine hemisulfate were purchased from Sigma. Heparin–Sepharose CL-6B (derived from unfractionated high molecular weight porcine intestinal mucosa heparin) was obtained from Pharmacia—LKB (Uppsala, Sweden). Prestained high molecular weight protein standards were from Bethesda Research Laboratories.

Proteins. Purified human α-thrombin (Mertens et al., 1985), having a protein concentration of 4.5 mg/mL (Bradford, 1976), was subjected to active-site titration using *p*-guanidinobenzoate (Chase & Shaw, 1970). The latter analysis yielded a concentration of 5 mg/mL, approximating the protein determination indicated above. Thrombin, radiolabeled with ^{125}I using the Iodogen method, was shown to retain its fast rate of complex formation with antithrombin III in the presence of heparin (data not shown). Two-chain Bowes melanoma t-PA (specific activity 910 000 units/mg) was purchased from Biopool (Umea, Sweden). Recombinant PAI-1 was purified to apparent homogeneity from extracts of *Escherichia coli* K12 strain 1046, cells transformed with plasmid pMBL11/PAI as previously described (Ehrlich et al., 1992). Metabolically labeled PAI-1 was prepared from 250-mL cultures of *E. coli* K12 strain 1046, transformed with pMBL11/PAI, grown in 2 × YT medium until a density of 6×10^8 cell/mL. Subsequently, the bacteria were pelleted by centrifugation, suspended in 250 mL of M9 medium supplemented with 1 × RPMI, lacking methionine and tryptophan, and 250 μCi of [^{35}S]methionine, and grown under vigorous shaking overnight at 37 °C.

Activation of PAI-1 and Titration of Its Activity. The activation of PAI-1 with guanidine hydrochloride and the determination of the active fraction of PAI-1 with purified t-PA were done as outlined before (Ehrlich et al., 1990).

Partial Depolymerization of Heparin with Nitrous Acid. The treatment of heparin from porcine intestinal mucosa (Sigma; grade I, 177 USP units/mg) with nitrous acid, the fractionation of the digestion products by chromatography on a Bio-Gel P-10 column, and the determination of the concentration of heparin species by the carbazole assay were performed as described before (Maimone & Tollefsen, 1988).

Thrombin Inhibition by PAI-1 in the Presence of Glycosaminoglycans. Thrombin (0.3 nM) was incubated with 5 nM active PAI-1 in 30 μL containing 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 0.1% (v/v) Tween-80 (TST buffer), and increasing concentrations (0–100 μg/mL) of various glycosaminoglycans. After incubation for 1 h at 37 °C, 200 μL of a 0.6 mM sample of the thrombin-specific chromogenic substrate S-2238 was added, and the residual thrombin activity was determined from the linear increase of the optical density at 405 nm, recorded with a Titertek twinreader (Flow

Laboratories). The absorbance of a sample, due to thrombin in the absence of PAI-1, was taken as 100% and did not change in the presence of various glycosaminoglycan concentrations.

Formation of SDS-Stable Complexes of Thrombin and PAI-1 in the Presence of Glycosaminoglycans. ^{125}I-Labeled thrombin (2.5 nM) was incubated with 5 nM active PAI-1 in 30 μL of 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 0.1% (v/v) Tween-80 supplemented with an optimal concentration of a particular glycosaminoglycan, as determined by the thrombin inhibition assay outlined above. After 1 h at 37 °C, the reactions were terminated by adding 7 μL of 0.25 M Tris-HCl (pH 6.8), 40% (v/v) glycerol, 10% (w/v) SDS, and 0.05% (w/v) bromophenol blue and boiling for 2 min. The mixture was subjected to electrophoresis on a 10% (w/v) SDS–polyacrylamide gel, and radiolabeled material was visualized by autoradiography.

