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Functional Interaction of Plasminogen Activator Inhibitor Type 1 (PAI-1) and Heparin

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ABSTRACT: Plasminogen activator inhibitor type 1 (PAI-1), the fast-acting inhibitor of tissue-type plasminogen activator (t-PA) and urokinase (u-PA), is a member of the serpin superfamily of proteins. Both in plasma and in the growth substratum of cultured endothelial cells, PAI-1 is associated with its binding protein vitronectin, resulting in a stabilization of active PAI-1. Recently, it has been demonstrated that the PAI-1-binding site on vitronectin is adjacent to a heparin-binding site (Preissner et al., 1990). Furthermore, it can be deduced that the amino acid residues, proposed to mediate heparin binding in the serpins antithrombin III and heparin cofactor II, are conserved in PAI-1. Consequently, here we have investigated whether PAI-1 also interacts with heparin. At pH 7.4, PAI-1 quantitatively binds to heparin–Sepharose and can be eluted with increasing [NaCl]. Binding of PAI-1 to heparin–Sepharose can be efficiently competed with heparin in solution (IC_{50}, 7 μM). In the presence of heparin, the protease specificity of PAI-1 toward thrombin is substantially increased. This is shown by (i) quenching of thrombin activity of PAI-1 in the presence of heparin and (ii) induction of the formation of SDS-stable complexes between thrombin and PAI-1 by heparin. In a dose response curve, both effects reached a maximum at approximately 1 unit/mL and then diminished again upon further increasing the heparin concentration, strongly suggesting a template mechanism as an explanation for the observed effect. In contrast to vitronectin, heparin does not stabilize the active conformation of PAI-1. We propose that PAI-1, like antithrombin III, heparin cofactor II, and protease nexin 1, belongs to the group of heparin-dependent serpins. The binding of PAI-1 to heparin suggests that heparin may contribute to the localization of PAI-1 at particular sites, thus being involved in the regulation of plasminogen activation. Furthermore, we provide evidence that heparin has the potential to locally enhance plasminogen activation by catalyzing the thrombin-induced neutralization of PAI-1.

Serpins comprise a family of over 40 highly homologous proteins, most of which function as specific inhibitors of selected target serine proteases by forming a tight, equimolar, inactive complex (Travis & Salvesen, 1983; Huber & Carrell, 1989). The interaction between a serpin and a target protease is characterized by a fast association rate ($>10^5$ M^{-1} s^{-1}) and an extremely slow dissociation rate ($<10^{-5}$ s^{-1}). Protease specificity of a serpin is mainly determined by the amino acid sequence of the carboxyl terminally located reactive center, which functions as an exposed "bait" for the protease by mimicking a putative cleavage site (Travis & Salvesen, 1983; Carrell & Boswell, 1986).

Additional factors may contribute toward target protease specificity, since some serpins were shown to have dramatically increased association rates with certain proteases in the presence of sulfated glycosaminoglycans such as heparin. Heparin has been used for many years as a potent anticoagulant for the treatment of thromboembolic disorders in man. Its anticoagulant effect has been attributed to the acceleration of inhibition of thrombin and other proteases of the coagulation cascade by the serpin antithrombin III (Rosenberg & Damus,

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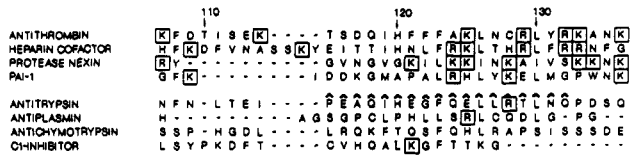

FIGURE 1: Alignment of amino acid sequences of the putative heparin-binding site around the proposed helix D of the human serpins antithrombin III (ANTITHROMBIN), heparin cofactor II (HEPARIN COFACTOR), protease nexin (PROTEASE NEXIN), PAI-1 (PAI-1), α_1-antitrypsin (ANTITRYPSIN), α_2-antiplasmin (ANTIPLASMIN), α_1-antichymotrypsin (ANTICHYMOTRYPSIN), and C1-esterase inhibitor (C1-INHIBITOR), as described (Huber & Carrell, 1989). Amino acid residues are presented in the one-letter code. The numbering refers to mature antithrombin III. In the serpins for which an interaction with heparin has been described, i.e., antithrombin III, heparin cofactor II, and protease nexin, all arginine and lysine residues are boxed in this area. In PAI-1, α_1-antitrypsin, α_2-antiplasmin, α_1-antichymotrypsin, and C1-esterase inhibitor, only arginines and lysines that are conserved in comparison with the heparin-dependent serpins are boxed. A circumflex indicates amino acid residues in α_1-antitrypsin which were proposed to comprise the helix D subdomain upon analysis of the crystallized, cleaved form of the inhibitor (Loebermann et al., 1984).

1973). In addition to antithrombin III, the serpins heparin cofactor II (Tollefsen & Blank, 1981), protease nexin I (Baker et al., 1980), and protein C inhibitor (Suzuki et al., 1984) were shown to exhibit greatly accelerated reactivities with certain serine proteases in the presence of heparin or heparin-like molecules. Under normal physiological conditions, heparin does not circulate in blood. Heparin has, however, been described as an integral component of the luminal surface of vascular endothelium (Marcum & Rosenberg, 1984). This localized presence of heparin appears to contribute to the nonthrombogenic properties of the vessel wall.

