Stability of plasminogen activator inhibitor-1: role of tyrosine^{221}

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Abstract Using site-directed mutagenesis, changes of Tyr^{221} in plasminogen activator inhibitor-1 (PAI-1) have provided mutants with normal activity, but with increased stability. At physiological conditions, the transition of the PAI-1 mutants Tyr^{221}His and Tyr^{221}Ser to the latent form was significantly prolonged (half-lives 14.8 and 4.1 h, respectively) as compared to wild-type PAI-1 (2.0 h). Their half-lives, especially for the Tyr^{221}Ser mutant, were even more prolonged in the presence of vitronectin (23.8 and 53.7 h, respectively). While wild-type PAI-1 was more stable at lower pH, the PAI-1 mutants Tyr^{221}His and Tyr^{221}Ser had stability optima at about pH 6.5, but displayed shorter half-lives at pH 5.5.

Key words: Plasminogen activator inhibitor-1; Mutation; Stability

1. Introduction

Plasminogen activator inhibitor-1 (PAI-1) is the physiological inhibitor of tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA). Activation of the fibrinolytic system by plasminogen activators is important for the physiological removal of fibrin deposits in vivo, and perhaps in preventing occlusive thrombotic disease [1].

PAI-1 is a glycoprotein, consisting of 379 amino acids [2,3]. It has a molecular weight of 50 000, while the recombinant non-glycosylated PAI-1 expressed in prokaryotic cells has a molecular weight of about 43 000. PAI-1 is a member of the serpin superfamily of proteins [4]. Unlike most other serpins, PAI-1 spontaneously converts to an inactive, 'latent', form with a half-life of about 2 h at 37°C and neutral pH [5,6]. Latent PAI-1 can be reactivated by protein denaturing agents, such as guanidinium chloride or urea, followed by a slow refolding, e.g. by dialysis against buffers without denaturing agents [7], preferably at a slightly decreased pH [8]. It is also known that active PAI-1 is somewhat stabilized when associated with vitronectin [6]. The bait peptide bond, Arg^{346}-Met^{347}, makes it possible for PAI-1 to form SDS-stable complexes with tPA or uPA with a second-order rate constant of about 2×10^7 mol^{-1} l s^{-1} [9].

Many studies about the function of the PAI-1 molecule have been focussed on the vicinity of the bait peptide bond [10–14], but other studies have shown that residues remote from the reactive center loop of PAI-1 are also of importance for the functional activity of PAI-1 [15,16].

In the present work, we have produced several mutations of Tyr^{221} in PAI-1 and studied the effects on different PAI-1 functions, including stability.

2. Materials and methods

2.1. Materials

Bacteria were cultured in BHI medium from Difco (USA). T_4 DNA ligase was purchased from Life Technologies AB (Täby, Sweden). The restriction enzymes *Eco*RI, *Hin*dIII, *Sac*I, *Xho*I were from New England Biolabs Inc. (Beverly, MA, USA). The expression vector, pBV220, contains two heat-inducible promoters, λP_R and λP_L [17]. The full-length cDNA for PAI-1 was a kind gift of Prof. Paul Declerck, University of Leuven, Belgium. In PCR amplification, the thermostable DNA polymerase, DyNAZyme, was from Finnzymes Oy (Espoo, Finland). The deoxynucleoside phosphate set (A, C, G and T) was purchased from Boehringer Mannheim AB (Bromma, Sweden). QIAquick kit used in PCR product purification was from Qiagen GmbH (Germany). Heparin-Sepharose CL-6B was purchased from Pharmacia (Uppsala, Sweden). Acrylamide, *N*,*N*′-methylenebisacrylamide and anhydrotrypsin agarose were from Sigma-Aldrich (Stockholm, Sweden). Kits for measuring PAI-1 antigen or activity (Imulyse PAI-1 and Spectrolyze PL PAI-1 kits) were from Biopool AB (Umeå, Sweden). The monoclonal antibody towards PAI-1 (MAI-12) was also obtained from Biopool. Synthetic oligonucleotides were synthesized using a Gene Assembler Special system (Pharmacia). The kit used in DNA sequencing was the Dye primer cycle sequencing, Ready reaction-21M13, from Perkin-Elmer AB (Stockholm, Sweden). Vitronectin was purified as described previously [18].

