Functional effects of single amino acid substitutions in the region of Phe¹¹³ to Asp¹³⁸ in the plasminogen activator inhibitor 1 molecule

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Thirteen amino acid substitutions have been introduced within the stretch Phe¹¹³ to Asp¹³⁸ in the plasminogen activator inhibitor 1 (PAI-1) molecule by site-directed mutagenesis. The different proteins and wild-type (wt) PAI-1 have been overexpressed in *Escherichia coli* and purified by chromatography on heparin–Sephacrose and on anhydrotypsin–agarose. The PAI-1 variants have been characterized by their reactivity with tissue plasminogen activator (tPA), interactions with vitronectin or heparin, and stability. Most PAI-1 variants, except for Asp¹²⁵ → Lys, Phe¹²⁶ → Ser and Arg¹³³ → Asp, displayed a high spontaneous inhibitory activity towards tPA, which did not change greatly on reactivation with 4 M guanidinium chloride, followed by dialysis at pH 5.5. The variants Asp¹²⁵ → Lys and Arg¹³³ → Asp became much more active after reactivation and they were also more rapidly transformed to inactive forms ($t_{1/2}$ 22–31 min) at physiological pH and temperature than the other variants. However, in the presence of vitronectin they were both almost equally

stable ($t_{1/2}$ 2.3 h) as wtPAI-1 ($t_{1/2}$ 3.0 h). The mutant Glu¹³⁰ → Lys showed an increased stability, both in the absence and in the presence of vitronectin compared with wtPAI-1. Nevertheless a similar affinity between all the active PAI-1 variants and vitronectin was observed. Further, all mutants, including the three mutants with low activity, were to a large extent adsorbed on anhydrotypsin–agarose and were eluted in a similar fashion. In accordance with these data, the three variants with a low activity were all to a large extent cleaved as a result of their reaction with tPA, suggesting that they occurred predominantly in the substrate conformation. Our results do not support the presence of a binding site for vitronectin in this part of the molecule, but rather that it might be involved in controlling the active PAI-1 to substrate transition. Partly, this region of the PAI-1 molecule (Arg¹¹⁵ to Arg¹¹⁸) seems also to be involved in the binding of heparin to PAI-1.

INTRODUCTION

Plasminogen activator inhibitor 1 (PAI-1) is the physiological inhibitor of the tissue plasminogen activator (tPA) and urokinase. It is a major regulator of the fibrinolytic enzyme system *in vivo* and increased levels are correlated with the development of thrombotic disease, both myocardial infarction [1,2] and deep vein thrombosis [3,4]. PAI-1 belongs to the serpin superfamily of proteins and consists of a single polypeptide chain of 379 amino acid residues [5–7]. The peptide bond Arg³⁴⁶–Met³⁴⁷ constitutes the ‘bait’ region [8]. The functionally active form of PAI-1 converts spontaneously to a ‘latent’ form [9] and has a half-life of approx. 2 h under physiological conditions [10,11]. The latent form can be reactivated by denaturation by, for example, guanidinium chloride, followed by refolding during dialysis at slightly acidic pH. In addition, a third form of PAI-1 has been suggested, acting as a substrate for the plasminogen activators but without the ability to form stable complexes [12–14]. In plasma, virtually all active PAI-1 is bound to vitronectin [15,16]. This increases the stability of the active PAI-1 molecule, causing an approximate doubling of its half-life in the conversion to the latent form [11]. The reaction between PAI-1 and plasminogen activators is very fast, with a second-order rate constant of approx. $2 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$ [17]. Besides the interaction between the active site of tPA and the ‘bait’ peptide bond in PAI-1, interactions between other sites in the molecules also seem to be of importance for the high reaction rate [17,18]. In analogy with some of the other serpins [19], PAI-1 also binds heparin [20,21].

The reaction rate with thrombin is thereby increased by about two orders of magnitude [22], making this reaction fast enough to become physiologically significant. However, the reaction rate of PAI-1 with plasminogen activators seems not to be affected by the presence of heparin.

In the present study we have used site-directed mutagenesis to introduce 13 single amino acid substitutions in the region Phe¹¹³ to Asp¹³⁸ in PAI-1. This region constitutes one of two clusters of negatively charged residues present in the PAI-1 sequence. It has also been suggested to contain a vitronectin-binding site [23,24]. After expression in *Escherichia coli* and purification, the functional properties of the different PAI-1 variants were studied, to increase our knowledge about the different physiological functions of PAI-1.