Several positively charged amino acid residues in and around the proposed helix D subdomain of human antithrombin III have been postulated to compose the heparin-binding domain (Chang, 1988; Huber & Carrell, 1989). An alignment of this region with other serpins shows that these residues are conserved in the other heparin-binding inhibitors heparin cofactor II and protease nexin I (Figure 1). In contrast, these residues are not encountered in the serpins α_1-antitrypsin, α_1-antichymotrypsin, α_2-antiplasmin, and C1-esterase inhibitor, for which an interaction with heparin has not been described. Most notably, however, these amino acid residues are also partially conserved in PAI-1, the major protein involved in the regulation of plasminogen activation [see recent reviews by Sprengers and Kluft (1987) and Schleef and Loskutoff (1988)]. PAI-1 inhibits t-PA and u-PA by forming serpin-typical, SDS-resistant, inactive complexes with the proteases. The extremely fast association between PAI-1 and these plasminogen activators is not dependent on the presence of effector molecules such as heparin (Chmielewska et al., 1988) or vitronectin (Declerck et al., 1988). The latter protein has recently been identified as the PAI-1-binding protein from plasma (Declerck et al., 1988; Wiman et al., 1988) and growth substratum of endothelial cells in culture (Mimuro & Loskutoff, 1989), that stabilizes the activity of PAI-1 in vitro (Mimuro et al., 1987; Declerck et al., 1988). Furthermore, we found that vitronectin endows PAI-1 with additional target-protease specificity (Ehrlich et al., 1990).

As stated before, crucial positively charged amino acid residues for binding of serpins to heparin are also encountered in the PAI-1 protein. Significantly, it has recently been shown that PAI-1 and heparin bind to adjacent sites in the highly positively charged glycosaminoglycan-binding domain of vitronectin (Preissner et al., 1990). Subsequently, we investigated whether PAI-1 and heparin could also interact with each other. This putative interaction and the consequences arising from it could be of physiological relevance with respect to the effect of heparin on the localization and function of PAI-1. In this report, we provide evidence that PAI-1 reacts with heparin, as shown by the ability of the inhibitor to bind to immobilized heparin. The interaction of PAI-1 and heparin results in an increase in the target-protease specificity of the inhibitor toward thrombin. It is suggested that PAI-1 belongs to the group of heparin-dependent serpins and that heparin might be involved in the regulation of plasminogen activation by binding, thus localizing the major inhibitor of plasminogen activators. Finally, heparin may contribute toward the mutual neutralization of both PAI-1 and thrombin.

Experimental Procedures

Materials. Restriction endonucleases were obtained from New England Biolabs or from Bethesda Research Laboratories. T4-polynucleotide kinase was from Pharmacia (Uppsala, Sweden) and the Klenow fragment of DNA polymerase I from Boehringer Mannheim Biochemicals (Mannheim, FRG). The synthetic tripeptide substrates H-D-isoleucylprolylarginyl-*p*-nitroanilide (S2288) and H-D-phenylalanylpipecolylarginyl-*p*-nitroanilide (S2238) were purchased from KabiVitrum (Stockholm, Sweden) and Q-Sepharose Fast Flow and heparin–Sepharose from Pharmacia (Uppsala, Sweden). Plasmid pMBL11, carrying the promoter–operator of the *Escherichia coli* tryptophan operon and the gene for ampicillin resistance, was the generous gift of Dr. B. E. Enger-Valk (Medical Biological Laboratory TNO, Rijswijk, The Netherlands). *Escherichia coli* K12 strain 1046 (recA$^-$) was obtained from the Phabagen Collection (State University of Utrecht, The Netherlands). Heparin from porcine intestinal mucosa (H-3125, lot 29F-0314), having a stated specific activity of 178 units/mg and an average molecular weight of 15 000–18 000, and low molecular weight heparin (H-5640, lot 128F-0683), having an average molecular weight of 4000–6000, were purchased from Sigma. Prestained protein high molecular weight standards were from Bethesda Research Laboratories.

Proteins. Two-chain t-PA (specific activity 910 000 IU/mg), purified from conditioned media of Bowes melanoma cells, was purchased from Biopool (Umea, Sweden). Purified human α-thrombin, having a protein concentration of 4.5 mg/mL as determined by the Bradford procedure (Bradford, 1976), was kindly donated by Dr. K. Mertens (Department of Blood Coagulation, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands). Active-site titration using *p'*-guanidinobenzoate (Chase & Shaw, 1970) resulted in a concentration of 5 mg/mL: this value, in good agreement with the protein concentration, was used throughout the experiments. Thrombin was radiolabeled with ^{125}I by utilizing the Iodogen procedure. The radiolabeled thrombin was shown to retain its fast rate of complex formation with antithrombin III in the presence of heparin (data not shown).

The murine and anti-human PAI-1 monoclonal antibody 1C3 was obtained by using standard hybridoma techniques as described (Lambers et al., 1988), and the murine anti-human PAI-1 monoclonal antibody MAI-13, coupled to Sepharose, was purchased from Biopool (Umea, Sweden).

Endothelial Cell Culture and Metabolic Labeling. Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical cords and cultured as described (Jaffe et al., 1973; Willems et al., 1982). Conditioned medium was prepared by incubating confluent cells for 24 h with serum-free medium

containing 0.03% (w/v) human serum albumin. Metabolic labeling of proteins de novo synthesized by HUVECs was carried out by incubating the cells for 48 h with serum-free media containing [^{35}S]methionine as described (van Mourik et al., 1985).

