Identification of positively charged residues contributing to the stability of plasminogen activator inhibitor 1

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Abstract Plasminogen activator inhibitor 1 (PAI-1), a member of the serpins, has a unique conformational flexibility. A typical characteristic is its intrinsic lability resulting in the conversion of the active conformation to a latent conformation. In the present study, we have evaluated the effect of substitution of positively charged residues located at the turn connecting strand s4C with strand s3C, either with negatively charged or with neutral residues, on the functional stability of PAI-1. The following mutants were constructed, purified and characterized in comparison to wild-type (wt) PAI-1: PAI-1-R186E,R187E ($Arg^{186} \rightarrow$ Glu and $Arg^{187} \rightarrow$ Glu), PAI-1-H190E,K191E ($His^{190} \rightarrow$ Glu and $Lys^{191} \rightarrow$ Glu) and PAI-1-H190L,K191L ($His^{190} \rightarrow$ Leu and $Lys^{191} \rightarrow$ Leu). In contrast to wtPAI-1 the mutants exhibited no inhibitory activity. Whereas latent wtPAI-1 can be reactivated (up to a specific activity of 78 ± 19%) by treatment with guanidinium chloride, a similar treatment applied to these mutants resulted in a significant but relatively small increase of specific activity (i.e. to 14%). Evaluation of the functional stability (at 37°C, pH 5.5, 1 M NaCl) revealed a strongly decreased functional stability compared to wtPAI-1 (i.e. 3–9 h for the mutants vs. > 24 h for wtPAI-1). Further characterization by heat denaturation studies and plasmin susceptibility confirmed that removal or reversal of the positive charge on the turn connecting s4C with s3C results in PAI-1 mutants with a highly accelerated conversion of active to latent forms. We can therefore conclude that the pronounced positive charge in the turn connecting s4C with s3C is of the highest importance for the functional stability of PAI-1.

Key words: Plasminogen activator inhibitor 1; Serpin; Functional stability; Mutagenesis

1. Introduction

Plasminogen activator inhibitor 1 (PAI-1) is a member of the serine proteinase inhibitor (serpin) superfamily. All serpins have the same highly ordered tertiary structure consisting of three β-sheets, α-helices A–I and a reactive site loop (P16–P10′) [1,2] that contains the active center (P1–P1′) that mimics the normal substrate of the target proteinase [3]. In PAI-1, the Arg^{346}–Met^{347} bond has been identified as the P1–P1′ bond [4].

PAI-1 is synthesized as an active molecule that spontaneously converts into a latent conformation. The latent conformation, in which the P1–P1′ bond is not accessible due to the insertion of strand s4A into β-sheet A [5], cannot interact with the target proteinases but can be reactivated by denaturants such as guanidinium chloride, sodium dodecyl sulfate or urea [6]. In addition, a stable non-inhibitory conformation with substrate properties has been identified [7–9]. Conformational studies [5,7,10–13] and evaluation of the heat denaturation properties of the various PAI-1 forms have revealed that the differences in functional properties are associated with the existence of these different conformations. In addition, contrary to the active and the substrate conformations, latent PAI-1 typically contains an exposed plasmin susceptible cleavage site resulting in the generation of two degradation products of ∼24 kDa upon incubation with plasmin [14].

Comparison of the crystal structure of a cleaved non-inhibitory substrate mutant PAI-1-P12 (Ala → Pro at P12) [15] with that of latent PAI-1 [5] revealed major positional differences among loop 1 (His^{185}–Pro^{200}, the turn connecting strand s4C with strand s3C) and loop 2 (Glu^{242}–Pro^{246}, the turn connecting strand s3B with helix hG) [15]. From this comparison it was also deduced that the mobility of 'loop 1' is required for transition to the latent form [15,16] and it was subsequently hypothesized that the presence of positively charged residues in this region (i.e. Arg^{186}, Arg^{187}, His^{190} and Lys^{191}) plays a critical role in the functional stability of PAI-1. In the present study, we describe the characterization of three mutants of PAI-1 in which the following mutations were introduced: $Arg^{186} \rightarrow$ Glu and $Arg^{187} \rightarrow$ Glu (PAI-1-R186E,R187E); $His^{190} \rightarrow$ Glu and $Lys^{191} \rightarrow$ Glu (PAI-1-H190E,K191E), $His^{190} \rightarrow$ Leu and $Lys^{191} \rightarrow$ Leu (PAI-1-H190L,K191L). Comparison of the properties of these mutants with those of wtPAI-1 demonstrated that this positively charged region is of the utmost importance for the stability of PAI-1.