MATERIALS AND METHODS

Materials

Acrylamide, *N,N'*-methylene bisacrylamide and anhydrotypsin–agarose were purchased from Sigma-Aldrich (Stockholm, Sweden). Coomassie Brilliant Blue G-250 Protein Assay kit was from Bio-Rad (Hercules, CA, U.S.A.). Imulyse PAI-1 kit (for PAI-1 antigen determination), Chromolize PAI-1 activity kit, Spectrolyse/PL PAI kit and single-chain tPA (sctPA) were from Biopool AB (Umeå, Sweden). Restriction enzymes and T₄ DNA ligase were from New England BioLabs (Beverly, MA, U.S.A.). DyNAzyme, the thermostable DNA polymerase for PCR amplification, was from Finnzymes Oy (Espoo, Finland).

Abbreviations used: PAI-1, plasminogen activator inhibitor 1; sctPA, single-chain tPA; tPA, tissue plasminogen activator; wt, wild-type.

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T₇ Sequencing Kit, heparin–Sephadex CL-6B, CM Sepharose CL-6B and Sephadex G-75 were all from Pharmacia AB (Uppsala, Sweden). The chromogenic tPA substrate S-2288 (*H*-D-isoleucyl-L-prolyl-L-arginine-*p*-nitroanilide dihydrochloride), was from Chromogenix AB (Mölnådal, Sweden). The expression vector used, pBV220, has two heat-inducible promoters, λP_R and λP_L [25]. Vitronectin was purified from plasma as described previously [26].

Expression and purification of recombinant PAI-1

Expression of recombinant PAI-1 was performed as described [27], with some modifications. The *E. coli* strain XL1 Blue transformed by the expression plasmid pBV220/PAI-1 was grown overnight at 30 °C in BHI medium (Detroit, MI, U.S.A.) containing 50 mg/l ampicillin and diluted at a ratio of 1:50 into 1 litre of the same medium. The diluted culture was grown at 30 °C until the attenuation at 650 nm reached 0.5 (approx. 2 h of incubation); the temperature was then increased sharply to 42 °C to induce PAI-1 synthesis. After 4 h (attenuance at 650 nm reached 1.5–1.6) the bacteria were harvested by centrifugation for 15 min at 4000 *g* and 4 °C. The bacterial pellet was digested by 200 μ g/ml lysozyme and sonicated (Sonifier B-12; Branson Sonic Power Co, Danbury, CT, U.S.A.) five times for 1 min at 90 W and 0 °C in 0.05 M sodium acetate buffer, pH 5.5, containing 0.1 M NaCl, 0.1 g/l Tween 80 and 0.5 g/l glycerol to break the bacterial cell walls. After another centrifugation at 12000 *g* for 30 min at 4 °C, the supernatant was used as the starting material for purification of recombinant PAI-1. The supernatant was applied to a heparin–Sephadex CL-6B column (bed volume approx. 10 ml) equilibrated with the buffer described above, but without glycerol. The unadsorbed components were washed out by at least 100 ml of the buffer at a flow rate of 12 ml/h. Gradient elution was performed by increasing the concentration of NaCl from 0.1 to 1.1 M in 200 ml of the equilibration buffer. The chromatograms were monitored by the absorbance at 280 nm and by measuring the PAI-1 antigen content. The fractions with a high concentration of PAI-1 antigen were pooled, and after dialysis against 0.05 M sodium acetate buffer, pH 5.2, containing 0.1 g/l Tween 80, the sample was loaded on a CM Sepharose CL-6B column (bed volume approx. 5 ml) equilibrated with the same buffer at a flow rate of 12 ml/h. The column was washed by at least 50 ml of the same buffer, then eluted with a 100 ml gradient of 0–0.8 M NaCl in the same buffer. Alternatively, to purify the 'active' PAI-1 variants, affinity chromatography on anhydrotypsin–agarose was employed, basically as described [28] but with some modifications. The anhydrotypsin–agarose column (bed volume 1 ml) was run and equilibrated with 0.15 M sodium phosphate buffer, pH 6.6, containing 0.1 M NaCl. After the sample (about 0.2 mg of PAI-1 variant) had been loaded on the column and washed the 'active' PAI-1 was eluted with 0.2 M sodium acetate buffer, pH 5.5, containing 0.1 M NaCl and 0.1 g/l Tween 80. Unless specifically stated, the material obtained from the anhydrotypsin–agarose chromatography was used in the subsequent experiments of functional characterization.