Purification of Endothelial PAI-1. PAI-1 was purified from conditioned media of labeled or unlabeled endothelial cells by a one-step immunoaffinity purification procedure (Wagner et al., 1989), utilizing the murine monoclonal antibody 1C3 which predominantly recognizes the latent form of PAI-1 (Lambers et al., 1988). The purity of the PAI-1 preparations was assessed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE), followed either by staining with Coomassie brilliant Blue (unlabeled PAI-1) or by fluorography (metabolically labeled PAI-1). A single band of the expected molecular weight (47 000) was visualized in each case. The purified proteins were further identified as PAI-1 by reverse-fibrin autography (Erickson et al., 1984), revealing an opaque zone of lysis resistance comigrating with the purified protein.

Purified PAI-1 antigen was measured by using an immunoradiometric assay as described (Ehrlich et al., 1990). The specific radioactivity of the purified ^{35}S-labeled PAI-1 was determined to be 300 cpm/ng.

Expression of Recombinant PAI-1 in Escherichia coli and Purification of the Gene Product. The construction of a plasmid suitable for expression of biologically functional recombinant PAI-1 in transformed *Escherichia coli* has been described (Ehrlich et al., 1990). Briefly, full-length human PAI-1 cDNA (Pannekoek et al., 1986) was inserted into vector pMBL11, containing the tryptophan promoter/operator, designed for expression of recombinant proteins in *Escherichia coli* under conditions of derepression. The resulting plasmid, denoted pMBL11/PAI-1, enables the synthesis of a PAI-1 protein with an amino-terminal extension of the following amino acid residues: Met-Gln-Thr-Gln-Lys-Pro-Thr-Arg-Asp-Leu, fused to the second residue (His) of mature PAI-1. Expression of the gene product in *Escherichia coli* K12 strain 1046 was carried out as described (Broekhuizen et al., 1986). The recombinant protein was purified from *Escherichia coli* lysates to apparent homogeneity by ammonium sulfate precipitation (precipitate of 25–45% saturation), chromatography on Q-Sepharose Fast Flow, and immunoaffinity chromatography, using Sepharose-coupled murine monoclonal antibody MAI-13, which is directed against human PAI-1, as described previously (Ehrlich et al., 1990).

Activation of PAI-1 and Titration of PAI-1 Activity. PAI-1 (20–40 μg/mL) was activated with 4 M guanidine hydrochloride (2 h at 20 °C), followed by extensive dialysis against 20 mM Tris-HCl (pH 8.0), containing 100 mM NaCl and 0.1% (v/v) Tween 80 (TST buffer). The concentration of PAI-1 was then adjusted to 10 μg/mL with TST buffer. Increasing amounts (2.5–50 ng) of activated PAI-1 were incubated in microtiter wells for 30 min at 20 °C with 5 ng of two-chain t-PA in a total volume of 50 μL. Residual t-PA activity was recorded in a Titertrek twinreader (Flow Laboratories) after the addition of 200 μL of 0.6 mM S2288 and calculated from a linear plot of the increase of absorbance at 405 nm over time. Linear ($r < 0.99$) dose responses were obtained in a plot of PAI-1 concentration (antigen) versus residual t-PA activity, facilitating the calculation of PAI-1 activity from the intercept with the x axis.

Binding of Purified PAI-1 to Heparin–Sepharose. Five micrograms of nonactivated, ^{35}S-labeled, purified PAI-1 from conditioned media of HUVECs was incubated in a total volume of 1 mL of TT buffer [20 mM Tris-HCl (pH 7.4) and 0.1% (v/v) Tween 80] with 50 μL of packed beads of heparin–Sepharose by end-over-end rotation at 4 °C. After 2 h, the incubation was terminated by centrifugation and careful removal of the supernatant. Subsequently, the beads were washed twice with 1 mL of TT buffer, and PAI-1 was eluted in 500-μL fractions with TST buffer, containing concentrations of NaCl increasing from 100 mM to 1 M (in steps of 100 mM). The content of radioactivity in the eluted fractions was quantified by using a liquid scintillation counter.

The binding of guanidine hydrochloride activated PAI-1 was studied in an identical fashion. After activation (2 h at 20 °C), PAI-1 was dialyzed overnight against TT buffer and then incubated with heparin–Sepharose as described above.

Effect of Soluble Glycosaminoglycans on the Binding of PAI-1 to Heparin–Sepharose. Five nanograms of ^{35}S-labeled PAI-1, purified from conditioned media of cultured HUVECs, was incubated with heparin–Sepharose (diluted 1:400) in TST buffer (pH 7.4), containing 4 mg/mL ovalbumin and increasing concentrations of heparin or low molecular weight heparin. Incubations were carried out at 20 °C in a total volume of 375 μL, by rotating the mixtures end-over-end in Eppendorf tubes. Binding reached equilibrium within 2 h, at which time the tubes were briefly centrifuged. More than 90% of the respective supernatants were removed; the pellets were washed once with 1 mL of TST buffer (pH 7.4) and then transferred to glass vials for liquid scintillation counting.

The apparent dissociation constant for the interaction between ^{35}S-labeled PAI-1 and heparin was derived from the IC_{50} value, i.e., the concentration of solution-phase heparin that caused reduction of binding by 50%.

Inhibition of Thrombin by PAI-1. Recombinant PAI-1, activated and titrated as described above, was incubated with α-thrombin in the presence of increasing amounts of heparin. The incubations were carried out in a total volume of 50 μL of TST buffer for 1 h at 37 °C using the following concentrations: active PAI-1, 5 nM; α-thrombin, 0.15 nM; heparin, 0.0003–30 units/mL. Experiments in the absence of heparin served as control and did not reveal any detectable inhibition of thrombin. Incubations were terminated by the addition of 200 μL of 0.6 mM S2238, and the residual thrombin activity was determined from a linear plot of the increase of absorbance at 405 nm versus time. Kinetic analysis of thrombin inhibition by PAI-1 in the presence of heparin was studied as follows. Thrombin (2.5 nM) was incubated at 37 °C in TST buffer with 1 unit/mL heparin and 10 mM active PAI-1. The reaction was allowed to proceed for 4–12 min, at which time it was terminated by diluting the mixture 30-fold with TST buffer containing 0.6 mM S2238. Residual thrombin activity was determined from the initial increase of optical density at 405 nm, recorded in a Cary 219 spectrophotometer. The second-order association rate constant (k_1) was calculated by applying a pseudo-first-order model as well as a standard equation for a second-order reaction as described (Ehrlich et al., 1990), both resulting in virtually identical values.