Competition between Solid-Phase Heparin and Soluble Glycosaminoglycans for Binding to PAI-1. Metabolically labeled ^{35}S-PAI-1 (160 ng) was incubated for 2 h at room temperature with heparin–Sepharose in 20 mM Tris-HCl (pH 7.4), 100 mM NaCl, 0.1% (v/v) Tween-80, 4 mg/mL bovine serum albumin, and increasing concentrations of various glycosaminoglycans. Incubations were carried out in Eppendorf tubes in a volume of 400 μL, and mixing was ensured by "end-over-end" rotation. Subsequently, the beads were pelleted by centrifugation and washed once with 1 mL of 20 mM Tris-HCl (pH 7.4), 100 mM NaCl, and 0.1% (v/v) Tween-80, and radioactivity was determined by liquid scintillation counting.

Dye Displacement from Heparin by Thrombin or by PAI-1. The binding of thrombin and of PAI-1 to soluble heparin was determined by the dye displacement method described (Li et al., 1970, 1974). Briefly, difference spectra of heparin/proflavine vs proflavine were obtained at room temperature, using a Cary Varian 219 spectrophotometer. To that end, both cuvettes contained 30 μM proflavine in 10 mM Tris-HCl (pH 7.5)/20 mM NaCl, and the sample cuvette contained in addition either 6 μg/mL fractionated high molecular weight heparin or fractionated low molecular weight heparin (fraction 105, 10 monosaccharide units; see Figure 3) or no heparin.

RESULTS

Inhibition of Thrombin by PAI-1 in the Presence of Various Glycosaminoglycans. We have shown previously that heparin from porcine intestinal mucosa, with an average molecular weight of 15 000–18 000, accelerates the inhibition of human α-thrombin by recombinant human PAI-1 about 100-fold (Ehrlich et al., 1991a; Keijer et al., 1991). Here, we have assessed the effect of various glycosaminoglycans on thrombin inhibition in the presence of a 15-fold molar excess of active PAI-1 over thrombin. To that end, both the residual amidolytic activity of thrombin was measured, using the specific chromogenic substrate S-2238, and the formation of serpin-typical SDS-stable complexes between radiolabeled thrombin and PAI-1 was measured. The selected glycosaminoglycans, that were tested at concentrations between 0 and 1000 μg/mL, are heparin, heparan sulfate, dermatan sulfate, chondroitin 6-sulfate, chondroitin 4-sulfate, keratan sulfate, and hyaluronic acid. The structure of these glycosaminoglycans has been outlined previously (Lindahl & Höök, 1978).

The determination of the residual thrombin activity in the presence of different concentrations of various glycosaminoglycans has been depicted in Figure 1. We confirm our previous results (Ehrlich et al., 1991a) that efficient thrombin inhibition by PAI-1 is optimally promoted by unfractionated

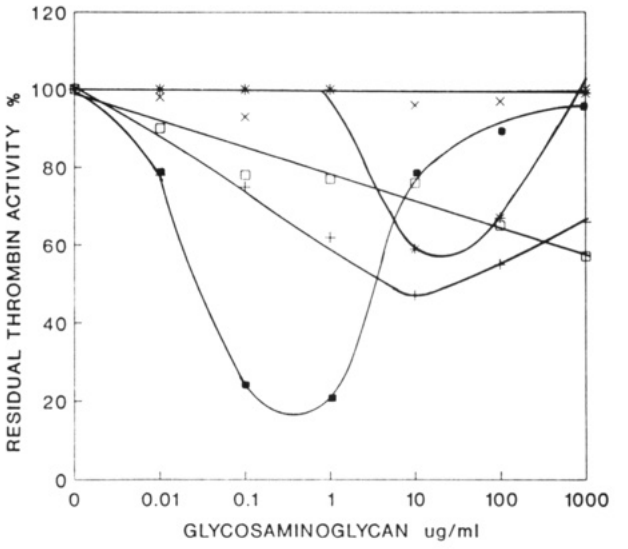

FIGURE 1: Effect of various glycosaminoglycans on the inhibition of thrombin by PAI-1. The conditions for the inhibition of 0.3 nM α-thrombin by 5 nM active PAI-1 in the presence of the indicated glycosaminoglycans have been outlined under Experimental Procedures. The amidolytic activity of thrombin, in the absence of PAI-1, remained unchanged over the indicated range of glycosaminoglycan concentrations. (■) Heparin; (+) heparan sulfate; (☆) dermatan sulfate; (□) chondroitin 4-sulfate; no effect was observed for (×) keratan sulfate, chondroitin 6-sulfate, and hyaluronic acid.