Characterization of recombinant PAI-1

The PAI-1 variants expressed in *E. coli*, without carbohydrate moieties, obviously had a different response to the monoclonal antibody in the Imulyse PAI-1 kit from the PAI-1 from HT1080 cells. Therefore wild-type (wt) PAI-1 was used as a standard in all PAI-1 antigen measurements. To determine the specific absorption coefficient of wtPAI-1, a solution of purified wtPAI-1, containing approx. 50% active and 50% latent material, was

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

Sequences are shown from 5' to 3'. Mutated nucleotides are printed in bold in all primers. The location of each 5' nucleotide is given; + and – indicate the coding and anti-sense strands respectively. All the oligonucleotides were synthesized as described in the Materials and methods section.

Primer	Application	Sequences of the oligonucleotides	Position
1	Phe ¹¹³ → Ser	AACAGCCTGAAGGAGTGGGGC	–353
2	Phe ¹¹⁴ → Ser	GAACAGCCTGGAGAAGTGGGG	–354
3	Arg ¹¹⁵ → Asp	GTGCTCCGGACAGATCGAAGAAG	–362
4	Phe ¹¹⁷ → Ser	CCGTGCTCCGGACAGCCTGAAG	–364
5	Arg ¹¹⁸ → Asp	TGCTTGACCGTGTATCGAACAGCC	–371
6	Asp ¹²⁵ → Lys	CTCCACCTCTGAAAATTTCACTTGCTTGAC	–393
7	Phe ¹²⁶ → Ser	CCACCTCTGAAGAGTCCACTTG	–391
8	Glu ¹²⁸ → Lys	GGCTCTCTCCACCTTTGAAA	–399
9	Glu ¹³⁰ → Lys	TCTGGCTCTTCCACCTCTGA	–402
10	Arg ¹³¹ → Asp	GATGAATCTGGCATCTCCACCTCTG	–408
11	Arg ¹³³ → Asp	TGATGATGAATCGGCTCTCTCA	–409
12	Phe ¹³⁴ → Ser	GTCATTGATGATGATCTGGC	–417
13	Asp ¹³⁸ → Lys	TGCTTCACCCATTATTGATGATG	–429
14	PCR	GTTTCCCACTGACGAC	on pUC 19
15	PCR	CCCAGGTTCTCGAGGGGCTTCTGAG	–884
16	DNA sequencing	GTACAAGGAGCTCATGGGGC	+237

scanned between 250 and 350 nm with a Kontron 800 spectrophotometer. The protein concentration was determined on the same solution by Coomassie Brilliant Blue G-250 binding. The specific absorption coefficient for wtPAI-1 at its absorption maximum (280 nm) was determined as 0.77 l·g⁻¹·cm⁻¹. This is in accordance with the content of aromatic amino acids in PAI-1. It has been reported that active and latent PAI-1 might have different absorption coefficients [29]. To investigate whether this also applied to our material, fully active PAI-1 (0.3 mg/ml) was incubated at pH 7.5 and 37 °C for 5 h, after which time approx. 80% of the PAI-1 had been converted to the latent form. Simultaneous recordings showed that the absorbances at 280 and 320 nm increased slightly during this period, but when the A_{320} was subtracted from the A_{280} , no significant change was observed during the incubation period. Thus the same absorption coefficient can be used for both latent and active PAI-1.

Mutagenesis of PAI-1 cDNA

The PAI-1 cDNA sequence coding mature wild-type human PAI-1, modified by the introduction of a *Xho*I site at bp 871 by a synonymous mutation (adenine nucleotide at position 873 replaced by a cytosine), was subcloned into the *Eco*RI and *Hind*III sites of pUC19. The plasmid generated, pUC19/PAI-1, was used as the template in the PCR amplification in which site-directed mutations were introduced by synthesized oligonucleotides (primers 1–15; Table 1). Oligonucleotides were synthesized by using a Gene Assembler Special system (Pharmacia) in accordance with the instructions of the manufacturer.