Formation of SDS-Resistant Complexes between PAI-1 and Thrombin. ^{125}I-Labeled thrombin (2.5 nM) was incubated in a total volume of 20 μL in TST buffer with recombinant PAI-1 (7 nM) in the presence of increasing concentrations of heparin. After 1 h at 37 °C, 5 μL of sample buffer [0.25 M Tris-HCl (pH 6.8), 10% (w/v) SDS, 40% (v/v) glycerol, 0.05% (w/v) bromophenol blue, and 200 mM dithiothreitol] was added. The samples were boiled for 2 min, followed by electrophoresis on a 10% (w/v) SDS–polyacrylamide gel (Laemmli, 1970). Visualization of the position of the ^{125}I-radiolabeled material was achieved by autoradiography, and

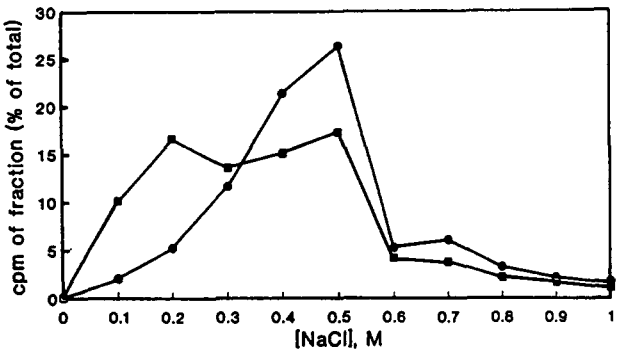

FIGURE 2: Elution of ^{35}S-labeled PAI-1 from heparin–Sepharose. PAI-1, bound to heparin–Sepharose, was eluted by a stepwise increase of the NaCl concentration as described under Experimental Procedures. (●) Nonactivated PAI-1 (■) activated PAI-1. Three experiments gave similar results.

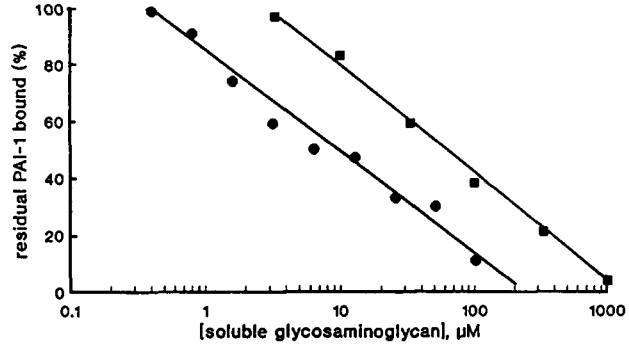

FIGURE 3: Competition with serial dilutions of soluble heparin and low molecular weight heparin for binding of ^{35}S-labeled PAI-1 to heparin–Sepharose. Five nanograms of ^{35}S-labeled PAI-1 was incubated (in triplicate) at 20 °C with heparin–Sepharose (diluted 1:400) in TST buffer (pH 7.4), containing 4 mg/mL ovalbumin and increasing concentrations of heparin (●) or low molecular weight heparin (■) as indicated. After 2 h, the heparin–Sepharose was washed and analyzed for the amount of PAI-1 bound. Three experiments gave similar results.

the positions were compared with those of prestained molecular weight markers.

Determination of the Half-Life of Active PAI-1. Recombinant PAI-1, activated and titrated against t-PA as described above, was incubated at 37 °C in the absence and presence of heparin (1.0 unit/mL) or arginine (100 mM). The concentration of active PAI-1 in TST buffer was 20 nM. At indicated times, aliquots were withdrawn and immediately chilled on melting ice. PAI-1 activity in each aliquot was then calibrated by adding increasing amounts of the respective sample of PAI-1 to 5 ng of t-PA, present in microtiter wells in a total volume of 50 μL. After an incubation for 30 min at 20 °C, 200 μL of 0.6 mM S2288 was added, and residual t-PA activity was determined from the linear plot of the increase of absorbance at 405 nm over time as described above.

Neutralization of PAI-1 by Thrombin. Active PAI-1 and thrombin were incubated at equimolar concentrations (18 nM) in a total volume of 110 μL in the presence or absence of heparin (1 unit/mL). Control experiments were carried out in the absence of thrombin. After 15 min at 37 °C, the activity of thrombin was quenched by the addition of hirudin (final concentration, 50 units/mL), resulting in inhibition of more than 99% of the amidolytic activity of thrombin. Subsequently, the residual PAI-1 inhibitory activity was determined by titration of aliquots (2–12 μL) on t-PA (50 μL; 1.5 nM), using S2288 as described above. Since the amidolytic activity of thrombin on S2288 was not completely blocked by hirudin, the increase of the optical density at 405 nm in the samples containing both t-PA and (hirudin-inactivated) thrombin was corrected for the values measured with (hirudin-inactivated) thrombin alone.

