Elucidation of Structural Requirements on Plasminogen Activator Inhibitor 1 for Binding to Heparin*

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Plasminogen activator inhibitor 1 (PAI-1), a member of the serpin superfamily of proteins, has been demonstrated previously to interact functionally with the glycosaminoglycan heparin (Ehrlich, H. J., Keijer, J., Preisser, K. T., Klein Gebbink, R., and Pannekoek, H. (1981) Biochemistry 30, 1021-1028). Heparin specifically enhances the rate of association between PAI-1 and thrombin by about 2 orders of magnitude, whereas no effect is detected with other serine proteases (e.g. factor Xa). For the heparin-dependent serpins antithrombin III and heparin cofactor II, basic amino acid residues in and around the helix D subdomain were proposed to be involved in the binding of glycosaminoglycans. Here we employed site-directed mutagenesis of full-length PAI-1 cDNA to identify the amino acid residues that mediate heparin binding. To that end, 15 single-point mutants of PAI-1, each having individual arginyl, lysyl, or histidyl residues replaced by a neutral (alanyl) residue ("ala-scan"), and one double mutant were constructed, expressed in Escherichia coli, and purified to apparent homogeneity. The purified biologically active proteins were subjected to the following analyses: (i) heparin-dependent inhibition of thrombin; (ii) heparin-dependent formation of sodium dodecyl sulfate-stable complexes with thrombin; and (iii) binding to and elution from heparin-Sepharose. Based on the data presented, we propose that the amino acid residues Lys190, Lys192, Arg193, Lys194, and Lys198 constitute major determinants for heparin binding of PAI-1. These residues are located in and around the helix D domain and are conserved in the other heparin-dependent thrombin inhibitors, antithrombin III and heparin cofactor II.

Heparin is a sulfated glycosaminoglycan that interacts with a variety of proteins such as growth factors (Maciag et al., 1984; Shing et al., 1984), coagulation factors (Fujikawa et al., 1973), fibronectin (Stathakis and Mosesson, 1977), vitronectin (Preissner and Müller-Berghaus, 1987), tissue-type plasminogen activator (t-PA)1 (Andrade-Gordon and Strickland, 1986), and the serine protease inhibitors (serpins) antithrombin III (Rosenberg and Damus, 1973), heparin cofactor II (Tollefsen and Blank, 1981), protease nexin I (Baker et al., 1980), and plasminogen activator inhibitor 1 (PAI-1) (Ehrlich et al., 1991a). One of the most dramatic consequences resulting from these interactions is the potent anticoagulant effect observed in vivo after heparin administration (Brinkhous et al., 1939). Extensive studies on the underlying molecular mechanism revealed that highly negatively charged heparin molecules accelerate the inhibition of thrombin and other serine proteases of the coagulation system by antithrombin III (Rosenberg and Damus, 1973). The cofactor function of heparin has been explained by the observations that (i) heparin provides for a template in the assembly of both the protease and the serpin (Pomerantz and Owen, 1978; Griffith, 1982; Nesheim, 1983); and (ii) heparin induces a conformational change in the reactive site of the serpin, thereby enhancing the reactivity toward thrombin (Rosenberg and Damus, 1973; Olson et al., 1981).

