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PAI-1 stability: the role of histidine residues

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Abstract The role of the 13 histidine residues in plasminogen activator inhibitor 1 (PAI-1) for the stability of the molecule was studied by replacing these residues by threonine, using site-directed mutagenesis. The generated mutants were expressed in *Escherichia coli*, purified and characterized. All variants had a normal activity and formed stable complexes with tissue-type plasminogen activator. Most of these PAI-1 variants displayed a similar pH-dependency in stability as wild-type PAI-1, with increased half-lives at lower pH. However, the variant His364Thr had a half-life of about 50 min at 37°C and had almost completely lost its pH-dependency. Therefore, our data suggest that His^{364}, in the COOH-terminal end of the molecule might be responsible for the pH-dependent stability of PAI-1.

Key words: Plasminogen activator inhibitor 1; Site-directed mutagenesis; Stability

1. Introduction

Plasminogen activator inhibitor 1 (PAI-1) is the physiological inhibitor of tissue-type plasminogen activator (tPA) and urokinase. In vivo, PAI-1 seems to be a major regulator of the fibrinolytic system [1] and increased levels are correlated with the development of thrombotic disease, principally myocardial infarction [2,3] but also deep vein thrombosis [4,5] and stroke [6]. PAI-1 is a member of the serine protease inhibitor superfamily of proteins and consists of a single polypeptide chain of 379 amino acid residues [7–9]. PAI-1 spontaneously converts from the functionally active form into an inactive, so-called latent form, with a half-life of about 2 h at neutral pH and 37°C [10,11]. In plasma most of the active PAI-1 is bound to vitronectin, resulting in an increased stability of about 4 h [10,11]. The latent form of the inhibitor can be reactivated by treatment with guanidinium chloride or urea in high concentrations, followed by dialysis at slightly acidic pH [12,13]. It has also been reported that vitronectin [14,15] and phospholipid [16] slowly may reactivate latent PAI-1. During the conversion to the latent form there is a complete insertion of the reactive center loop (RCL) into β-sheet A [17]. It has been found that substitution of residues in different parts of the molecule, such as on the turn connecting s3C and s4C [18], at the opposite end of the RCL [19], on the s2B and s3B strands [20,21], may affect its transformation from the active to the latent form. A combination of changes of several residues in different parts of the molecule gives an even more pronounced stability [22]. It has also recently been shown that Triton X-100 significantly accelerates conformational transitions in PAI-1 [23,24]. In addition, PAI-1 is found in a cleaved form, where it obviously has functioned as a substrate to tPA rather than as an inhibitor [25–27]. The stability (i.e. conversion of the active to the latent form) of PAI-1 is normally pH-dependent. The native glucosylated protein is considerably more stable at slightly decreased pH. Thus, its half-life at pH 5.5 and 37°C is about 16 h [28]. The decrease in pH from neutral to slightly acidic is accompanied by protonation of imidazole groups. It is therefore possible that one or several histidine residues in PAI-1 might be involved in stabilizing the active form. It was speculated that His^{143} might be responsible for this effect [29]. However, by introducing four different mutations at this position in PAI-1, we recently demonstrated that protonation of His^{143} is not directly involved in the pH-dependent stability [30]. In our present work, we have systematically mutated the remaining 12 histidine residues to see if any of these would be responsible for the observed stability of PAI-1 at slightly acidic pH.