RESULTS

Binding of Purified PAI-1 to Heparin–Sepharose. Five micrograms of metabolically labeled PAI-1, purified from conditioned media of cultured HUVECs, was incubated in a total volume of 1 mL for 4 h at 4 °C with 50 μL of packed beads of heparin–Sepharose. For these experiments, we separately employed both latent and activated ^{35}S-PAI-1. Under these conditions, the majority of PAI-1, i.e., 98% of the nonactivated PAI-1 and 88% of the guanidine hydrochloride activated PAI-1, bound to the heparin–Sepharose. The elution profiles, in response to increasing concentrations of NaCl, are depicted in Figure 2. Nonactivated PAI-1 is eluted as a single peak, with the maximum of PAI-1 desorbing from the heparin–Sepharose between 400 and 500 mM NaCl. Approximately 90% of the radioactive material was recovered in the elution, while about 10% of PAI-1 was still bound to the resin after the last elution step (1 M NaCl). Compared with nonactivated PAI-1, part of the guanidine hydrochloride activated PAI-1 desorbed already at lower concentrations of NaCl from the heparin–Sepharose, with most of the material eluting in a broad peak between 100 and 500 mM NaCl. Apparently, guanidine hydrochloride induced activation of PAI-1 compromises the integrity of the heparin-binding site in a substantial fraction of PAI-1 molecules. Similar findings were reported for antithrombin III, which exhibited a largely diminished effect of heparin on the rate of thrombin inhibition after guanidine hydrochloride induced unfolding and subsequent renaturation (Villanueva & Allen, 1983). As observed for nonactivated PAI-1, the total recovery of activated PAI-1 was also 100%, with about 90% of the PAI-1 recovered in the elution fractions.

To further characterize the interaction between PAI-1 and heparin, the effect of heparin and low molecular weight heparin in solution on the binding of PAI-1 to heparin–Sepharose was studied. The competition between increasing concentrations of heparin or low molecular weight heparin in solution and heparin–Sepharose for binding of nonactivated ^{35}S-labeled PAI-1 is depicted in Figure 3. Both fluid-phase competitors caused a linear (r = 0.987 ± 0.009) decrease of binding of PAI-1 to heparin–Sepharose when residual binding was plotted against the logarithm of the competitor concentration. Half-maximal inhibition of PAI-1 binding was achieved with 7.0 ± 1.9 μM heparin (n = 3) and with 73 ± 14 μM low molecular weight heparin (n = 3).

Heparin-Mediated Inhibition of Thrombin by PAI-1. It has been demonstrated that the association rate constants of the serpins antithrombin III, heparin cofactor II, and protease nexin with thrombin are dramatically increased in the presence of heparin. To investigate whether heparin would have a similar effect on PAI-1, recombinant PAI-1 (rPAI-1), purified to apparent homogeneity from lysates of transformed *Escherichia coli* cells, was incubated with thrombin in the presence of serial dilutions of heparin. rPAI-1 from *Escherichia coli* was utilized for these experiments, since it is readily available in apparently pure form and in a large quantity and, most significantly, because rPAI-1 is identical with PAI-1 synthesized in endothelial cells with respect to (i) dependence on in vitro activation, (ii) specific activity of the activated preparations, and (iii) inhibition kinetics toward thrombin (Ehrlich et al., 1990). After a preincubation of 1 h, the residual amidolytic activity of thrombin was determined,

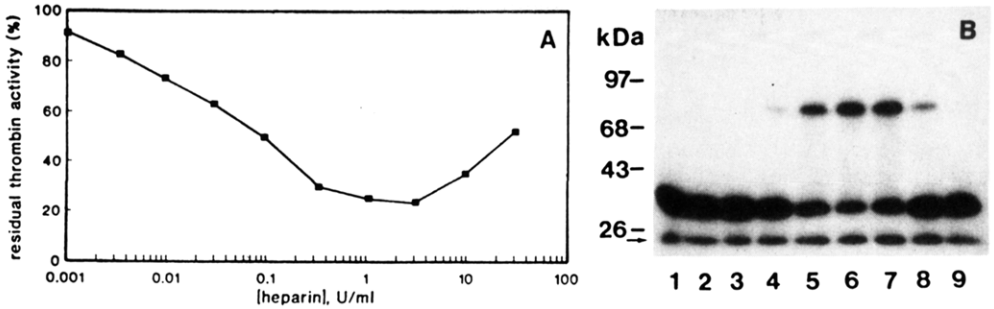

FIGURE 4: Effect of heparin on the interaction between PAI-1 and thrombin. (A) Inhibition of thrombin by PAI-1 in the presence of increasing heparin concentrations. Thrombin (0.15 nM) was incubated at 37 °C with PAI-1 (5 nM of active inhibitor) in the presence of increasing heparin concentrations as indicated. After 60 min, residual thrombin activity was determined by adding the chromogenic substrate S2238 (0.5 mM) and recording the linear increase of absorbance at 405 nm. (B) Formation of SDS-stable complexes between ^{125}I-labeled thrombin (2.5 nM, all lanes) and PAI-1 (5 nM active inhibitor, lanes 2–9) after incubation (1 h, 37 °C) in the presence of the following heparin concentrations: lanes 1 and 2, no heparin; lane 3, 0.001 unit/mL heparin; lane 4, 0.01 unit/mL heparin; lane 5, 0.5 unit/mL heparin; lane 6, 1 unit/mL heparin; lane 7, 10 units/mL heparin; lane 8, 100 units/mL heparin, lane 9, 1000 units/mL heparin. The arrow indicates the position of the dye front.

using the synthetic substrate S2238. The results are shown in Figure 4A. In the absence of heparin, no inhibition of thrombin by rPAI-1 was observed (data not shown). However, upon increasing heparin concentrations, the residual thrombin activity declined, with half-maximal inhibition observed at 0.03 unit/mL. Full inhibition was reached between 0.3 and 3 units/mL and then diminished upon further increasing the heparin concentration. The second-order association rate constant for the inhibition of thrombin by PAI-1 in the presence of an optimal concentration of heparin (1 unit/mL) was $(1.0 \bullet 0.3) \times 10^5$ M^{-1} s^{-1} ($n = 3$).