In addition to its anticoagulant effects, heparin was shown to enhance fibrinolysis by binding to t-PA, thereby stimulating the plasminogen activating activity of this enzyme in the absence of fibrin (Andrade-Gordon and Strickland, 1986; Pâques et al., 1986; Stein et al., 1989). Moreover, as we have shown recently, there is also a profibrinolytic effect resulting from a functional interaction between heparin and the serpin PAI-1, the fast inhibitor of t-PA and urokinase-type plasminogen activator (urokinase-type PA) (Ehrlich et al., 1991a). The striking selectivity of PAI-1 for the inhibition of t-PA and urokinase-type PA is compromised in the presence of either heparin (Ehrlich et al., 1991a) or vitronectin (Ehrlich et al., 1990), resulting in an enhancement of the association rate between PAI-1 and thrombin of 2–3 orders of magnitude. The effect of heparin on PAI-1-mediated inhibition is apparently restricted to thrombin inasmuch as no enhancement was observed with other serine protease tested, e.g. factors Xa and XIIa (Keijer et al., 1991). The interaction between PAI-1 and thrombin ultimately leads to neutralization of PAI-1 because of the formation of inactive, SDS-stable thrombin-PAI-1 complexes and the proteolytic cleavage of PAI-1 by thrombin (Ehrlich et al., 1991a, 1991b). The molecular mechanism of the effect of vitronectin, the PAI-1-binding protein in plasma (Declerck et al., 1988; Wiman et al., 1988) and in the endothelial cell matrix (Mimuro and Loskutoff, 1989; Preissner et al., 1990), on this reaction remains to be elucidated. For heparin, however, experimental evidence suggests that this glycosaminoglycan functions as a template for the assembly of both the protease and the protease inhibitor

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1 The abbreviations used are: t-PA, tissue-type plasminogen activator; PAI-1, plasminogen activator inhibitor 1; SDS, sodium dodecyl sulfate; RF, replicative form.
Purification of PAI-II and PAI II Mutants—Expression of the recombinant proteins was carried out as described previously (Ehrlich et al., 1990), except that heparin-Sepharose was utilized instead of an immunoadfinity matrix for the final purification step. The supernatants of the sonicated bacterial pellets from a 250-ml culture were treated with pancreatic DNase I and subsequently fractionated by ammonium sulfate precipitation (25% saturation). The resulting precipitate was resuspended in 20 mm Tris- HCl (pH 8.0), 0.1% (v/v) Tween 80 and, after extensive dialysis against the same buffer, adsorbed batchwise onto 2 ml of Q-Sepharose Fast Flow matrix that was then packed into a column, washed with 10 ml of 20 mm Tris- HCl (pH 8.0), 0.1% (v/v) Tween 80, and eluted with 5 ml of 20 mm Tris- HCl (pH 8.0), 0.1% (v/v) Tween 80, 200 mm NaCl. The eluates were dialyzed against 20 mm Tris- HCl (pH 7.4), 0.1% (v/v) Tween 80, 50 mm NaCl, and adsorbed for 16 h at 4 °C by end-over-end rotation to 1.5-ml packed beads of heparin-Sepharose. Finally, the matrix was packed into a column, washed with 5 column volumes of 20 mm Tris- HCl (pH 7.4), 0.1% (v/v) Tween 80, 100 mm NaCl, and eluted with 5 column volumes of 20 mm Tris- HCl (pH 7.4), 0.1% (v/v) Tween 80, 400 mm NaCl. The eluates were then dialyzed extensively against 20 mm Tris- HCl (pH 8.0), 0.1% Tween 80, 100 mm NaCl (TST buffer), and PAI II: antigen in the purified fractions was determined using an immunoradiometric assay that utilized two different monoclonal antibodies raised against human PAI II, as described previously (Lambers et al., 1988; Ehrlich et al., 1990). PAI II mutant K154A could not be detected using this assay: its concentration was estimated from the relative intensity of the PAI II-related band on a silver-stained gel. SDS-polyacrylamide gel electrophoresis followed by silver staining essentially showed a single band of the molecular weight of about 42,000 for the purified PAI II preparations.

Activation of PAI II and PAI II Mutants and Titration of their Respective Activities—PAI I or PAI II mutants (100–500 ng/ml) were activated by incubation for 2 h at room temperature in 4 ml guanidine HCl (4 M, Cakir and Loskutoff, 1985). The denaturation was removed by overnight dialysis at 4 °C against TST buffer. Increasing amounts of activated PAI I or PAI II mutants were incubated at 37 °C in a total volume of 50 ml with 1.5 nm two-chain t-PA in TST buffer. Then, 200 ml of the chromogenic substrate S2288 (0.6 mm in TST buffer) was added, and residual t-PA activity was determined from a linear plot of the increase of absorbance at 405 nm over time.