2. Materials and methods

2.1. Materials

In the PCR amplification, the thermostable DNA polymerase, DyNAZyme, was from Finnzymes Oy (Espoo, Finland) and the GeneAmp PCR System 2400 was from Perkin-Elmer AB (Stockholm, Sweden). PCR products were purified by QIAquick kit (Germany). All restriction enzymes were purchased from New England BioLabs Inc. (Beverly, MA, USA) and T_4 DNA ligase was from Life Technologies AB (Täby, Sweden). In DNA sequencing, the BigDye Terminator Cycle Sequencing Ready Reaction kit and the ABI PRISM 310 Genetic Analyzer were also from Perkin-Elmer AB. The vector, pBV220, used to produce PAI-1 variants in *Escherichia coli* is a temperature-inducible expression vector with λP_R and λP_L as promoters [31]. In the protein purification, heparin-Sepharose CL-6B was from Pharmacia AB (Uppsala, Sweden) and anhydrotrypsin agarose was purchased from Sigma-Aldrich (Stockholm, Sweden). Imulyse PAI-1 kit and Chromolize PAI-1 activity kit (Biopool AB, Umeå, Sweden) were used to measure PAI-1 antigen and activity, respectively. Melanoma cell single-chain tPA was a kind gift from Biopool (courtesy of Dr. Nils Bergsdorf).

2.2. Mutagenesis of PAI-1 cDNA

Site-directed mutations were introduced into PAI-1 cDNA by synthesized oligonucleotides (primer 1–12, Table 1). The following mutations were made, using a two-step PCR procedure [19], His2Thr, His3Thr, His10Thr, His77Thr, His112Thr, His185Thr, His190Thr, His219Thr, His229Thr, His261Thr, His316Thr and His364Thr. The full-length PAI-1 cDNA with a *Xho*I site introduced at bp 871 by silent mutation, encodes mature wild-type human PAI-1. This PAI-1 cDNA was cloned between the *Eco*RI and *Hin*dIII sites of pBV220

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Abbreviations: PAI-1, plasminogen activator inhibitor 1; tPA, tissue-type plasminogen activator; wt, wild-type; RCL, reactive center loop; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis

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(for mutants His2Thr, His3Thr, His10Thr and His77Thr, respectively) or cloned between the *Eco*RI and *Hin*dIII sites of pUC19 (for the remaining mutants). These two generated plasmids were used as templates in the first PCR. The mutation primers were used together with a second primer, specific for different mutations (primers 13–17, Table 1). The products were purified by agarose gel electrophoresis and subsequently used as primers together with a second primer (primers 17–19, Table 1) in a second PCR. The *Eco*RI-*Hin*dIII fragment of PAI-1 cDNA was used as a template. The products in the second PCR were digested by *Eco*RI and *Xho*I (His2Thr, His3Thr, His10Thr and His77Thr, respectively), *Sac*I and *Xho*I (His112Thr, His185Thr, His190Thr, His219Thr, His229Thr and His261Thr, respectively) or *Sac*I and *Bam*HI (His316Thr and His364Thr respectively). The digested products were subsequently used to replace the corresponding fragment in PAI-1 cDNA in order to generate expression plasmids for the PAI-1 mutants. The sequences in the mutated regions were confirmed by DNA sequencing, using primers 15 and 17, together with one of the primers 20–23 (Table 1).

The mutation primers were designed to avoid the possibility of unwanted mutations caused by an extra nucleotide added by DyNAZyme to the 3′-terminus of DNA. The conditions used in the PCR amplifications were also optimized to diminish the errors caused by the non-proof DNA synthesis of DyNAZyme.

2.3. Expression and purification of recombinant PAI-1

The PAI-1 variants were expressed and purified according to the procedure described previously [19,32]. Briefly, the expression plasmids based on pBV220 were induced to express recombinant PAI-1 proteins in the *E. coli* strain XL1 Blue at 42°C in BHI medium containing 50 mg/l ampicillin. After 4 h of cultivation at this temperature (A_{650} = 1.5–1.6), the bacteria were harvested by centrifugation. The pellet was quickly re-suspended in 0.05 mol/l sodium acetate buffer, pH 5.5, containing 0.1 mol/l sodium chloride, 0.1 g/l Tween-80 and 0.5 g/l glycerol. Lysozyme digestion and sonication were used to disrupt the bacteria. After another centrifugation for 30 min at 12 000 × *g* and 4°C, the supernatants of these PAI-1 variants were used as the sources for chromatographic purification.