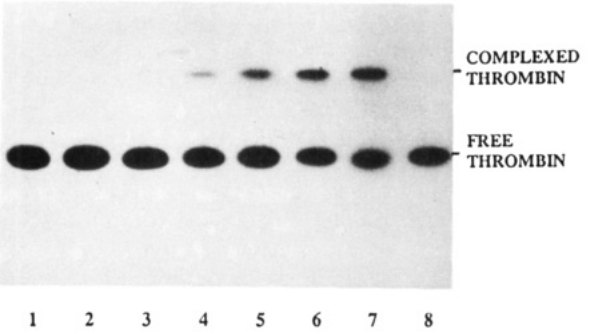

FIGURE 2: Analysis of the generation of SDS-stable complexes between thrombin and PAI-1. The reactions between 2.5 nM ^{125}I-labeled thrombin and 5 nM active PAI-1, in the presence of various glycosaminoglycans, are described under Experimental Procedures. The analysis was done by electrophoresis on 10% (w/v) SDS–polyacrylamide gels, followed by autoradiography. Lane 1, 10 μg/mL chondroitin 6-sulfate; lane 2, 10 μg/mL hyaluronic acid; lane 3, 10 μg/mL keratan sulfate; lane 4, 10 μg/mL heparan sulfate; lane 5, 10 μg/mL dermatan sulfate; lane 6, 100 μg/mL chondroitin 4-sulfate; lane 7, 0.1 μg/mL heparin; lane 8, no glycosaminoglycan.

high molecular weight heparin at concentrations between 0.1 and 1 μg/mL. Inhibition of thrombin by PAI-1 was obtained with dermatan sulfate, heparan sulfate, and chondroitin 4-sulfate at concentrations that are at least 1 order of magnitude higher than that required for maximal thrombin inhibition in the presence of heparin. It should be noted, however, that the extent of thrombin inhibition in the presence of dermatan sulfate did not reach the optimal level of inhibition obtained with heparin. Clearly, none of the other glycosaminoglycans supported thrombin inhibition by PAI-1.

We employed SDS–polyacrylamide gel electrophoresis, followed by autoradiography, to qualitatively assess the equimolar complex formation between ^{125}I-labeled thrombin and PAI-1 in the presence of various glycosaminoglycans (Figure 2). Efficient complex formation was obtained with 0.1 μg/mL heparin, whereas at this concentration virtually no complex formation occurs with the other glycosaminoglycans. However, by using substantially higher concentrations,

Table I: Competition between Solid-Phase Heparin and Various Soluble Glycosaminoglycans for Binding to PAI-1[a]

glycosaminoglycan	IC_{50} (μg/mL)
heparin	4
chondroitin 4-sulfate	200
dermatan sulfate	200
keratan sulfate	300
chondroitin 6-sulfate	>500
hyaluronic acid	>500
heparan sulfate	>500

[a] The data are expressed as half-maximal inhibition values (IC_{50}) for the binding of metabolically, ^{35}S-labeled PAI-1 to heparin bound to Sepharose beads. The procedure is given under Experimental Procedures.

in some cases complex formation was observed. Notably, complexes were formed with 100 μg/mL chondroitin 4-sulfate, with 10 μg/mL dermatan sulfate, and with 10 μg/mL heparan sulfate, whereas no complexes were seen with high concentrations of chondroitin 6-sulfate, hyaluronic acid, and keratan sulfate.