Heparin-mediated Inhibition of Thrombin by PAI I and PAI II Mutants—Thrombin (0.3 nm) was incubated with PAI I or PAI II mutants (5 nm active PAI I, titrated on t-PA as described above) in a total volume of 30 ml of TST buffer in the absence or in the presence of 45 nm vitronectin or 1 unit/ml heparin. After 1 h at 37 °C, 200 ml of the synthetic substrate S2288 was added, and thrombin activity was determined from the linear increase of absorbance at 405 nm. The increase of absorbance measured for the sample containing only thrombin was taken as 100%.

Oligonucleotide-directed Mutagenesis—Oligonucleotide-directed, site-specific mutagenesis was carried out as described (Kramer et al., 1984), utilizing three different M13 vectors as single-stranded template. First, a 3'-nonradioactive pair EcoRI-AsI restriction fragment of 94 base pairs from pM13/PBLII/PAI-I covering part of the multiple cloning site of phage M13mp18am4 RF DNA, yielding M13mp18am4 RF DNA. A 300-base pair SacI-SacI restriction fragment from full-length, human PAI I cDNA (corresponding to positions 1045 and 1375, respectively) was then inserted into the M13mp18am4 RF DNA (corresponding to positions 52-436), was inserted into M13mp18am4 RF DNA. Second, a 609-base pair SacI-SacI restriction fragment, corresponding to positions 436 and 1045, was inserted into M13mp18am4 RF DNA. For the construction of the third vector, both strands of a linker (sequence of "upper" strand, 5'-GAATTCCGATGCGCGCCACTGGCCATCCAGGTTCGAC-3'), containing the restriction sites EcoRI-NsiI-SacI, was synthesized and ligated to the 5'-portion of the dDNA coding for mature human PAI I (positions 25-436), was inserted into M13mp18am4 RF DNA. For the construction of the individual mutants are summarized in Table I. The fidelity of the entire PAI I coding sequences in the mutated EcoRI-SacI fragment of the multiple cloning site of phage M13mp18am4 RF DNA, yielding M13mp18am4 RF DNA. A 300-base pair SacI-SacI restriction fragment from full-length, human PAI I cDNA (corresponding to positions 1045 and 1375, respectively) was then inserted into the M13mp18am4 RF DNA (corresponding to positions 52-436), was inserted into M13mp18am4 RF DNA.
polyacrylamide gel (Laemmli, 1970). The relative position of the 

radiolabeled material, as compared with molecular weight markers, 

was visualized by autoradiography.

One ml of PAI-1 wild-type or mutants (100 pg/ml in 20 mM Tris-
HCl (pH 7.4), 0.1% (v/v) Tween 80) was incubated for 16 h at 4°C 

by end-over-end rotation with 100 p1 of a slurry of heparin-Sepharose.

was then carried out by washing the beads with 0.5 ml of 20 mM Tris-
HCl (pH 7.4), 0.1% (v/v) Tween 80) and then by stepwise increasing 

the NaCl concentration (steps of 50 mM). The amounts of wild-type 

PAI-1 or mutants in each fraction were assessed using the immuno-

as shown in Table I. Our choice for mutating basic (arginyl, 

lypyrl residues related on: (i) a comparison between the amino acid sequences of antithrombin III and PAI-1, in particular of the region of antithrombin III implicated either in heparin binding (helix D) or in a heparin-induced conformational change (Arg187/Arg188 and Lys191); and (ii) a computer program that predicts surface probability. Each 250-ml culture of transformed E. coli cells yielded at least 500 µg of purified protein, irrespective of the nature of the PAI-1 variant. Upon activation with guanidinium Cl, the purified PAI-1 proteins displayed similar specific activities in a titration assay, measuring inhibition of the amidolytic activity of t-PA (data not shown). These observations indicate that none of the mutations grossly affected the structure of the respective PAI-1 proteins.