Mutations in the region Phe¹¹³ to Asp¹³⁸ were conducted by two-step PCR amplifications, as demonstrated schematically in Figure 1. The first PCR was performed on pUC19/PAI-1, by using primer 14 together with each of the primers 1 to 13 respectively. The products were purified by agarose gel electrophoresis and subsequently used as primers together with primer 15 in the second PCR. The *Eco*RI–*Hind*III fragment of PAI-1 cDNA was used as template. The PCR products were then digested by *Sac*I (at bp 244 of PAI-1 cDNA) and *Xho*I, and used to replace the corresponding fragment in PAI-1 cDNA in the

Figure 1 Schematic presentation of the procedure to introduce single-site mutations in PAI-1

The restriction enzymes are represented as E (*EcoRI*), S (*SalI*), X (*XhoI*) and H (*HindIII*).

expression vector. All the sequences in the mutated regions were confirmed by DNA sequencing with primer 16.

It is well known that *Taq* DNA polymerase has the property of frequently adding an extra adenosine nucleotide to the 3' terminus of double-stranded DNA. DyNAzyme has, in this respect, the same property. As a result, in the first PCR step, some of the PCR products occasionally had an extra 'A' at their 3' terminus, which might have generated an unwanted mutation in the PAI-1 cDNA in the second PCR step. With primers 1, 4, 8, 9, 12 and 13 (Table 1) this problem might be real, but for the other mutation primers the introduction of an extra A matches a T on the template, causing no changes, or causes a synonymous mutation. For the mutation primers in risk of obtaining unwanted mutations several clones were screened and sequenced to ensure that PCR products without an extra A were used in the subsequent PCR step.

Determination of PAI-1 antigen and activity

Determination of PAI-1 antigen concentration was performed with an ELISA method, utilizing a commercially available kit (Imulyse PAI-1). Purified wtPAI-1 was used as a standard. It was found that wtPAI-1 expressed in *E. coli* had a somewhat lower response with this kit than PAI-1 from plasma or from HT1080 cells. Determination of PAI-1 activity was performed in three different ways. Measurement of 'fast-acting' PAI-1 was performed with either the Chromolize PAI-1 activity kit or the Spectrolyse PL kit. In addition, a titration assay was also performed, with higher concentrations of the reactants and long

incubation time, to identify PAI-1 variants with a potentially decreased reaction rate. The tPA concentration was kept constant at 2 mg/l, and the concentration of most of the PAI-1 mutants was varied from 0 to 1 mg/l. Occasionally, with some of the PAI-1 variants with low activity, concentrations as high as 15 mg/l were used (only with material obtained after CM Sepharose chromatography). After incubation in microtitre plates for 20 min at ambient temperature, the residual tPA activity was measured by the addition of the chromogenic substrate S-2288 to a final concentration of 0.5 mM and recording the increase in absorbance at 405 nm. The residual tPA activity was plotted against the PAI-1 concentration. The straight lines obtained were extrapolated to zero tPA activity to get the PAI-1 concentration equal to the tPA concentration.

Reactivation of PAI-1

Most functional studies were performed on the purified PAI-1 variants before any reactivation. However, for comparison all mutants were also reactivated by treatment with 4 M guanidinium chloride at pH 5.5, followed by refolding during dialysis at 5 °C against 0.05 M sodium acetate buffer, pH 5.5, containing 0.1 M NaCl and 0.1 g/l Tween 80 [11]. The 'reactivated' PAI-1 mutants were subsequently subjected to the same functional studies.

Interaction of PAI-1 with vitronectin

The affinity of PAI-1 for vitronectin was tested by using vitronectin-coated microtitre plates, essentially as described earlier [30]. In brief, PAI-1 mutants at different concentrations (0–400 units/ml on a titration activity basis, 0–10 µg/ml on an antigen basis) were added to microtitre plates coated with vitronectin. After incubation for 1 h at ambient temperature the plates were washed and horseradish peroxidase-conjugated antibodies against PAI-1 were added. After another incubation for 1 h the plates were developed with *o*-phenylene diamine and H₂O₂. The absorbance at 492 nm was recorded and used as a measure of PAI-1 binding to vitronectin.

Stability of PAI-1 mutants

The stability of PAI-1 mutants was determined by incubating the PAI-1 mutants at pH 7.3 and 37 °C. At intervals, samples were collected, acidified slightly and subsequently analysed for PAI-1 activity. The procedure has been described in detail previously [11].