2.2. Expression procedure of rPAI-1 in Escherichia coli

The expression of rPAI-1 was carried out as described [19] with some modifications. The expression plasmid, pBV220/PAI-1, was transformed to the *E. coli* host XL1 Blue. The bacteria were cultivated in BHI medium containing 50 mg/l ampicillin at 30°C overnight and it was diluted by 1:50 into 1 l of the same medium. When the diluted bacteria had grown at 30°C for about 2.5 h ($A_{650}\sim0.5$), the production of rPAI-1 was initiated by increasing the temperature to 42°C as rapidly as possible. After 4 h cultivation at this temperature (A_{650}=1.5–1.6), the bacteria were harvested by centrifugation. The pellet was quickly resuspended 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. The bacteria were disrupted by addition of 200 μg/ml lysozyme, followed by incubation and 5×1 min sonication (at 90 W and 0°C) in the same buffer. After another centrifugation for 20 min at $12\,000\times g$ and 4°C, the supernatant was subjected to the purification procedure as described below.

2.3. Purification of rPAI-1 by chromatographies

Purification of the PAI-1 variants was performed as described previously [20]. Briefly, the supernatant was applied to a heparin-Sepharose CL-6B column equilibrated 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. After washing, the rPAI-1 was eluted by a 200 ml gradient increasing the sodium chloride concentration from 0.1 to 1.1 mol/l in the same acetate buffer. The fractions containing highest PAI-1 antigen concentrations were pooled and subsequently subjected to chromatography on anhydrotrypsin agarose.

The anhydrotrypsin agarose column (bed volume about 1.0 ml) was equilibrated with 0.075 mol/l sodium phosphate buffer, pH 6.6, containing 0.1 mol/l sodium chloride. About 0.1 mg of rPAI-1 was applied to the column and the unadsorbed protein, mostly latent PAI-1, was washed out by the equilibration buffer. Then, elution was carried out by 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 eluted material was collected and stored frozen at −70°C.

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2.4. Mutagenesis of PAI-1 cDNA

The full-length PAI-1 cDNA with a *Xho*I site introduced at bp 871, by synonymous mutation, encodes mature wild-type (wt) human PAI-1. This PAI-1 cDNA was cloned between the *Eco*RI and *Hin*dIII sites of pUC19. The generated plasmid, pUC19/PAI-1, was used as a template in the following PCR amplifications to introduce mutations in PAI-1 cDNA.

The mutation primers (primers 1–5, Table 1) were used to produce five different PAI-1 mutants with Tyr^{221} substituted by different types of amino acids: His, Ser, Phe, Lys and Asp. The primers were designed to minimize the possibility of unwanted mutations caused by an extra adenosine nucleotide added by DyNAZyme to the 3′-terminus of DNA [21,22]. Also the conditions of PCR amplification were optimized in order to reduce the risk of generating unwanted mutations, by using minimal concentrations of deoxynucleotides and DyNAZyme.

The procedure to introduce mutations at Tyr^{221} consisted of two-step PCR amplifications [20]. In the first PCR, the mutation primers and primer 6 were used to get PCR products on pUC19/PAI-1. The second step PCR employed the products of the first step PCR as primers, together with primer 7, and PAI-1 cDNA as template. The products in the second PCR were digested by *Sac*I and *Xho*I and subsequently used to replace the corresponding *Sac*I-*Xho*I region in the original PAI-1 cDNA. The sequence in the *Sac*I-*Xho*I regions was confirmed by DNA sequencing for all the PAI-1 mutants, while for the mutants Tyr^{221}Lys and Tyr^{221}Asp the sequences were confirmed for the whole coding region.

2.5. Determination of PAI-1 antigen and activity

PAI-1 antigen was measured by the Imulyse PAI-1 kit, using purified wtPAI-1 expressed in *E. coli* as a standard. The absorbance coefficient of 7.7 for a 10 g/l wtPAI-1 solution was used [20]. PAI-1 activity was determined by inhibition of single-chain tPA, using the Spectrolyze PL PAI-1 kit.