Purification by heparin-Sepharose CL-6B chromatography was performed by equilibrating the column (bed volume about 5 ml) with 0.05 mol/l sodium acetate buffer, pH 5.5, containing 0.1 mol/l sodium chloride and 0.1 g/l Tween-80. The unadsorbed proteins were washed out by the same buffer and the PAI-1 adsorbed to the column was eluted by a gradient of sodium chloride from 0.1 to 1.1 mol/l in 200 ml of the same acetate buffer. The fractions containing the highest PAI-1 antigen concentrations were pooled and dialyzed against 0.15 mol/l sodium phosphate buffer, pH 6.6, containing 0.1 mol/l sodium chloride. Subsequently, the samples were applied to an anhydrotrypsin agarose column (bed volume about 1.0 ml) equilibrated with the same phosphate buffer [33]. The adsorbed material was eluted from the column by 0.3 mol/l of arginine chloride dissolved in the same buffer. The eluted PAI-1 variants were collected and stored frozen at −70°C.

2.4. Determination of PAI-1 antigen and activity

PAI-1 antigen was measured by the Imulyse PAI-1 kit, using purified wild-type PAI-1 (wtPAI-1) produced in *E. coli* as a standard. The absorbance coefficient used was 7.7 for a 10 g/l wtPAI-1 solution [19]. PAI-1 activity was determined with the Chromolize PAI-1 kit.

2.5. Stability of PAI-1 mutants

The procedure to study the stability of PAI-1 variants was performed as previously described [20]. PAI-1 stability was studied at three different pH intervals; 5.5, 6.5 and 7.5. The temperature was constantly kept at 37°C. The buffer systems used were: 0.05 mol/l sodium acetate buffer, pH 5.5, 0.05 mol/l sodium phosphate buffer at pH 6.5 or at pH 7.5. The three buffers also contained 0.1 mol/l sodium chloride, 0.5 mg/ml bovine serum albumin and 0.1 g/l Tween-80. The PAI-1 variants were diluted by these buffers to a final concentration of 800 U/ml in a final volume of 1 ml. Samples of 100 μl were taken during the time interval from 0 to 8 h. The samples were kept on ice until analysis.

2.6. Analysis of the reactions between PAI-1 variants and tPA by SDS–PAGE

About 30 pmol of each PAI-1 variant was incubated with about 50 pmol of single-chain tPA at ambient temperature for 10 min. The samples were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), performed with a Mini-PROTEAN II Electrophoresis cell (Bio-Rad) according to the procedure described by Laemmli [34]. A polyacrylamide gel concentration of 10% was used and the gel was subsequently stained with Coomassie Brilliant Blue R-250.

3. Results

3.1. Expression and purification of PAI-1 variants

Recombinant PAI-1 expressed in *E. coli* with the vector pBV220 is found both soluble in the cytoplasm and precipi-

Table 1
Oligonucleotides for introducing mutations in PAI-1 cDNA and sequencing PAI-1 variants