2. Materials and methods

2.1. Materials

Restriction enzymes were obtained from Pharmacia (Uppsala, Sweden) or from Boehringer Mannheim (Brussels, Belgium). T_4 DNA ligase, the Klenow fragment of *Escherichia coli* DNA polymerase I and alkaline phosphatase were purchased from Boehringer Mannheim (Brussels, Belgium). M13KO7 helper phage was obtained from Promega (Leiden, The Netherlands). The expression vector pIGE20 was kindly provided by Innogenetics (Ghent, Belgium), together with the bacterial strains *E. coli* DH1λ for cloning and *E. coli* MC1061 for

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Abbreviations: PAI-1, plasminogen activator inhibitor 1; PAI-1-R186E,R187E, PAI-1 in which Arg^{186} and Arg^{187} have been replaced by Glu; PAI-1-H190E,K191E, PAI-1 in which His^{190} and Lys^{191} have been replaced by Glu; PAI-1-H190L,K191L, PAI-1 in which His^{190} and Lys^{191} have been replaced by Leu; wt, wild-type; serpin, serine proteinase inhibitor; t-PA, tissue-type plasminogen activator; u-PA, urokinase-type plasminogen activator; PBS, phosphate buffered saline; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

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expression as well as the pAcI plasmid encoding the thermolabile repressor.

The chromogenic substrate S-2403 was obtained from Chromogenix (Mölndal, Sweden). t-PA (predominantly single-chain) was a kind gift from Boehringer Ingelheim (Brussels, Belgium); single-chain u-PA (scu-PA) from which low molecular weight two-chain u-PA (tcu-PA) was prepared and plasmin were kindly provided by Dr. Lijnen (University of Leuven, Belgium).

2.2. Construction, expression and purification of PAI-1 and PAI-1 mutants

In vitro site-directed mutagenesis of PAI-1 was performed as described earlier [17] using the pMa/c system ([18], kindly provided by Corvas, Ghent, Belgium) and the following synthetic oligonucleotides to obtain the desired mutation: PAI-1-R186E,R187E, 5′-GA TTT GTG GAA GAG CTC TTC GTG GGT GCT GGA G-3′; PAI-1-H190E,K191E, 5′-CT GCC GTC TGA TTC TTC GAA GAG GCG GCG-3′; PAI-1-H190L,K191L, 5′-GT GCT GCC GTC ACT TAA GAG GAA GAG GCG G-3′. Specific restriction sites, i.e. a *Sac*I site (GAGCT↓C) and a *Ban*II site (G(A,G)GC(C,T)↓C) for PAI-1-R186E,R187E; a *Nsp*V site (TT↓CGAA) for PAI-1-H190E,K191E; a *Afl*II site (C↓TTAAG) for PAI-1-H190L,K191L were simultaneously created to allow confirmation of the desired mutation by restriction analysis. *Sac*I-*Xba*I fragments from pMa-PAI-1-H190E,K191E and pMa-PAI-1-H190L,K191L and *Stu*I-*Xba*I fragments from the mutant pMa-PAI-1-R186E,R187E were recovered and substituted for the *Sac*I-*Xba*I and *Stu*I-*Xba*I fragments, respectively, in pIGE20-wtPAI-1. Nucleotide sequencing using A.L.F. (Pharmacia) was used to confirm the presence of the desired mutation. Expression and purification of wtPAI-1 and PAI-1 mutants was performed in *E. coli* as described earlier [14].