Cofactor-dependent Thrombin Inhibition by PAI-1 and PAI-1 Mutants—The effect of heparin on the inhibition of thrombin by PAI-1 and PAI-1 mutants was analyzed as described (Ehrlich et al., 1991a). Experiments, carried out in the presence of vitronectin or in the absence of either cofactor, served as controls. In the absence of heparin or vitronectin, no inhibition of thrombin by PAI-1 wild-type or by any of the PAI-1 mutants was observed (data not shown). The results obtained in the presence of either one of the cofactors are depicted in Fig. 1. Upon adding vitronectin to the reaction mixtures, PAI-1 wild-type and each of the PAI-1 mutants were endowed with thrombin inhibitory properties. Under the conditions employed, approximately 50–75% inhibition of the amidolytic activity of thrombin was observed, indicating that both wild-type and any of the PAI-1 mutants can inhibit thrombin in the presence of vitronectin. In contrast, the effect of heparin as a cofactor for thrombin inhibition is markedly different for the various PAI-1 derivatives. A similar thrombin inhibition in the presence of heparin is detected for wild-type

![Fig. 1. Cofactor-induced inhibition of thrombin by wild-type PAI-1 and mutants.](https://example.com/fig1.png)
PAI-1 and for the variants PAI-1 K28A, K154A, R186A/R187A, K191A, K207A, K243A, K263A, K277A, and K288A. However, hardly any inhibition of thrombin in the presence of heparin is observed using the mutant proteins PAI-1 R76A, K80A, and K88A, whereas these proteins fully inhibit thrombin in the presence of vitronectin. Partial inhibition of thrombin in the presence of heparin is detected with the mutant proteins PAI-1 K65A and PAI-1 K69A: again full inhibition of thrombin by these latter mutant proteins is apparent in the presence of vitronectin. It should be noted that three mutants, i.e. PAI-1 H10A, PAI-1 K28A (H19 and Lys26) comprise the two positively charged amino acid residues in the helix A subdomain of PAI-1, the counterpart of which in antithrombin III contains the Arg47 residue, proposed to be involved in heparin binding (Owen et al., 1987)), and PAI-1 H77A (the latter histidy1 residue is located between the crucial Arg60 and Lys60 residues in PAI-1) displayed similar thrombin inhibitory properties in the presence of either vitronectin or heparin as wild-type PAI-1 (data not shown).

Cofactor-dependent Formation of SDS-stable Complexes between PAI-1 or PAI-1 Mutants and Thrombin—A typical feature of the inhibition of a serine protease by its cognate serpin is the ultimate generation of equimolar, inactive complexes that do not dissociate in the presence of SDS. Consequently, we examined the formation of complexes between 125I-labeled thrombin and PAI-1 (derivatives) in the presence of either heparin or vitronectin, SDS-polyacrylamide gel electrophoresis. The results are depicted in Fig. 2. Both heparin and vitronectin efficiently promoted the generation of SDS-stable complexes between thrombin and either PAI-1 wild-type or the mutants PAI-1 K28A, K154A, R186A/R187A, K207A, and K243A. In addition, the mutants PAI-1 H10A, H77A, K263A, K277A, and K288A also displayed no significant difference in complex formation from wild-type PAI-1 (data not shown). In contrast, hardly any complex formation is observed with the mutant proteins PAI-1 R76A, K80A, and K88A, whereas only part of the 125I-labeled thrombin formed complexes with the mutants PAI-1 K65A and K69A in the presence of heparin. Again, it should be noted that complex formation of thrombin with any of the PAI-1 derivatives was comparable in the presence of vitronectin. These observations are consistent with the data presented on the inhibition of thrombin by the different PAI-1 species in the presence of either heparin or vitronectin (Fig. 1). An exception is represented by the mutant protein PAI-1 K191A: this protein, although fully inhibitory in a suitable amidolytic assay toward t-PA, urokinase-type PA, and thrombin in the presence of either heparin or vitronectin, reproducibly failed to form SDS-stable complexes with thrombin in the presence of either cofactor.