No.	Application	Sequences of the oligonucleotides	Position
1	His2Thr	GG GGG ATG **AGT** CAC CAT GAA TTC C	−17
2	His3Thr	GA TGG GGG A**GT** GTG CAC CAT GAA T	−20
3	His10Thr	GA GGC CAG G**GT** **A**GC CAC GTA GGA	−41
4	His77Thr	AG CTC CTT GTA CAG A**GT** CCG **A**AG GGC	−248
5	His112Thr	AG CCT GAA GAA G**GT** GGG CAT GAA GC	−350
6	His185Thr	GAC TCC AGC ACC **AC**C CG**T** CGC CTC	+544
7	His190Thr	CGC CTC TTC **AC**C AAA TCA GAC GG	+562
8	His219Thr	ACG CCC GAT GGC **AC**T TAC TAC GAC	+646
9	His229Thr	GAA CTG CCC TAC **ACT** GGG GAC ACC	+676
10	His261Thr	CAG CTC ATC AGC **AC**C TGG AAA GG	+772
11	His316Thr	GC CTG CGC GAC G**GT** GAG AGG CTC T	−962
12	His364Thr	GT TCC TGT GGG GTT **AGT** CCG AAC CA	−1109
13	PCR	GGA ACA ACG CAT AAC CCT GAA AG	on pBV220
14	PCR	GCA CCA TCC CCC ATC CTA	+6
15	PCR, sequencing	TCG TGA AGT CAG CCT GAA AC	−925
16	PCR	GGT CAG GGT TCC ATC ACT ACT T	−1148
17	PCR, sequencing	GAG AAA CCC AGC AGC AGA T	+158
18	PCR	CCC AGG TTC TCG AGG GGC TTC CTG AG	−884
19	PCR	GTT TTC CCA GTC ACG AC.	on pUC19
20	sequencing	AG CTC CTT GTA CAG AGT CCG AAG GGG	−248
21	sequencing	TGT AAA ACG ACG GCC AGT GAA GAA GTG GGG CAT GAA	−363
22	sequencing	CAA CTT GCT TGG GAA AGG AG	+450
23	sequencing	TGT AAA ACG ACG GCC AGT GAC CTC AGG AAG CCC CTC	+856

The sequences are shown from 5′ to 3′. The mutated nucleotides are printed in bold in all primers and the mutated codons are underlined. Location of each 5′ nucleotide is given and the signs + and − indicate the coding and antisense strands, respectively.

Table 2
Specific activity of all the PAI-1 variants

PAI-1 mutants	Specific activity ($\times 10^6$ U/mg)
wtPAI-1	1.08 ± 0.09
His2Thr	0.88 ± 0.06
His3Thr	0.79 ± 0.13
His10Thr	0.86 ± 0.06
His77Thr	0.55 ± 0.06
His112Thr	0.76 ± 0.10
His143Thr[a]	0.98 ± 0.15
His185Thr	0.88 ± 0.14
His190Thr	0.86 ± 0.16
His219Thr	1.24 ± 0.08
His229Thr	0.84 ± 0.03
His261Thr	1.14 ± 0.03
His316Thr	0.80 ± 0.06
His364Thr	0.49 ± 0.05

The data were obtained from at least three separate experiments and expressed as mean ± S.D.
[a]Published previously, see [30].

tated in inclusion bodies. Since there is a higher risk that the protein in the inclusion bodies is denatured, the soluble proteins in the cytoplasm have been used as the source for protein purification. Most of the PAI-1 variants were expressed in a soluble form in quite a high yield. When His[364] was changed to Thr, the soluble form of the protein repetitively displayed a somewhat lower yield in the cytoplasm than did the other PAI-1 variants. Most PAI-1 variants, including wtPAI-1, were eluted from the heparin-Sepharose column at a concentration of about 0.73 mol/l of sodium chloride. The four variants His3Thr, His77Thr, His112Thr and His229Thr were eluted somewhat earlier during the heparin-Sepharose chromatography (at 0.62, 0.59, 0.63 and 0.63 M NaCl, respectively), suggesting a slightly decreased affinity for heparin.

3.2. Inhibitory activity of PAI-1 variants

The specific activities of the different PAI-1 variants purified from anhydrotrypsin agarose are shown in Table 2. The data were obtained by dividing the activity values with the protein concentration calculated from the absorbance values (280 nm). Calculation of specific activities by dividing activity and antigen concentrations gave very similar results (data not shown). wtPAI-1 had an inhibitory activity of about 1.1×10^6 U/mg. As can be seen, most PAI-1 variants show a small decrease in activity as compared to wtPAI-1. The specific activity for the mutant His364Thr was only 45% of the activity of wtPAI-1. However, as can be seen in SDS–PAGE (Fig. 1) this variant to a large extent forms a stable complex with tPA. The somewhat decreased activity is most likely due to the presence of impurities in the PAI-1 preparation, supported by PAI-1 antigen measurements.