Binding of PAI-1 to Various Glycosaminoglycans. A competition assay was used to determine the capacity of the various glycosaminoglycans to bind to PAI-1 (Ehrlich et al., 1991a). For that purpose, we monitored the binding of metabolically labeled ^{35}S-PAI-1 to heparin–Sepharose in the presence of increasing amounts of the different glycosaminoglycans. The data are expressed as half-maximal inhibition values (IC_{50}) for the binding of PAI-1 to solid-phase heparin (Table I). An average IC_{50} value of 4 μg/mL was calculated for heparin, whereas for dermatan sulfate and chondroitin 4-sulfate a value of approximately 200 μg/mL was deduced. Concentrations exceeding 200 μg/mL were required for the other glycosaminoglycans to half-maximally prevent the binding of PAI-1 to heparin–Sepharose. We conclude that, except for heparin, the glycosaminoglycans are unable to support the inhibition of thrombin by PAI-1 at low concentrations, due to a lack of binding to PAI-1.

Inhibition of Thrombin by PAI-1 in the Presence of Heparin Species of a Defined Size. To gain insight into the contribution of heparin in the ternary reaction with thrombin and PAI-1, and in particular whether it performs a template function for the assembly of both components, we partially depolymerized heparin using nitrous acid (Maimone & Tollefsen, 1988). Nitrous acid cleaves heparin at the α1–4 glycosidic linkages between N-sulfated D-glucosamine and uronic acid, resulting in fragments with an even number of monosaccharide units. The partial digest was fractionated on a Bio-Gel P-10 filtration column, and distinct peaks were observed for digestion products spanning between 2 and 14 monosaccharide units (Figure 3). Various concentrations of several selected fractions were tested for their ability to sustain the inhibition of thrombin by PAI-1. Residual amidolytic activity at a 15-fold molar excess of active PAI-1 over thrombin is plotted against different concentrations of the heparin species (Figure 4). In terms of extent of inhibition, fractionated high molecular weight heparin (fraction 58) clearly acts as the most effective cofactor for PAI-1. A gradually lower optimal inhibition, at similar concentrations, is observed when heparin fractions are used of a smaller size. Low molecular weight oligosaccharides (e.g., fractions 98, 105, and 113), having a size below about 14 monosaccharides units, do not support the inhibition of thrombin by PAI-1. It is conceivable that the lack of low molecular weight heparins to promote inhibition of thrombin by PAI-1 is due to inability of PAI-1 to bind to these species. This is supported by results of the competition assay (done as outlined in Table I) between metabolically labeled ^{35}S-PAI-1

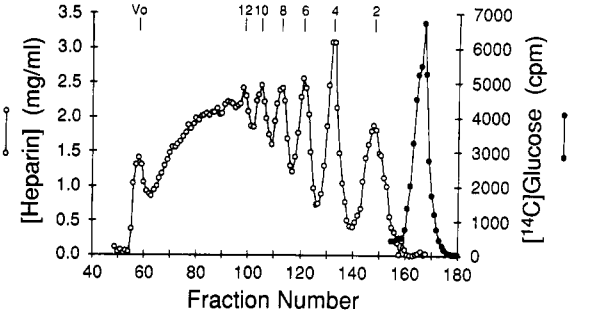

FIGURE 3: Fractionation of heparin treated with nitrous acid by chromatography on Bio-Gel P-10. Partial depolymerization and fractionation were carried out as described previously (Maimone & Tollefsen, 1988). [^{14}C]Glucose was used as the monosaccharide marker (●). The number of monosaccharide units per oligosaccharide is indicated above each peak. V_0, void volume. The concentration of different heparin species, indicated by (○), was determined by the carbazole assay for uronic acid, standardized with known amounts of unfractionated heparin (Maimone & Tollefsen, 1988).