Interaction of PAI-1 and PAI-1 Mutants with Heparin-Sepharose—It is thought that the function of heparin in the acceleration of the inhibition of thrombin by its cognate heparin-dependent serpin antithrombin III is to provide for a template to assemble both the enzyme and the inhibitor and/or to induce a conformational change that increases the reactivity of the inhibitor. In either case, efficient binding of heparin to the serpin is a prerequisite for the observed enhanced inhibition. To determine relative affinities of wild-type PAI-1 and the various PAI-1 mutants for heparin, these species were separately bound to heparin-Sepharose and eluted from the gel by increasing the ionic strength. The results are summarized in Table II. PAI-1 wild-type and the mutants PAI-1 K28A, K65A, K69A, R186A/R187A, K191A, K207A, and K243A eluted at a concentration between 293 and 318 mM NaCl. No significant difference in the affinity for heparin-Sepharose was detected between the mutants PAI-1 K65A and PAI-1 K69A, being partially defective in heparin-induced inhibition of thrombin, and e.g. wild-type PAI-1. In contrast, the mutants PAI-1 R76A, K80A, and K88A, being fully defective in heparin-induced stimulation of thrombin inhibition as well as in complex formation with the protease, eluted at a considerably lower ionic strength (between 175 and 238 mM NaCl) than wild-type PAI-1.

**DISCUSSION**

Evidence for the involvement of specific amino acid residues in the interaction between the serpins antithrombin III/heparin cofactor II and heparin originates from three different experimental approaches: (i) chemical modification of specific residues in proteins (Rosenberg and Damus, 1973; Chang, 1989); (ii) analysis of the antithrombin III variants of patients suffering from thrombotic episodes caused by defective heparin binding (for review, see Huber and Carrell, 1989); and
been interpreted as evidence for a conformational change of antithrombin I11 after binding to heparin. The corresponding residue Lys65 of antithrombin I11 is exposed upon binding of those of antithrombin I11 and heparin cofactor I11. No evidence for the interaction between heparin and antithrombin I11 which has been shown to become exposed inasmuch as they require a lower ionic strength to dissociate from heparin-Sepharose than wild-type PAI-1. Reduced affinity for heparin presumably results in a deficiency of thrombin inhibition and a lack of heparin-induced complex formation between thrombin and the respective PAI-1 mutants. The control experiments, carried out with vitronectin as a cofactor, clearly demonstrate that these variants interact with thrombin in the presence of vitronectin. It is concluded that at least the amino acid residues Arg76, Lys65, and Lys80, located in the helix D subdomain of PAI-1, are part of its heparin binding site. Interestingly, the mutant proteins PAI-1 K65A and PAI-1 K69A are partially defective in thrombin inhibition in the presence of heparin and in SDS-stable complex formation. Nevertheless, under the conditions employed, their affinity for heparin is similar to wild-type PAI-1. It should be noted, however, that each PAI-1 species was allowed to interact with heparin-Sepharose for an extensive period of time to reach equilibrium of binding. Currently, more detailed kinetic experiments are being performed with PAI-1 K65A and PAI-1 K69A to determine whether the association and dissociation constants with heparin differ from those of wild-type PAI-1. Based on our observations on the partial inhibition of thrombin and SDS-stable complex formation in the presence of heparin, we tentatively propose that, next to Arg76, Lys65, and Lys80, residues Lys60 and Lys89 are also part of the heparin binding site of PAI-1. The involvement of (at least) 5 basic residues of PAI-1 with a similar number of negatively charged groups on the heparin molecule has been proposed previously to constitute the high affinity heparin binding site on antithrombin III (Olson et al., 1981). We conclude that PAI-1 is a member of the heparin-dependent thrombin inhibitors: the location on helix D and the nature of the amino acid residues that constitute the PAI-1 heparin binding site are similar to those of antithrombin III and heparin cofactor II. No evidence can be advanced for the participation of His80 and Arg89 in heparin binding, indicating that in contrast to antithrombin III and heparin cofactor II, PAI-1 may not contain a site in the helix A subdomain which would provide the molecule with additional heparin binding properties.