2.6. Interaction of PAI-1 with vitronectin

To quantify the interaction of PAI-1 variants with vitronectin, vitronectin-coated microtiter plates were used, essentially as described earlier [23]. Briefly, PAI-1 variants, as well as reactivated native PAI-1 (from HT1080 cells), in different concentrations (final concentrations 0–600 U/ml) were added to microtiter plates coated with vitronectin. After incubation for 1 h at ambient temperature the plates were washed and horseradish peroxidase-conjugated monoclonal antibodies towards PAI-1 (MAI-12) were added. After incubation for another hour, the plates were developed with *o*-phenylenediamine and H_2O_2. The absorbance at 492 nm was recorded and used as a measure of the PAI-1 binding to vitronectin.

2.7. Stability of PAI-1 mutants

The procedure was performed as previously described [20]. PAI-1 stability was studied as a function of pH (pH 5.5–8.5) at 37°C and temperature (0–37°C) at pH 7.5. The buffers used were 0.05 mol/l sodium acetate buffer, pH 5.5; sodium phosphate buffer, pH 6.5 or pH 7.5; or Tris-HCl buffer, pH 8.5. All buffers also contained 0.1 mol/l sodium chloride, 0.5 mg/ml bovine serum albumin and 0.1 g/l Tween 80. When needed, vitronectin was added to a final concentration of about 50 μg/ml. The PAI-1 variants were diluted to final concentrations of about 550 U/ml in the buffers and incubated at specified conditions. At various time intervals (0–144 h), 100 μl samples were removed and slightly acidified with 8 μl of 1 mol/l sodium acetate buffer, pH 3.9, followed by addition of 25 μl of PAI-1 depleted plasma. The samples were then stored at −70°C until analysis of PAI-1 activity.

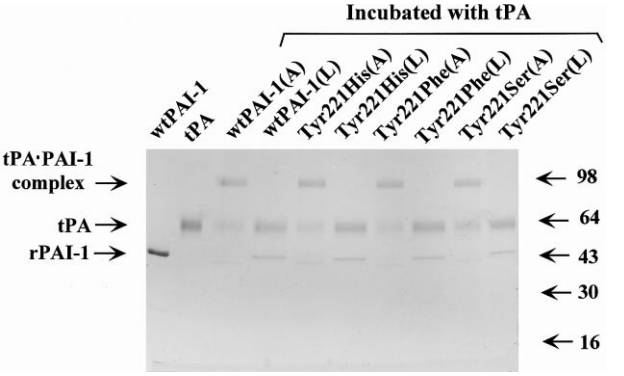

Fig. 1. SDS-polyacrylamide gel electrophoresis of mixtures of the PAI-1 variants and tPA. About 25 pmol of each PAI-1 variant was mixed with about 45 pmol sctPA in a phosphate buffer of pH 7.3 (in a final volume of 100 μl). After 20 min of incubation at ambient temperature, 25 μl of the mixture solution for each sample was analyzed by SDS-polyacrylamide gel electrophoresis which was performed as described in methods. (A) means that the active PAI-1 variants which were eluted from the anhydrotrypsin agarose column at pH 5.5, while (L) means that the latent PAI-1 variants which went through the column at pH 6.6.

2.8. SDS-PAGE

SDS-PAGE was performed using a Mini-PROTEAN II Electrophoresis cell (Bio-Rad) according to the procedure described by Laemmli [24]. 10% polyacrylamide gels were used and staining was performed with Coomassie brilliant blue R-250.

3. Results

3.1. Expression, purification and activity of PAI-1 variants

The PAI-1 mutants Tyr^{221}His, Tyr^{221}Ser and Tyr^{221}Phe, as well as wtPAI-1, were found in a high yield, soluble in the cytoplasm. In contrast, the PAI-1 mutants Tyr^{221}Lys, and Tyr^{221}Asp repeatedly gave a very low yield in the supernatant after bacterial lysis, but were rather found in insoluble inclusion bodies. This suggested that these two mutants had much lower solubility than the other PAI-1 variants, possibly due to major conformational changes. Therefore, these two PAI-1 mutants were not further characterized in this paper.