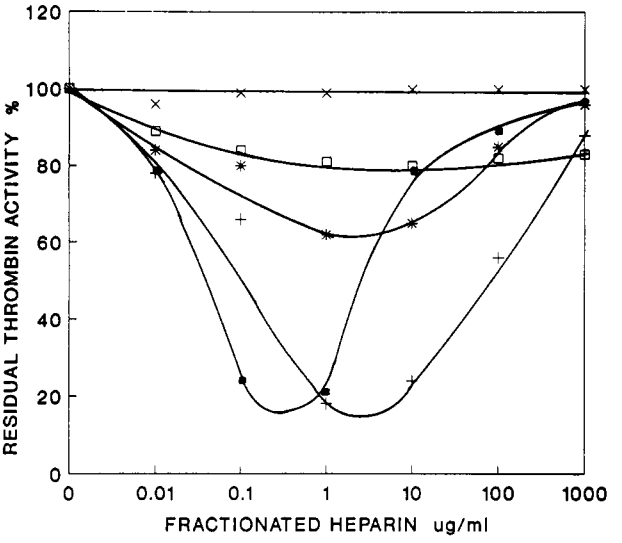

FIGURE 4: Effect of the size of heparin on the inhibition of thrombin by PAI-1. The conditions for inhibition of thrombin by PAI-1 in the presence of various species of size-fractionated heparin are outlined under Experimental Procedures. The fractions that were used are derived from the experiment presented in Figure 3. Fraction 113 corresponds with 8 monosaccharide units, fraction 105 with 10, and fraction 98 with 12 whereas fractions 80, 69, and 58 contain increasing numbers of monosaccharide units. (■) Unfractionated heprin; (+) fraction 58; (☆) fraction 69; (□) fraction 80; no effect was observed with fraction 98; (×) fractions 105 and 113.

and either unfractionated heparin or fraction 105 (10 monosaccharide units) for binding to heparin–Sepharose. While an IC_{50} value of 4 μg/mL was determined for unfractionated heparin, an about 2 orders of magnitude higher concentration of fraction 105 was required for half-maximal inhibition of binding (data not shown).

Our observations on the formation of SDS-stable complexes between ^{125}I-labeled thrombin and PAI-1, in the presence of defined low molecular weight oligosaccharides and of high molecular weight heparin, parallel the data reported above (Figure 5). Fractionated high molecular weight heparin, at concentrations between 0.1 and 1 μg/mL, acts as an efficient catalyst for the formation of SDS-stable complexes, whereas heparin species of a lower molecular weight are less effective.

Thrombin and PAI-1 Occupy Separate Binding Sites on High Molecular Weight Heparin. We employed a dye displacement method to investigate whether thrombin and PAI-1 have different binding sites on high molecular weight

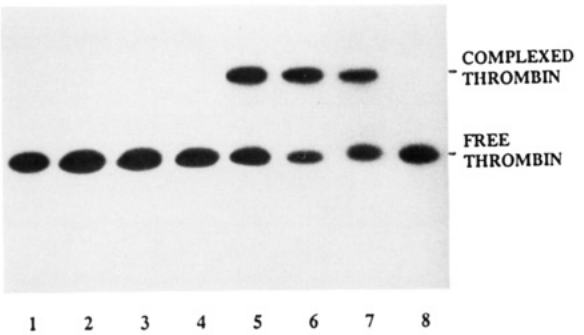

FIGURE 5: Analysis of the generation of SDS-stable complexes between thrombin and PAI-1 in the presence of size-fractionated heparin. The reaction between 2.5 nM ^{125}I-labeled thrombin and 5 nM active PAI-1 in the presence of glycosaminoglycans has been described under Experimental Procedures as has been the protocol for gel electrophoresis and autoradiography. The following concentrations were used: lane 1, 1 μg/mL fraction 113; lane 2, 1 μg/mL fraction 105; lane 3, 1 μg/mL fraction 98; lane 4, 1 μg/mL fraction 80; lane 5, 1 μg/mL fraction 69; lane 6, 1 μg/mL fraction 58; lane 7, 0.1 μg/mL unfractionated heparin; lane 8, no glycosaminoglycan.