Heparin-Mediated Formation of SDS-Stable Complexes between Thrombin and PAI-1. To explain the effect of heparin on the inhibition of thrombin by rPAI-1, we subsequently determined whether heparin promotes, in a dose-dependent manner, the formation of SDS-resistant complexes between ^{125}I-labeled thrombin and rPAI-1. The results of these experiments are shown in Figure 4B. The radiolabeled thrombin migrates as a single band with the expected molecular weight of approximately 36 000 (lane 1). The pattern on the autoradiogram is unaltered upon addition of purified rPAI-1 (lane 2). However, upon including serial dilutions of heparin in the incubation mixes of thrombin and rPAI-1, the appearance of a band of higher molecular weight can be noted. This novel band, exhibiting a molecular weight which is consistent with a 1:1 molar complex of thrombin and rPAI-1, is very faint at a heparin concentration of 0.01 unit/mL, of increased intensity at higher heparin concentrations with a maximum at 1 unit/mL, and finally decreases upon further raising the heparin concentration to 100 or 1000 units/mL. Thus, the heparin-dependent formation of SDS-stable complexes between PAI-1 and thrombin coincides with the heparin-dependent inhibition of thrombin activity by PAI-1.

Half-Life of PAI-1 Activity in the Absence and Presence of Heparin. PAI-1 is unique among serpins in that the active form, capable of inhibiting t-PA, spontaneously decays into a latent form, which no longer exhibits this inhibitory function (Kooistra et al., 1986; Levin & Santell, 1987; Chmielewska et al., 1987; Hekman & Loskutoff, 1988a). The latent form of PAI-1 can be distinguished from the typical inactive, cleaved form of serpins, since it can be converted into an active form. This is achieved by exposing PAI-1 either to denaturants such as SDS or guanidine hydrochloride (Hekman & Loskutoff, 1985) or to negatively charged phospholipids (Lambers et al., 1988). The half-life of PAI-1 activity at 37 °C is rather short, with reported values ranging from 1 to 4 h (Mimuro et al., 1987; Declerck et al., 1988; Hekman & Loskutoff, 1988a; Lindahl et al., 1989). However, it has been shown that the

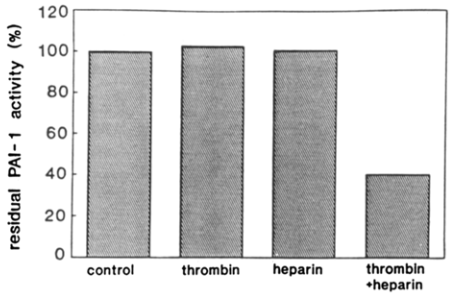

FIGURE 5: Heparin-mediated neutralization of PAI-1 activity by thrombin. Eighteen nanomolar active PAI-1 was incubated at 37 °C with either 1 unit/mL heparin or 18 nM thrombin or with both thrombin and heparin. After 15 min, residual PAI-1 activity in the samples was determined by titration on t-PA. Residual PAI-1 activity in the control, incubated for 15 min at 37 °C without heparin and thrombin, was taken as 100%.

active form of PAI-1 is considerably stabilized by association with the binding protein vitronectin (Declerck et al., 1988) and, as we recently demonstrated, in the presence of arginine (Keijer et al., 1990).

To establish whether the association between PAI-1 and heparin could also result in a similar effect, the spontaneous decay of PAI-1 at 37 °C was studied in the presence and absence of 1.0 unit/mL heparin and, as a control, in the presence of 100 mM arginine. Aliquots of PAI-1, preincubated at 37 °C for different time intervals, were subsequently titrated for protease-inhibiting activity by using the enzyme t-PA. The dose response curve of t-PA inhibition were linear in all cases ($r > 0.98$), enabling us to establish the amount of PAI-1 in these aliquots required for complete inhibition of t-PA (IC_{100} values). PAI-1 activity steadily declined upon incubation at 37 °C, and residual PAI-1 activities in the presence and absence of heparin (1.0 unit/mL) were virtually identical at each time point measured (data not shown). The half-life of active PAI-1, determined from a semilogarithmic plot of residual PAI-1 activity over time ($r > 0.99$), was 65 min irrespective of the presence of heparin. In contrast, PAI-1 was stabilized in control experiments by 100 mM arginine, consistent with data reported earlier (Keijer et al., 1990).

Heparin-Mediated Neutralization of PAI-1 by Thrombin. To study whether heparin would enhance the neutralization of PAI-1 by thrombin, activated and titrated PAI-1 was incubated with an equimolar amount of thrombin in the presence or absence of heparin. Experiments in the absence of thrombin were performed in parallel. After 15 min at 37 °C, the activity of thrombin was quenched with hirudin, and the concentration of residual active PAI-1 in the samples was determined by

titration on t-PA. Linear dose response curves ($r > 0.99$) were obtained in all cases. The results, calculated from the amounts of PAI-1 required for half-maximal inhibition of t-PA, are shown in Figure 5. After preincubation with either thrombin or heparin, residual PAI-1 activity was virtually identical (103% and 101%, respectively) with the control, i.e., preincubation of PAI-1 in the absence of either compound. In contrast, when PAI-1 was preincubated for 15 min with both thrombin and heparin, residual PAI-1 activity was decreased to 40% of the control, lacking both thrombin and heparin.