The specific activity data of the purified PAI-1 variants were very similar and about 1×10^6 U/mg for all, utilizing the standards included in the kits for PAI-1 activity and antigen measurements. The specific activity roughly doubled as a result of purification on the anhydrotrypsin agarose column,

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

No.	Application	Sequences of the oligonucleotides	Position
1	Tyr^{221}His	GGCCATTAC**C**ACGACATCCTGG	+655
2	Tyr^{221}Phe	GGCCATTACT**T**CGACATCCTGG	+655
3	Tyr^{221}Ser	GGCCATTACT**C**CGACATCCTGG	+655
4	Tyr^{221}Lys	GGCCATTAC**AA**AGACATCCTGG	+655
5	Tyr^{221}Asp	GGCCATTAC**G**ACGACATCCTGG	+655
6	PCR	CCCAGGTTCTCGAGGGGCTTCCTGAG	−884
7	PCR	CAGCAGCAGATTCAAGCAG	+166

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

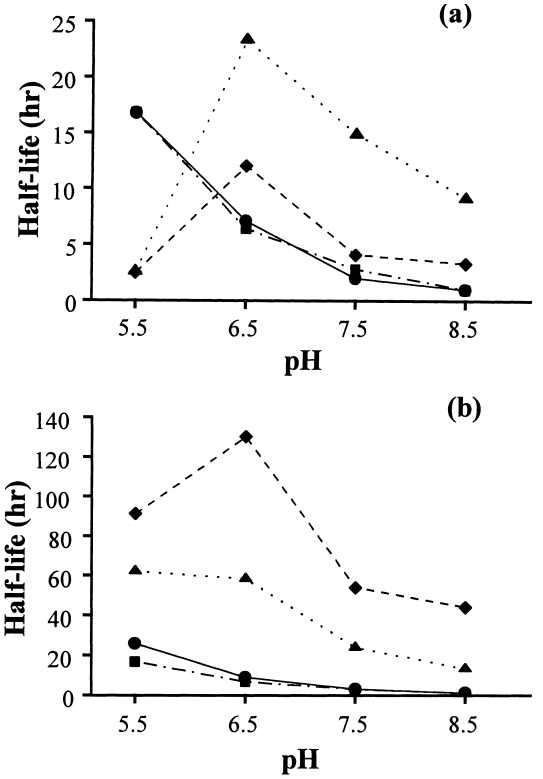

Fig. 2. PAI-1 stability at 37°C and different pH. a: In the absence of vitronectin; b: in the presence of vitronectin. The PAI-1 variants are indicated by: wtPAI-1 (●); Tyr^{221}His (▲); Tyr^{221}Phe (■) and Tyr^{221}Ser (◆).

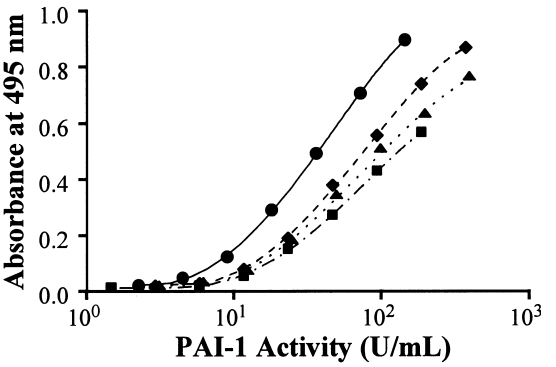

Fig. 3. The binding of some of the PAI-1 variants to vitronectin-coated microtiter plates as a function of PAI-1 activity concentration. The PAI-1 variants are shown by: wtPAI-1 (●); Tyr^{221}His (▲); Tyr^{221}Phe (■) and Tyr^{221}Ser (◆).

which mainly removes latent PAI-1 material. The similar specific activities obtained for the different PAI-1 variants suggest that the residue Tyr^{221} is of little importance for PAI-1 inhibitory function.