Finally, it should be emphasized that none of the mutations grossly affected the general structure of the inhibitor as evidenced by similar specific inhibitory activity toward either t-PA or urokinase-type PA. Moreover, as shown in Figs. 1 and 2, the ability of the mutants to inhibit thrombin in the presence of vitronectin and to form SDS-stable complexes is unaltered. However, we consistently found that the mutant PAI-1 K191A, although fully able to inhibit thrombin and to bind to heparin efficiently, displays significantly reduced SDS-stable complex formation with thrombin in the presence of both heparin and vitronectin. The position of the mutation (Lys191 replaced by Ala) coincides with the Arg296 residue of antithrombin III which has been shown to become exposed upon binding of antithrombin III to heparin and which may play a direct role in the interaction between the heparin-bound inhibitor and thrombin (Chang, 1989). The properties of residues of PAI-1, i.e. either Arg187-Arg188 or Lys191, were thus also part of the analysis presented. Fifth, six lysyl residues (Lys154, Lys207, Lys243, Lys246, Lys277, and Lys289) that were predicted to display either high or low surface probability were separately replaced by an alanine residue and served as controls.

Our data provide direct evidence that the amino acid residues Arg76, Lys65, and Lys80 are of critical importance for the interaction between PAI-1 and heparin. Clearly, the mutant proteins PAI-1 R76A, K80A, and K88A have a significantly reduced affinity for heparin inasmuch as they require a lower ionic strength to dissociate from heparin-Sepharose than wild-type PAI-1. Reduced affinity for heparin presumably results in a deficiency of thrombin inhibition and a lack of heparin-induced complex formation between thrombin and the respective PAI-1 mutants. The control experiments, carried out with vitronectin as a cofactor, clearly demonstrate that these variants interact with thrombin in the presence of vitronectin. It is concluded that at least the amino acid residues Arg76, Lys65, and Lys80, located in the helix D subdomain of PAI-1, are part of its heparin binding site. Interestingly, the mutant proteins PAI-1 K65A and PAI-1 K69A are partially defective in thrombin inhibition in the presence of heparin and in SDS-stable complex formation. Nevertheless, under the conditions employed, their affinity for heparin is similar to wild-type PAI-1. It should be noted, however, that each PAI-1 species was allowed to interact with heparin-Sepharose for an extensive period of time to reach equilibrium of binding. Currently, more detailed kinetic experiments are being performed with PAI-1 K65A and PAI-1 K69A to determine whether the association and dissociation constants with heparin differ from those of wild-type PAI-1. Based on our observations on the partial inhibition of thrombin and SDS-stable complex formation in the presence of heparin, we tentatively propose that, next to Arg76, Lys65, and Lys80, residues Lys60 and Lys89 are also part of the heparin binding site of PAI-1. The involvement of (at least) 5 basic residues of PAI-1 with a similar number of negatively charged groups on the heparin molecule has been proposed previously to constitute the high affinity heparin binding site on antithrombin III (Olson et al., 1981). We conclude that PAI-1 is a member of the heparin-dependent thrombin inhibitors: the location on helix D and the nature of the amino acid residues that constitute the PAI-1 heparin binding site are similar to those of antithrombin III and heparin cofactor II. No evidence can be advanced for the participation of His80 and Arg89 in heparin binding, indicating that in contrast to antithrombin III and heparin cofactor II, PAI-1 may not contain a site in the helix A subdomain which would provide the molecule with additional heparin binding properties.

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of the mutant PAI-1 K191A would be consistent with normal formation of a Michaelis-Menten (EI) complex, whereas the subsequent formation of an SDS-stable complex (so-called E' complex) could be hampered. Detailed kinetic experiments will provide more insight in the function of Lys<sup>190</sup> of PAI-1 in cofactor-dependent inhibition of thrombin.

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