3.3. Reactions of the PAI-1 variants with tPA

As visualized by SDS–PAGE, all of the PAI-1 variants purified by anhydrotrypsin agarose affinity chromatography formed a complex with single-chain tPA when incubated with a slight excess of this enzyme (Fig. 1). A small amount of the cleaved form of PAI-1 is seen after the incubation, most likely representing the substrate form of the inhibitor.

3.4. Stability of the PAI-1 variants

The stability represented by the decline in activity measured as half-lives of the PAI-1 variants is shown in Table 3. Most PAI-1 variants follow the same stability pattern as wtPAI-1, with longer half-lives as pH is decreased. The PAI-1 mutants His229Thr and His316Thr differ from wtPAI-1 by having a less pronounced pH-dependent stability. His2Thr, His10Thr, His112Thr and His219Thr have slightly longer half-lives at pH 6.5 than wtPAI-1 (12.3, 10.6, 12.0 and 15.6 h, respectively). His112Thr and His219Thr have slightly decreased half-lives at pH 5.5 (around 20 h for both). Two of the mutants, His185Thr and His190Thr, show largely decreased half-lives at all pH values studied, as compared to wtPAI-1. Most interestingly, the PAI-1 mutant His364Thr displayed an almost complete flat profile regarding stability at the three different pH's studied. Indeed this finding supports the idea that protonation of this histidine residue participates in the normal stabilization of the PAI-1 molecule.

4. Discussion

The PAI-1 molecule, native or recombinant, is synthesized as a fully active molecule but converts rapidly to an inactive so-called latent form at physiological conditions. The half-life for this transition is about 2 h at neutral pH and 37°C [10,11]. The mechanism is not fully understood but it seems that insertion of the RCL into the A β-sheet plays an important role [17]. In this way the bait peptide bond becomes inaccessible to plasminogen activators. PAI-1 is significantly more stable at

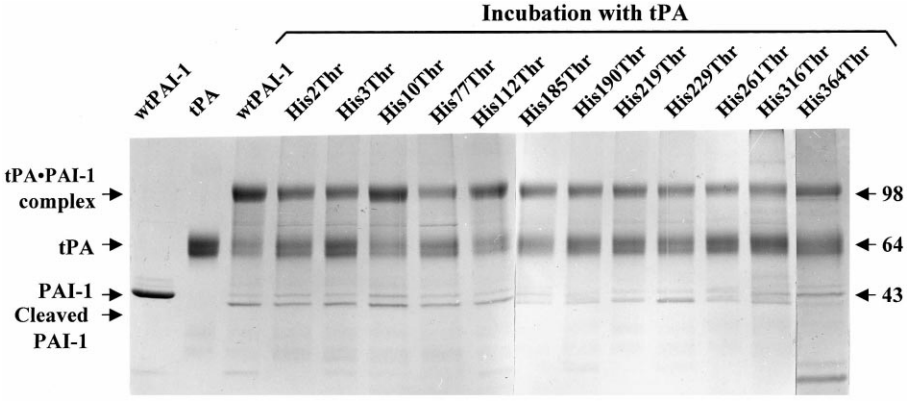

Fig. 1. SDS–PAGE of mixtures of the PAI-1 variants and tPA. About 30 pmol of each PAI-1 variant was mixed with about 50 pmol of single-chain tPA in a phosphate buffer of pH 7.3 (in a final volume of 100 μl). After 10 min of incubation at ambient temperature, 20 μl of the mixture was applied to the gel and analysis was performed as described in Section 2.