3.2. Formation of SDS-stable complexes between the PAI-1 variants and sctPA

When incubated with a slight excess of sctPA, virtually all of the PAI-1 purified on the anhydrotrypsin agarose column formed complexes with this enzyme (Fig. 1). Small amounts of a cleaved form of PAI-1 were generated from all PAI-1 variants during their reaction with sctPA, most likely due to the presence of small amounts of the substrate form of the inhibitor [25,26]. The portion of the different PAI-1 variants that were not adsorbed to the anhydrotrypsin agarose column also did not react with sctPA (Fig. 1).

3.3. Stability of PAI-1 variants

The stability of the PAI-1 variants as a function of temperature, in the absence or in the presence of vitronectin, is shown in Table 2. As can be seen, all PAI-1 variants displayed an increased stability at lower temperature. In the absence of vitronectin at 22°C, the half-lives of the PAI-1 variants were about 6–10-fold prolonged as compared to at 37°C. At 0°C, they were all almost completely stable (half-lives above 150 h).

The stability of the PAI-1 variants as a function of pH, in the absence or in the presence of vitronectin, is shown in Fig. 2a,b, respectively. At pH 6.5 and above, the PAI-1 mutants Tyr^{221}His and Tyr^{221}Ser were significantly more stable than wtPAI-1, both in the absence and in the presence of vitronectin. The half-lives of Tyr^{221}His were up to 7 times prolonged, both in the absence and in the presence of vitronectin. The half-lives of Tyr^{221}Ser were about doubled in the absence of vitronectin and 10–16-fold increased in its presence. However, at pH 5.5 in the absence of vitronectin, both these mutants were less stable (2.5 h) as compared to wtPAI-1 (16.8 h). Both in the absence and in the presence of vitronectin, wtPAI-1 was more stable at lower pH than at higher pH, which is in agreement with the behavior of 'native' PAI-1 in human plasma [6]. In contrast, however, the PAI-1 mutants Tyr^{221}His and Tyr^{221}Ser were maximally stable at pH 6.5, but lost quite dramatically in stability if pH was decreased to 5.5. In the presence of vitronectin, at pH 5.5, these two mutants gained considerably in stability, behaving almost as 'native' PAI-1. The PAI-1 mutant Tyr^{221}Phe had very similar stability properties to wtPAI-1 in the absence of vitronectin over the whole pH range studied. In contrast to the result with the other mutants, however, no effect on the stability properties could be found in the presence of vitronectin.

3.4. Binding of PAI-1 variants to vitronectin

The binding of PAI-1 mutants to vitronectin was studied utilizing vitronectin-coated microtiter plates. Fig. 3 shows the

Table 2
PAI-1 stability at pH 7.5 and 0, 22 or 37°C in the absence or presence of vitronectin

	In the absence of vitronectin			In the presence of vitronectin		
	0°C	22°C	37°C	0°C	22°C	37°C
wtPAI-1	>150	31.2	2	>150	67.6	3
Tyr^{221}His	>150	>150	14.8	>150	>150	23.8
Tyr^{221}Phe	>150	42.9	2.8	>150	50.4	2.8
Tyr^{221}Ser	>150	>150	4.1	>150	>150	53.7

For experimental details see text. The figures are given as half-lives (h).

binding curves of the different PAI-1 variants after purification on anhydrotrypsin agarose. Regarding the interaction with vitronectin, wtPAI-1 protein expressed in *E. coli* had an about 3-fold lower affinity to vitronectin-coated microtiter plates than reactivated PAI-1 obtained from HT1080 cells (data not shown). All the PAI-1 mutants studied in this report, Tyr^{221}His, Tyr^{221}Ser and Tyr^{221}Phe, seem to bind to vitronectin with a slightly decreased affinity as compared to wtPAI-1.