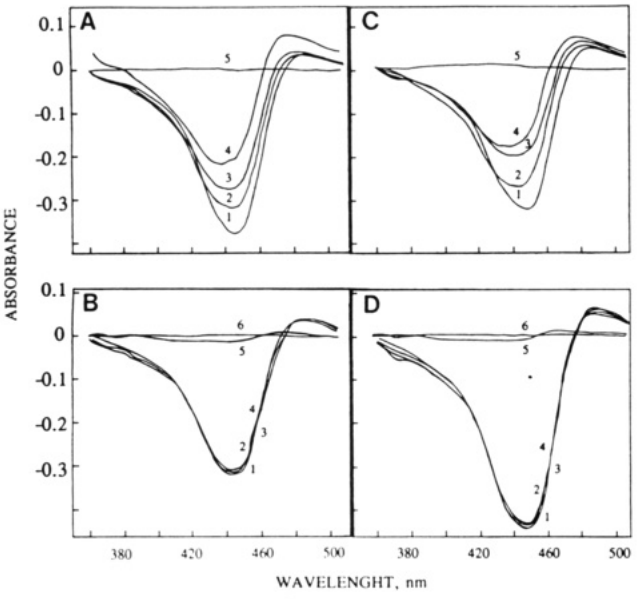

FIGURE 6: Dye displacement from heparin by thrombin or by PAI-1. The determination of difference spectra between heparin/proflavine vs proflavine was done as outlined under Experimental Procedures. (Panel A) 6 μg/mL fractionated high molecular weight heparin: 1, no thrombin; 2, 5 μg/mL thrombin; 3, 10 μg/mL thrombin; 4, 20 μg/mL thrombin; 5, baseline. (Panel B) 6 μg/mL high molecular weight heparin: 1, no PAI-1; 2, 2.5 μg/mL activated PAI-1; 3, 10 μg/mL activated PAI-1; 4, 20 μg/mL activated PAI-1; 5, 20 μg/mL activated PAI-1 without heparin; 6, base line. (Panel C) 6 μg/mL low molecular weight heparin (fraction 105; see Figure 3): 1, no thrombin; 2, 5 μg/mL thrombin; 3, 10 μg/mL thrombin, 4, 20 μg/mL thrombin; 5, base line. (Panel D) 6 μg/mL low molecular weight heparin (fraction 105): 1, no PAI-1; 2, 2.5 μg/mL activated PAI-1; 3, 10 μg/mL activated PAI-1; 4, 20 μg/mL activated PAI-1; 5, 20 μg/mL activated PAI-1 without heparin; 6, base line.

heparin. This method has been extensively used to study the interaction between heparin, thrombin, and heparin cofactor II (Li et al., 1970, 1974; Pomerantz & Owen, 1978). Here, difference spectra were recorded either between fractionated high molecular weight heparin/proflavine vs proflavine or between low molecular weight heparin/proflavine vs proflavine, in the presence of increasing amounts of thrombin or of PAI-1. The data are given in Figure 6. Addition of thrombin to the high molecular weight heparin/proflavine mixture causes a dose-dependent shift in the difference spectrum at 447 nm (Figure 6A). A smaller alteration is observed at 470 nm due to binding of the dye to the active site of thrombin (Li et al., 1974). In contrast, no spectral shift is measured upon addition of various concentrations of PAI-1 to heparin/proflavine (Figure 6B). Moreover, since no alterations are detected in the absence of heparin, it can be deduced that PAI-1 does not bind proflavine. Apparently, thrombin can bind to low molecular weight heparin, since in its presence the dye is displaced from heparin species consisting of only 10 monosaccharide units (Figure 6C). Finally, since PAI-1 does not bind to low molecular weight heparin (see Figure 4) and because no effect was seen on the binding of proflavine to high molecular weight heparin, it had been anticipated that PAI-1 also does not displace the dye from low molecular weight heparin (Figure 6D).