SDS/PAGE

SDS/PAGE was performed in a Mini-PROTEAN II electrophoresis apparatus (Bio-Rad) by the method of Laemmli [31]. Proteins were separated on 10 % (w/v) polyacrylamide gels and stained with Coomassie Brilliant Blue R-250.

RESULTS

Generation of PAI-1 mutants

Although DyNAzyme has higher fidelity than *Taq* DNA polymerase in the DNA synthesis of PCR [32,33], some unwanted mutations were still encountered in the screening and sequencing of PAI-1 mutants. In such cases the PAI-1 mutant cDNA species with unwanted mutations were discarded. Nevertheless, to make sure that no other unwanted changes existed in the PAI-1 mutants, all mutant molecules with obviously deviating inhibitory activity (i.e. Arg¹¹⁵ → Asp, Asp¹²⁵ → Lys, Phe¹²⁶ → Ser

Figure 2 Analysis of the recombinant wtPAI-1 expression in *E. coli* and the purified wtPAI-1

Lane 1, the protein molecular mass markers (in kDa); lanes 2 and 3, total cellular proteins expressed by the bacteria harbouring plasmid pBV220 or pBV220/PAI-1 respectively; lane 4, wtPAI-1 (1 μ g) purified by heparin–Sephacel and anhydrotypsin–agarose chromatography.

and Arg¹³³ → Asp) were completely sequenced in the coding cDNA regions. The other mutants were sequenced in the regions that were replaced by PCR fragments.

Expression, purification and characterization of PAI-1 variants

Recombinant PAI-1 expressed in *E. coli* with the pBV220 vector might exist both in the soluble cytoplasm and in inclusion bodies. Because the proteins in inclusion bodies are more likely to be denatured, the soluble PAI-1 in the cytoplasm was used as a source for purification. The soluble wtPAI-1 was determined as approx. 12 mg/l of culture fluid. The total purification yield of wtPAI-1 after heparin–Sephacel and CM Sepharose chromatography was approx. 40 % (approx. 5 mg from 1 litre of culture fluid). In heparin–Sephacel chromatography, wtPAI-1 was typically eluted at a NaCl concentration of approx. 0.8 M. The purified wtPAI-1 after anhydrotypsin–agarose chromatography was more than 95 % pure according to analysis by SDS/PAGE (Figure 2).

All PAI-1 variants could be purified by using the same combination of heparin–Sephacel and CM Sepharose or anhydrotypsin–agarose chromatography. The expression efficiencies and final yields varied somewhat between the different mutants. Most PAI-1 variants were expressed at high levels. The soluble forms of the two mutants Asp¹²⁵ → Lys and Phe¹²⁶ → Ser repetitively displayed a significantly lower yield in the cytoplasm than did the other variants. Interestingly, the two variants Arg¹¹⁵ → Asp (0.63 M NaCl) and Arg¹¹⁸ → Asp (0.58 M NaCl), but not Arg¹³¹ → Asp or Arg¹³³ → Asp, were eluted earlier under heparin–Sephacel chromatography than wtPAI-1 (0.80 M NaCl), suggesting a decreased affinity for heparin. With all mutants, including those with low activity (see below), approx. 50 % was adsorbed on the anhydrotypsin–agarose columns and eluted. The PAI-1 protein, not adsorbed on anhydrotypsin–agarose, did not have the ability to form stable complexes with tPA, nor was it cleaved by tPA (results not shown), presumably because it constituted ‘latent’ material.

Inhibitory activity of PAI-1 variants

The specific activities of the different purified PAI-1 variants (anhydrotypsin–agarose), as calculated from activity data ob-

Table 2 Relative inhibitory activity of PAI-1 variants towards tPA

The experiments were performed as described in the Materials and methods section. The inhibitory activity of the PAI-1 variants is expressed as percentage activity obtained compared with wtPAI-1, using data obtained with the Chromolize PAI-1 kit. All results are means $\pm$ S.D. for at least three separate experiments.