2.3. Immunological and functional determination of PAI-1

Comparative reactivity between the mutants and active and latent forms of wtPAI-1 was evaluated in an ELISA MA-19A6/MA-9E4-HRP exclusively sensitive to active and substrate PAI-1 [19]. PAI-1 activity was determined using the method described by Verheijen [20]. All PAI-1 activity data are expressed as percentage of the theoretical maximum activity, i.e. ∼745 000 U/mg (t-PA inhibitory units) and 120 000 U/mg (u-PA inhibitory units) [14].

2.4. Conformational distribution of PAI-1 mutants

PAI-1 samples were diluted with phosphate buffered saline (PBS) to a concentration of 0.3 mg/ml and incubated for 30 min at 37°C with a two-fold molar excess of t-PA, u-PA or plasmin. The reaction was terminated by adding SDS (final concentration 1%) and heating for 30 s at 100°C. Reaction products were analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10–15% gradient gels under non-reducing conditions with the Phast System (Pharmacia, Uppsala, Sweden). Proteins were visualized by staining with Coomassie brilliant blue and quantified by densitometric scanning with the Imagemaster (Pharmacia).

2.5. Determination of the stability of the reactivated wtPAI-1 and PAI-1 mutants

PAI-1 samples were diluted to 20 μg/ml in PBS containing 2 mM glutathione and 0.01% Tween 80 and incubated with 6 M guanidinium chloride for 25 min at 37°C, followed by extensive dialysis at 4°C against 50 mM sodium acetate buffer, pH 5.5, containing 1 M NaCl, 2 mM glutathione and 0.01% Tween 80 [21]. Reactivated PAI-1 samples were subsequently incubated at 37°C and aliquots were removed at various times and assayed for inhibitory activity against t-PA and u-PA.

2.6. Heat denaturation studies of wtPAI-1, PAI-1 mutants and derivatives

Samples containing latent PAI-1 or PAI-1 mutants were diluted to 20 μg/ml in 20 mM sodium acetate buffer pH 5.5 containing 1 M NaCl and 0.01% Tween 80 and incubated at a constant temperature between 30°C and 100°C for 2 ho. The samples were then cooled in ice and centrifuged for 15 min in a microfuge at 14 000 rpm to remove precipitated protein. The supernatants were carefully removed and either analyzed immediately or stored at −20°C. Residual PAI-1 protein in the supernatant was determined using the MA-15H12/MA-12A4 ELISA [22] and is expressed as percentage versus the initial value. The $Tm_{50\%}$ value is the temperature at which 50% of the antigen is precipitated.

2.7. Statistical analysis

The statistical significance of differences was evaluated using Student's *t*-test; *P*-values < 0.05 were considered significant.

3. Results

3.1. Determination of specific inhibitory activity against t-PA and u-PA

wtPAI-1 had a specific activity of 77 ± 14% (mean ± S.D., $n = 6$) of the theoretical maximum value towards t-PA. In contrast, PAI-1-R186E,R187E, PAI-1-H190E,K191E and PAI-1-H190L,K191L did not exhibit inhibitory activity (< 2%) towards t-PA or u-PA (Table 1). After inactivation followed by reactivation using guanidinium chloride, wtPAI-1 could be reactivated up to 78 ± 19% (towards t-PA) whereas under these conditions PAI-1-R186E,R187E, PAI-1-H190E,K191E and PAI-1-H190L,K191L were reactivated to a much lower extent, i.e. 14 ± 8%, 1.2 ± 0.5% and 12 ± 8%, respectively, towards t-PA (Table 1). Comparable data were obtained towards u-PA. These reactivated PAI-1 mutants revealed, at 37°C in sodium acetate buffer, pH 5.5, containing 1 M NaCl, half-lives of 9 ± 5 h (PAI-1-R186E,R187E) and 3 ± 3 h (PAI-1-H190L,K191L) whereas under these conditions wtPAI-1 had, as expected, a half-life > 24 h.