DISCUSSION

This study represents an extension of our previous finding that PAI-1 is endowed with thrombin-inhibitory properties in the presence of unfractionated high molecular weight heparin (Ehrlich et al., 1991a). Here, it is shown that at concentrations between 0.1 and 1 μg/mL, essentially none of the glycosaminoglycans tested could perform this function, besides high molecular weight heparin. Our initial observation that thrombin inhibition is maximal at about 1 μg/mL heparin and decreases at concentrations exceeding about 10 μg/mL had been indicative for a template mechanism (Ehrlich et al., 1991a). Such a mechanism suggests that the protease and the inhibitor both bind to separate sites on heparin, an event that subsequently results in an increased rate of association and inactivation of the protease. This hypothesis has now received support, since fractionated high molecular weight heparin efficiently promotes thrombin inhibition by PAI-1, whereas heparin species below about 14 monosaccharide units do not support this interaction. Moreover, our data on the displacement of a high molecular weight heparin-bound dye by thrombin, but clearly not by PAI-1, are indicative for distinct binding sites of the protease and the inhibitor. An alternative explanation of these experiments is that thrombin and PAI-1 share the same binding site that is not revealed due to a lower binding affinity of PAI-1 to heparin than proflavine. However, on the basis of chromatographic data, demonstrating that PAI-1 binds more tightly to heparin than thrombin (Ehrlich et al., 1991; Gitel, 1975), we discard this possibility and conclude that the data are consistent with a template function of high molecular weight heparin. In addition to its function as a template (Pomerantz & Owen, 1978; Griffith, 1983; Nesheim, 1984; Danielsson et al., 1986), it has been proposed that the interaction of heparin with antithrombin III results in a conformational change that favors the interaction with thrombin (Rosenberg & Damus, 1973; Olson et al., 1981; Chang, 1989). In view of the data presented here, it cannot be decided whether binding of PAI-1 to heparin also causes a conformational change of the inhibitor that might be essential for the interaction with thrombin. However, the option that heparin might "activate" PAI-1 toward thrombin seems unlikely, since we showed previously that heparin cannot activate PAI-1 toward t-PA (Lambers et al., 1987).

Heparin is extensively used as an anticoagulant for the management of thromboembolic disorders in man. Its effect is mainly attributed to an enhancement of the inhibition of thrombin and other serine proteases of the coagulation cascade by antithrombin III (Rosenberg & Damus, 1973). Furthermore, heparin-like molecules have been shown to be synthesized by vascular endothelial cells and contribute to the nonthrombogenic nature of the vessel wall (de Agostini et al., 1990).

Recently, it has been shown that heparin also has a profibrinolytic effect by enhancing systemic (fibrin-independent) t-PA-mediated plasminogen activation (Stein et al., 1989). The observation that heparin actually competes with fibrin for binding to t-PA may be relevant for the rare occurrence of bleeding episodes during thrombolytic therapy in conjunction with heparin. Finally, in view of heparin-mediated thrombin inhibition by PAI-1, the data presented here can be interpreted as an anticoagulant effect of PAI-1, although it remains to be established whether these events occur in the circulation or at sites that contain a high concentration of PAI-1, such as the platelet-rich thrombus (Erickson et al., 1984; Levi et al., 1992). At any rate, we recently demonstrated that thrombin activity is rapidly neutralized by PAI-1 associated with vibronectin in the subcellular matrix of cultured vascular endothelial cells (Ehrlich et al., 1991b). Preincubation of matrices with a polyclonal anti-vitronectin antiserum partially abolished thrombin–PAI-1 complex formation. It is possible that the presence of heparin in the subendothelial matrix may be responsible for any remaining thrombin inhibition by PAI-1 in the absence of vitronectin.

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