Discussion

Heparin has been shown to interact with a variety of plasma proteins, including serpins such as antithrombin III (Rosenberg & Damus, 1973), heparin cofactor II (Tollefsen & Blank, 1981), and protease nexin (Baker et al., 1980), coagulation factors (Fujikawa et al., 1973), t-PA (Andrade-Gordon & Strickland, 1986), growth factors (Maciag et al., 1984; Shing et al., 1984), apolipoproteins (Shelbourne & Quarfordt, 1977), fibronectin (Stathakis & Mosesson, 1977), and vitronectin (Preissner & Müller-Berghaus, 1987). The practical value of heparin, i.e., its clinical application as an anticoagulant which enhances the inhibition of coagulation proteases (most notably factor Xa and thrombin) by the serpin antithrombin III, initiated extensive investigations on the glycosaminoglycan and its interactions with coagulation factors during the last 2 decades. However, the precise mechanism by which heparin potentiates the rate of the interaction between thrombin and antithrombin III is still under discussion. There is increasing evidence suggesting that heparin functions as a template for the assembly of both antithrombin III and thrombin, resulting in a rapid formation of inactivate protease–protease inhibitor complexes (Pomerantz & Owen, 1978; Griffith, 1982; Nesheim, 1983). In addition, a heparin-induced conformational change of the inhibitor is proposed to contribute to the enhanced rate of protease inhibition (Rosenberg & Damus, 1973; Olson et al. 1981; Hoylaerts et al., 1983).

The role of heparin in fibrinolysis is less well established than its function in the coagulation system. Only recently it has been shown that heparin binds to t-PA and, subsequently, stimulates fibrin-independent plasminogen activation (Andrade-Gordon & Strickland, 1986; Pâques et al., 1986; Stein et al., 1989). This observation is of particular interest in light of concomitant application of t-PA and heparin for the treatment of thrombotic disorders, since it may contribute toward an explanation for unexpected, substantial systemic plasminogen activation. The data presented in this paper show that heparin also interacts with PAI-1, the physiological inhibitor of t-PA and u-PA. PAI-1 is very specific for the inhibition of the two plasminogen activators, since it exhibits second-order association rate constants with these target proteases of 10^7–10^8 M^{-1} s^{-1} (Kruithof et al., 1986; Chmielewska et al., 1988; Hekman & Loskutoff, 1988b; Thorsen et al., 1988), indicating that the inhibitory reactions are virtually diffusion controlled. In contrast to the inhibition of thrombin by antithrombin III, this very rapid inhibition of plasminogen activators by PAI-1 is not dependent on the presence of cofactors such as heparin (Chmielewska et al., 1988) or vitronectin (Declerck et al., 1988). The specificity of PAI-1 for the plasminogen activators t-PA and u-PA is further emphasized by the observation that association rates with other plasma proteases are several orders of magnitude slower (Hekman & Loskutoff, 1988; Berrettini et al., 1989). These findings, however, raise the possibility that cofactors, although not required for efficient inhibition of plasminogen activators, may influence the specificity of PAI by enhancing the reaction rates with serine proteases, initially considered as nontarget proteases. Thus, the restricted specificity of PAI-1 for plasminogen activators may be extended in the presence of cofactors such as vitronectin (Ehrlich et al., 1990) and, as we show in this paper, in the presence of heparin.

Fluid-phase heparin and also low molecular weight heparin were tested for their ability to compete for the binding of ^{35}S-labeled PAI-1 to heparin–Sepharose. Inhibition of this binding was half-maximal (IC_{50} values) with 7.0 μM soluble heparin and 73 μM low molecular weight heparin. A lower affinity of low molecular weight heparin as compared with heparin has also been described for antithrombin III and may be related to differences in the percentage of molecules carrying serpin-binding sites in these preparations (Dawes, 1988). In a similar experimental design, the concentration of soluble heparin required to half-maximally inhibit the binding of radiolabeled antithrombin III to heparin-Sepharose was found to be 1.0 μM (Dawes, 1988), indicating that PAI-1, although binding very strongly to heparin, has a lower affinity for the glycosaminoglycan than antithrombin III. This finding could reflect the fact that the heparin-binding site in PAI-1 may lack some of the structural features required for optimal heparin binding. Indeed, as can be deduced from Figure 1, two positively charged amino acid residues (Arg-123 and Lys-131; antithrombin III numbering), which are located in the proposed helix D of antithrombin III, are lacking in the corresponding homologous area in PAI-1. For the related serpin heparin cofactor II, the application of in vitro mutagenesis enabled direct proof for the involvement of Lys-185 (Blinder & Tollefsen, 1990), which is strictly conserved in the proposed glycosaminoglycan-binding site of the heparin-dependent serpins. Currently, we are also using site-directed mutagenesis to substitute several selected positively charged amino acid residues in PAI-1 for direct analysis of the importance of the altered residues for the interaction of PAI-1 with heparin.