3.2. Reaction products formed after incubation of wtPAI-1 and PAI-1 mutants with various serine proteinases

As expected, incubation of active wtPAI-1 with a two-fold molar excess of t-PA revealed the formation of t-PA/PAI-1 complexes (58 ± 8.6%, mean ± S.D., $n = 4$), small amounts of cleaved derivative (9.1 ± 4.5%) and residual non-reactive material (33 ± 4.3%) (data not shown). In the presence of a two-

Table 1
Specific activity towards t-PA (A) and u-PA (B) before and after inactivation at 37°C and after reactivation

	Start	Inactive	Reactivated
A			
wtPAI-1	77 ± 14[a]	1.6 ± 0.75	78 ± 19
PAI-1-R186E,R187E	0.54 ± 0.12	0.41 ± 0.32	14 ± 8
PAI-1-H190E,K191E	0.1 ± 0.02	< 0.1	1.2 ± 0.53
PAI-1-H190L,K191L	1.6 ± 0.4	< 0.1	12 ± 8
B			
wtPAI-1	79 ± 16	1.9 ± 0.7	70 ± 22
PAI-1-R186E,R187E	0.53 ± 0.17	< 0.1	13 ± 4.0
PAI-1-H190E,K191E	< 0.1	0.11 ± 0.14	2.3 ± 1.7
PAI-1-H190L,K191L	1.6 ± 1.6	< 0.1	12 ± 6.6

[a]Expressed as percentage of the theoretical maximum value; mean ± S.D., $n = 5$–9.

fold molar excess of u-PA, wtPAI-1 revealed similar reaction products. In contrast PAI-1-R186E,R187E, PAI-1-H190E, K191E and PAI-1-H190L,K191L were virtually completely non-reactive (>96%) towards t-PA as well as towards u-PA (Fig. 1), compatible with the absence of inhibitory activity in these mutants.

In the presence of a two-fold molar excess of plasmin, latent wtPAI-1 revealed the formation of two degradation products migrating with a molecular weight of approximately 24 kDa (Fig. 1). Under these conditions also PAI-1-R186E,R187E, PAI-1-H190E,K191E and PAI-1-H190L,K191L revealed the generation of two degradation products of 24 kDa (Fig. 1).

3.3. Recognition in 19A6/9E4 ELISA

Active wtPAI-1, latent wtPAI-1, PAI-1-R186E,R187E, PAI-1-H190E,K191E, and PAI-1-H190L,K191L were applied on the MA-19A6/MA-9E4-HRP ELISA. The relative reactivities of the mutants compared to active PAI-1 were 2±2% for latent PAI-1, <0.15% for PAI-1-R186E,R187E, 0.4±0.3% for PAI-1-H190E,K191E, and 0.9±0.6% for PAI-1-H190L,K191L.

3.4. Heat-induced denaturation of different conformations of wtPAI-1 and PAI-1 mutants

The heat denaturation profiles of active wtPAI-1, latent wtPAI-1, PAI-1-R186E,R187E, PAI-1-H190E,K191E, and PAI-1-H190L,K191L are shown in Fig. 2. The $Tm_{50\%}$ values are 54°C for active wtPAI-1, 86°C for wtPAI-1, 76°C for PAI-1-H190L,K191L, >90°C for PAI-1-R186E,R187E and PAI-1-H190E,K191E.