4. Discussion

The PAI-1 molecule, although synthesized as a fully active molecule, is rapidly converted to an inactive 'latent' form of the inhibitor at physiological conditions. For PAI-1 produced by the fibrosarcoma cell line HT1080 or native material in plasma samples, the half-life for this transition is about 2 h at neutral pH and 37°C in the absence of vitronectin, but is prolonged to about 4 h in the presence of vitronectin. The mechanism for this transition is not fully understood, but crystallographic data of the latent form [27] in connection with structural data from other serpins have suggested that insertion of the active site loop into the A β-pleated sheet structure present in serpins plays an important role. In this way the reactive center loop forms a new strand, between the original strands 2 and 3 of this β-pleated sheet structure, rendering the bait peptide bond in PAI-1 inaccessible to plasminogen activators. Recent data regarding the stabilization of complexes between serine proteinases and serpins have emphasized the importance of partial reactive site loop insertion as a result of the proteinase/serpin reaction [28–32].

In an elegant work by Berkenpas et al. [16], it was recently demonstrated with the aid of random mutagenesis and selection of clones with increased stability, that exchange of a large number of residues in different parts of the PAI-1 molecule did indeed result in a stabler molecule. However, none of the single-site changes caused any dramatic stabilization of the molecule, but with several changes in combination they reported a molecule with a half-life of about 150 h [16]. The changes found to stabilize the PAI-1 molecule were almost evenly distributed in the PAI-1 molecule, but with a tendency of a cluster, in the reactive center loop. Interestingly, no mutation was reported in strand 2 of the B β-sheet, but one was found in strand 1 and three in strand 3. $Tyrosine^{221}$, which is found in strand 2 of this β-structure, upon mutation to a His as reported in this paper, gives a molecule with a half-life of about 15 h. Mutation of the same tyrosine residue to serine gives a doubling of the stability in the absence of vitronectin. However, for this mutant an almost 20-fold increased stability is noted in the presence of vitronectin. The reason for the increased stability has not been clarified, but it is possible that introduction of a positive charge or hydrophilic side chain in this region, which is situated beneath the A β-structure, at about the point where insertion of the reactive center loop starts, might result in an increased difficulty for a spontaneous reactive center loop insertion into the A β-sheet. Whether the reason is a direct interaction with the reactive site loop, an increased stability in the β-structure, or just a steric hindrance for reactive peptide loop insertion is not known at present, but must be a subject of future studies. Nevertheless, since the activity of these mutants is not affected, insertion of the reactive site loop following the interaction with plasminogen activators must still proceed in a fairly normal way.

Another interesting observation is that the two mutants Tyr^{221}His and Tyr^{221}Ser have maximal stability at about pH 6.5 and that they again become more labile at pH 5.5. These data are in contrast to the results with wtPAI-1, native PAI-1 [6] and also regarding the PAI-1 mutant Tyr^{221}Phe, which are all more stable at pH 5.5 as compared to at higher pH. The anomalous behavior is mostly found in the absence of vitronectin. In the presence of vitronectin, all variants are very stable at pH 5.5. Only the mutant Tyr^{221}Ser is slightly more active at pH 6.5 as compared to pH 5.5. Due to this anomalous pH dependence it can be speculated that a histidine residue in the vicinity of Tyr^{221} might be of importance in maintaining the conformation in this portion of the molecule and that this plays a role for the active to latent PAI-1 transition. As seen in Fig. 2b, in the presence of vitronectin the pH dependence is much less pronounced, suggesting that changes induced in the PAI-1 mutants Tyr^{221}His and Tyr^{221}Ser are, at least partially, counteracted by the interaction with vitronectin.

It is also noted that the PAI-1 mutant Tyr^{221}Phe does not display an increased stability in the presence of vitronectin, in contrast to all other mutants studied, even if its binding to vitronectin is not dramatically altered. The reason for this is not known, but it can be speculated that a conformational change induced in PAI-1 by the interaction with vitronectin is blocked if Tyr^{221} is mutated to a phenylalanine.

Thus, our data suggest that Tyr^{221} and perhaps an interaction with a histidine residue in its vicinity is of importance for the transition of functional PAI-1 to the latent form. At present, work is being carried out to mutate the histidine residues His^{185}, His^{190}, His^{219} and His^{229}, which all are in quite close vicinity to Tyr^{221}, to find out if any one of these is of importance for the pH-dependent stability of PAI-1.

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