Both heparin and, as we described previously, vitronectin (Ehrlich et al., 1990) enhance the reactivity of PAI-1 toward thrombin. However, from a mechanistic point of view, these two cofactors of serpin specificity act strikingly different. Whereas vitronectin does not serve as a template for the interaction between PAI-1 and thrombin (Ehrlich et al., 1990), the effect of increasing heparin concentrations on this interaction is clearly consistent with a template mechanism, since with fixed concentrations of PAI-1 and thrombin, the reaction rate increases with increasing heparin concentrations up to maximum and then decreases. This type of response to increasing heparin concentrations is shown here both for thrombin inhibition by PAI-1 and for complex formation between thrombin and PAI-1, and has been described similarly for the effect of heparin on the interaction between antithrombin III and thrombin (Griffith, 1982b; Nesheim, 1983). At an optimal heparin concentration (1 unit/mL), the k_1 for thrombin inhibition by PAI-1 was determined to be 1.0×10^5 M^{-1} s^{-1}. Although this value represents an approximately 100-fold increase of the rate measured in the absence of heparin (Ehrlich et al., 1990), it is still considerably lower than the rates reported for the interaction between thrombin and other heparin-dependent serpins. At 37 °C and in the presence of heparin, the reported second-order association rate constants with thrombin are $(1.5–4) \times 10^7$ M^{-1} s^{-1} for antithrombin III (Jordan et al., 1979, 1980; Griffith 1982a,b), 8×10^6 M^{-1} s^{-1} for heparin cofactor II (Tollefsen et al., 1982), and 1×10^8 M^{-1} s^{-1} for protease nexin (Scott et al., 1985). However, it should be kept in mind that these rates were measured in purified systems, which may not always reflect the conditions

encountered physiologically. For example, it has been described that fibrin monomer (Hogg & Jackson, 1989) and also vitronectin (Preissner & Müller-Berghaus, 1986, 1987) protect thrombin from rapid inhibition by antithrombin III/heparin. In contrast, vitronectin does not slow down the heparin-mediated interaction between thrombin and PAI-1 (H. J. Ehrlich and H. Pannekoek, unpublished observation).

The physiological significance of the interaction between PAI-1 and heparin remains to be established. Here, we suggest two possibilities. First, this interaction could be related to the neutralization of either PAI-1 or thrombin in a microenvironment that is not accessible for the major thrombin inhibitor from plasma, i.e., antithrombin III. Second, it could indicate a function for heparin or related glycosaminoglycans in the localization of PAI-1, e.g., to the environment of granulosa cells, which have been shown to produce t-PA as well as PAI-1 (Canipari & Strickland, 1985; Ny et al., 1985). Local retention of PAI-1 may ascertain the regulation of the heparin-dependent activity of t-PA (Andrade-Gordon & Strickland, 1986). Thus, under those conditions, heparin might function similarly to fibrin in the fibrinolytic system (Wagner et al., 1989), in assembling both t-PA and PAI-1, ultimately leading to the formation of inactive t-PA/PAI-1 complexes.

Registry No. PAI, 105844-41-5; heparin, 9005-49-6; serpin, 96282-35-8; thrombin, 9002-04-4; plasminogen, 9001-91-6.

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Detection and Characterization of Intermediates in the Folding of Large Proteins by the Use of Genetically Inserted Tryptophan Probes†

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ABSTRACT: L-Lactate dehydrogenase from *Bacillus stearothermophilus* was rebuilt by using site-directed mutagenesis to produce an enzymically active, tryptophan-less enzyme by replacing all the wild-type tryptophans (80, 150, and 203) by tyrosines. Nine single tryptophan-containing active enzymes were constructed from this enzyme by genetically replacing one of the tyrosines 36, 85, 147, 190, 203, 237, 248, 279, or 285 by tryptophan. The equilibrium and the time-resolved tryptophan fluorescence intensity and anisotropy were used to report unfolding events in guanidine hydrochloride (GHCl) monitored from these nine defined positions. Three structural transitions, half complete at 0.55, 1.7, and 2.8 M GHCl, were identified and defined four folding intermediates, I (native), II (expanded monomer 1), III (expanded monomer 2), and IV (random coil), stable at 0, 1, 2.2, and 4 M GHCl, respectively. Intermediate II is a globular monomer. All the probed α-helices and most of the β-structure was intact. There was an increase in the rate but not the extent of the mobilities of six of the probed tryptophan side chains, indicating loss of tertiary structure. Circular dichroism (CD) showed all the secondary structure to be intact. Intermediate III is monomeric and still globular, but the tryptophan anisotropy indicated an increase mobility at positions 36, 85, 190, 203, 279, and 285. Helix α-B is further disrupted but helices α-1F, α-2G, and α-3G were still rigid. CD showed half the secondary structure to be still intact. Intermediate IV is a random coil in which all tryptophans have complete rotational freedom and the helix CD signal is lost. Intermediates II and III both have characteristics ascribed to a molten globule state. The time-resolved fluorescence anisotropy of a tryptophan on a helix in folding intermediates has a correlation time of 2–3 ns and a residual anisotropy of 0.08. Probes close together in the primary sequence (279 and 285) reported identical unfolding events. The nine probes suggest partial structures for the two monomeric intermediates in folding of *B. stearothermophilus* lactate dehydrogenase, which can be compared to two kinetic intermediates proposed by Jaenicke [(1987) *Prog. Biophys. Mol. Biol. 49*, 117–237].

There is increasing evidence [Ptitsyn, 1981; cf. Ptitsyn (1987) and Kuwajima (1989)] that proteins fold to their active conformation via a series of distinct intermediate structures in a defined pathway. A study of the process of folding requires characterization of the structures of intermediates at different stages along the pathway. A well-established method of trapping intermediates (Creighton, 1978) uses the formation of disulfide bonds between cysteine residues as proteins containing such bonds refold. This method has elucidated features of the folding process in bovine pancreatic trypsin inhibitor and the structure of one intermediate species has been characterized by NMR spectroscopy. Another approach makes use of the exchange reaction between the backbone amide

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