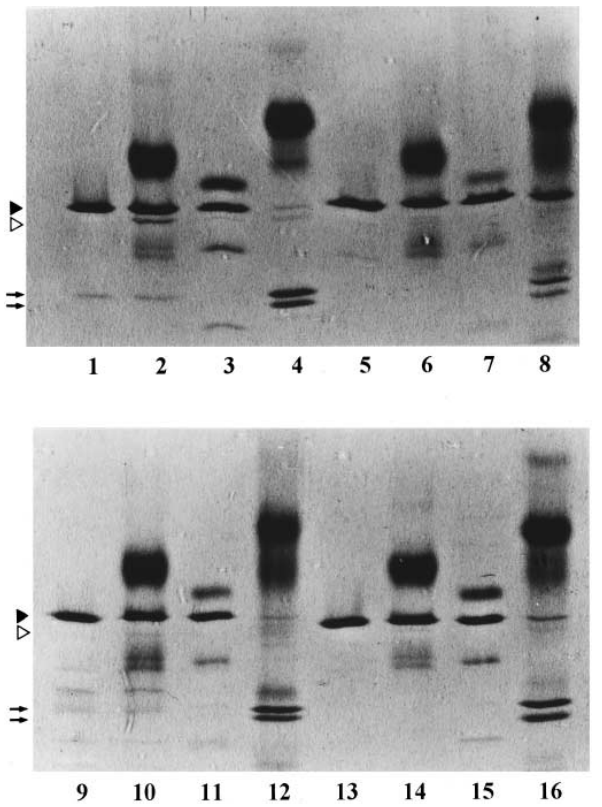

Fig. 1. SDS-PAGE of latent wtPAI-1 and PAI-1 mutants after addition of a two-fold molar excess of proteinase. Lanes 1–4: latent wtPAI-1; 2–8: PAI-1-R186E,R187E; 9–12: PAI-1-H190E,K191E; 13–16: PAI-1-H190L,K191L. Proteinase: t-PA (lanes 2, 6, 10, 14), u-PA (lanes 3, 7, 11, 15), and plasmin (lanes 4, 8, 12, 16). The closed arrowhead indicates the migration position of intact PAI-1. The open arrowhead indicates the migration position of the cleaved substrate form. The arrow indicates the position of the degradation products with molecular masses of ~24 kDa.

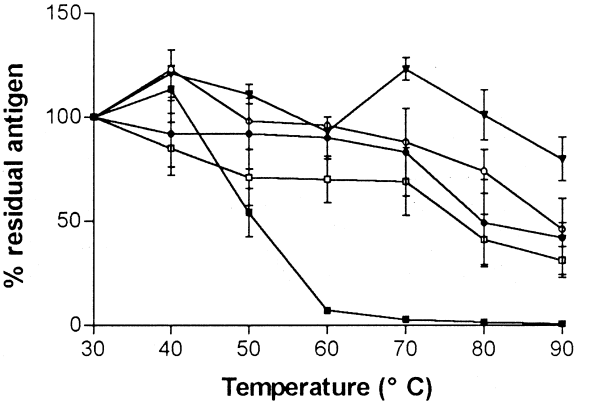

Fig. 2. Heat denaturation profiles of latent wtPAI-1 and PAI-1 mutants. Active wtPAI-1 (■), latent wtPAI-1 (●), PAI-1-R186E,R187E (○), PAI-1-H190E,K191E (▼), PAI-1-H190L,K191L (□). All data represent mean ± S.E.M., $n = 2–3$.

4. Discussion

PAI-1 is unique among the serpins because it is the only serpin that, under physiological conditions and also in vivo, converts spontaneously and reversibly into a non-reactive, latent conformation [6]. This conversion occurs much more slowly at pH 5.5 and at a ionic strength of 1 M NaCl [21]. Non-reactive conformations have been induced in antithrombin III [23] and α_1-antitrypsin [24] by treatment with 0.9 M guanidinium chloride or incubation at 60–70°C. Several studies on PAI-1 have been described where either site-directed mutagenesis or random mutagenesis has led to an increased functional stability [14,25–27]. Random mutagenesis suggested that only few single amino acid substitutions are capable of significantly increasing the stability of PAI-1 [26]. In addition, structural studies have shown that the conformational change accompanied with the conversion of active to latent serpins involves the formation of a central β-strand s4A by insertion of the N-terminal side of the reactive site loop into β-sheet A and a loss of strand s1C from sheet C followed by the transition of the C-terminal side of the reactive site loop through a gap composed of two loops [5,15,16,27]. The distance between the two loops, i.e. loop 1 (His^{185}–Pro^{200}; connecting strand s4C with s3C) and loop 2 (Glu^{242}–Pro^{246}; connecting strand s3B with helix hG) was found to be 14 Å in the latent conformation compared to only 7 Å in the reactive conformation. This gap widening is primarily due to a positional difference of loop 1 [15]. In some studies, it has been suggested that for most serpins strand s4C and strand s3C (connected by loop 1) is accommodated to a hydrophobic pocket consisting of residues equivalent to Pro^{276}, Phe^{278}, Leu^{280}, Phe^{358} and Pro^{379} in PAI-1. In contrast, in PAI-1, the C-terminus occupies this hydrophobic pocket, which possibly explains the mobility of strand s3C and s4C (and loop 1) [5,27]. However, engineering a disulfide bond between strand s3C and the C-terminus of PAI-1 to reduce the mobility of strands s3C and s4C only slightly increased the functional stability of PAI-1 [27]. Alter-