Table 3
The stability of the different PAI-1 variants

PAI-1 variant	pH 7.5 (h)	pH 6.5 (h)	pH 5.5 (h)
wtPAI-1	2.20 (100%)	7.83 (350%)	33.0 (1500%)
His2Thr	2.87 (100%)	12.3 (430%)	30.3 (1050%)
His3Thr	2.82 (100%)	9.16 (320%)	29.1 (1030%)
His10Thr	3.01 (100%)	10.6 (350%)	29.0 (960%)
His77Thr	2.49 (100%)	7.13 (290%)	26.0 (1050%)
His112Thr	2.08 (100%)	12.0 (580%)	20.7 (1000%)
His143Thr[a]	2.31 (100%)	6.61 (290%)	11.6 (500%)
His185Thr	0.93 (100%)	4.45 (480%)	14.2 (1530%)
His190Thr	0.76 (100%)	3.54 (470%)	6.21 (820%)
His219Thr	3.69 (100%)	15.6 (420%)	19.1 (520%)
His229Thr	2.06 (100%)	4.92 (240%)	6.23 (300%)
His261Thr	2.63 (100%)	9.98 (380%)	30.3 (1150%)
His316Thr	2.33 (100%)	5.35 (230%)	13.7 (590%)
His364Thr	0.63 (100%)	0.81 (130%)	0.63 (100%)

The half-life at pH 7.5 is set to 100%.
[a]Data published previously, see [30].

slightly acidic pH [19,28]. Kvassman et al. reported that the acidic stabilization was due to the protonation of a single group with a pK_a value of 7.6 [29]. They proposed that His^{143} in helix F, situated in the vicinity of the A β-sheet in the three-dimensional structure of PAI-1, could be involved. However, recently we were able to demonstrate that His^{143} is not responsible for the pH-dependent stability, since substitution of this histidine residue by threonine did not cause a decreased stability of PAI-1 at low pH [30]. In the present study we have substituted the remaining 12 histidine residues in PAI-1 by threonine, to see if any of these are responsible for the observed pH-dependence. If the protonated form of a histidine group would be involved in the stabilization of the active inhibitor, then exchange of this positively charged residue towards an uncharged residue (such as Thr) is expected to cause a decreased stability at low pH. We have previously found that modification of His^{229} to either Asp or Phe resulted in a clearly decreased pH-dependence regarding PAI-1 stability [21]. In our present work, most of the mutants did not display any significant changes in the pH-dependent stability. Most interestingly, however, the mutant His364Thr did not display any such pH-dependent stability. The half-life for the active to latent transformation of this mutant was found to be identical at pH 7.5 and 5.5. At pH 6.5 it was only marginally increased. Further, the stability of this mutant seems to be somewhat decreased over the whole pH range, as compared to wtPAI-1 at pH 7.5. From these data it seems likely that the protonation of His^{364} may be involved in the physiological stabilization of PAI-1 and that it indeed could be responsible for the increased stability of PAI-1 at slightly acidic pH. Crystallographic data have suggested that His^{364}, close to the COOH-terminal end of the protein, is situated in close vicinity to the B β-sheet, just beyond the A β-sheet where insertion of the RCL is believed to take place. Thus it is plausible that a positive charge at residue 364 might partially prevent this insertion.

The results in this study are achieved with non-glycosylated mutants produced in *E. coli*, whereas the native PAI-1 molecule contains more than 10% carbohydrate. Although wtPAI-1 produced in *E. coli* to a large extent has very similar properties as 'native' glycosylated PAI-1, we cannot be absolutely certain that our findings can be fully extrapolated to glycosylated PAI-1.

Another finding in the present work is that some of the PAI-1 variants with modified histidine residues displayed a slightly decreased binding to heparin (His3Thr, His77Thr, His112Thr and His229Thr). In the antithrombin molecule, the primary binding site for heparin has been found to exist on helix D [35]. Previously it has been suggested that the positively charged residues Lys^{65}, Lys^{69}, Arg^{76}, Lys^{80}, Lys^{88} [36] and Arg^{115}, Arg^{118} [19] are involved in the binding of PAI-1 to heparin. From the three-dimensional structure of PAI-1 it seems that residues His^3, His^{77} and His^{112} are situated on or in close vicinity to helix D and it is possible that these residues also are involved in the binding of PAI-1 to heparin.

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