natively, it has been suggested that an electrostatic repulsion between Arg^{356} (P10′) and positively charged residues on loop 1 (Arg^{186}, Arg^{187}, His^{190} and Lys^{191}) is in part responsible for the repositioning of the loops and consequently for the widening of the gap resulting in the transition from the active to the latent form [15].

The current study reveals the importance of the positively charged residues at the N-terminal region of 'loop 1' on the specific activity and functional stability of PAI-1. Three mutants in which the positively charged residues at the N-terminal region of 'loop 1' were substituted either with negatively charged or with neutral residues, i.e. PAI-1-R186E,R187E, PAI-1-H190E,K191E and PAI-1-H190L,K191L, revealed a lack of inhibitory activity towards t-PA and u-PA. Conformational distribution analysis confirmed the absence of complex formation and revealed the absence of substrate behavior indicating that no interaction occurs between the P1-P1′ site of the PAI-1 mutants and the target proteinases, compatible with the presence of a latent conformation. Indeed, previous studies [14] have shown that putative plasmin cleavage sites (i.e. Lys^{176}–Thr^{177}; Arg^{187}–Leu^{188}; Lys^{191}–Ser^{192}) are exclusively present in latent forms of PAI-1 typically resulting in the generation of two degradation products with molecular masses of 24 kDa upon incubation of latent forms with plasmin. In the current study all PAI-1 mutants yielded two degradation products of ~24 kDa upon incubation with plasmin, in keeping with the occurrence of a latent conformation. Alternatively, the occurrence of the latent conformation in these inactive mutants was further confirmed by their heat denaturation profile which was typically like that observed for latent serpins [12,14,23]. In addition, the three mutants studied exhibited a very low reactivity in an assay that specifically recognizes active forms of PAI-1. Taken together, these four characteristics clearly demonstrate that the non-reactive behavior of these mutants is explained by their latency. Consequently, it can be concluded that upon synthesis of these mutants the proteins are folded into the active conformation (as for wtPAI-1) which is then converted to the latent conformation. The latter conversion is strongly enhanced for these mutants as compared to that observed with wtPAI-1. This hypothesis is further supported by the denaturation/renaturation experiments together with functional stability tests. These experiments revealed indeed that the inhibitory activity can be restored in these mutants but that the active inhibitory conformation of these mutants had a much shorter half-life compared to that of active wtPAI-1. It is of interest to note that the positive charge at positions 187, 190 and 191 in PAI-1 is fully conserved between various species [28]. However, the charge at residue 186 is absent (i.e. replaced by Gln) in rat and murine PAI-1. Since rat and murine PAI-1 have similar stabilities as human PAI-1, one could speculate that the positive charge at position 186 has only a minimal, if any, influence on the stability of PAI-1.

In conclusion, we have identified a positively charged region in PAI-1 (i.e. residues 186, 187, 190 and 191) localized in an area previously suggested to play a role in the lability of PAI-1, that contributes significantly to the functional stability of PAI-1. Elucidation of the three-dimensional structure of these mutants will allow a detailed evaluation at a molecular level of the interactions involved between this region and other parts of the molecule. Thus, the current mutants constitute useful tools to obtain more insight into the underlying mechanisms involved in the conformational transitions in PAI-1. In addition, our current observations suggest that loop 1 might be a possible target for in vivo modulation of PAI-1 activity based on an enhanced conversion to the latent state. This may contribute to the development of pharmacological agents for the prevention of diseases associated with high PAI-1 levels.

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