PAI-1 variant	PAI-1 activity (%)
Wild-type	100
Phe ¹¹³ → Ser	89.9 $\pm$ 9.47
Phe ¹¹⁴ → Ser	65.2 $\pm$ 14.10
Arg ¹¹⁵ → Asp	< 0.1
Phe ¹¹⁷ → Ser	75.0 $\pm$ 7.42
Arg ¹¹⁸ → Asp	60.0 $\pm$ 10.46
Asp ¹²⁵ → Lys	4.9 $\pm$ 0.89
Phe ¹²⁶ → Ser	0.1 $\pm$ 0.05
Glu ¹²⁸ → Lys	136.5 $\pm$ 23.04
Glu ¹³⁰ → Lys	53.0 $\pm$ 4.91
Arg ¹³¹ → Asp	60.7 $\pm$ 12.26
Arg ¹³³ → Asp	8.4 $\pm$ 0.45
Phe ¹³⁴ → Lys	121.1 $\pm$ 14.41
Asp ¹³⁸ → Lys	120.5 $\pm$ 23.27

tained with the Chromolize PAI-1 activity kit, are shown in Table 2. All figures are expressed as a percentage of the specific activity calculated for wtPAI-1 [1092000 $\pm$ 29000 (S.D.) units/mg, using the standards included in the kit]. The activities of all variants purified by heparin–Sephacel and CM Sepharose chromatography were also investigated by the titration method and occasionally also by the Spectrolyse PL PAI kit.

Three of the mutants, Asp¹²⁵ → Lys, Phe¹²⁶ → Ser and Arg¹³³ → Asp, had a drastically decreased activity in all assays used. However, for the mutant Arg¹¹⁵ → Asp a low specific activity was obtained with one of the activity kits (Chromolize PAI-1; less than 0.1 %) but a normal specific activity was found by using the titration assay or the Spectrolyse PL kit [115 % $\pm$ 12.5 (S.D.)]. The explanation for these results is probably that the replacement of Arg¹¹⁵ by Asp affected the binding of PAI-1 to the monoclonal antibody used in the Chromolize PAI-1 kit. All other mutants had similar, or only slightly changed, specific activities, compared with wtPAI-1.

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

The ability of the different ‘active’ PAI-1 variants to form stable complexes with sctPA was studied by SDS/PAGE after incubation in a slight excess of sctPA (on an activity basis). The results are shown in Figure 3. With wtPAI-1, as well as with most mutants, nearly all of the PAI-1 formed stable complexes with sctPA. In agreement with activity data, no stable complexes, or very small amounts of them, were observed after the reaction between sctPA and the PAI-1 variants Asp¹²⁵ → Lys, Phe¹²⁶ → Ser or Arg¹³³ → Asp. With all these variants, the PAI-1 moiety was almost completely transformed into a degraded form of the inhibitor. This was confirmed by mixing with some latent wtPAI-1 before SDS/PAGE (results not shown). Small amounts of this form could also be seen with some of the other mutants. This probably represents the cleaved form, obtained from the so-called substrate form of the inhibitor. In fact, on using different incubation periods, ranging from 1 min to 6 h, almost identical results were obtained (results not shown), suggesting that the formation of the degraded form is not a result of dissociation or

Figure 3 SDS/PAGE of mixtures of the PAI-1 variants and tPA

Approx. 25 pmol of each PAI-1 variant was mixed with approx. 45 pmol of scfPA in a phosphate buffer of pH 7.3 (in a final volume of 100 μ l). After 30 min of incubation at ambient temperature, 25 μ l of the mixture solution for each sample was analysed by SDS/PAGE performed as described in the Materials and methods section.

Table 3 PAI-1 stability at pH 7.3 and 37 °C in the absence or presence of vitronectin

For experimental details see the text. The figures are given as half-lives. Results are means $\pm$ S.D. for at least three separate experiments. Abbreviation: n.d., not determined.

PAI-1 variant	$t_{1/2}$ in the absence of vitronectin (h)	$t_{1/2}$ in the presence of vitronectin (h)
Wild-type	2.0 ± 0.08	3.0 ± 0.25
Phe ¹¹³ $\rightarrow$ Ser	1.6 ± 0.10	2.6 ± 0.10
Phe ¹¹⁴ $\rightarrow$ Ser	2.0 ± 0.16	3.3 ± 0.24
Arg ¹¹⁵ $\rightarrow$ Asp	2.0 ± 0.07	2.6 ± 0.12
Phe ¹¹⁷ $\rightarrow$ Ser	2.0 ± 0.07	2.7 ± 0.18
Arg ¹¹⁸ $\rightarrow$ Asp	2.2 ± 0.18	3.2 ± 0.17
Asp ¹²⁵ $\rightarrow$ Lys*	0.37 ± 0.03	2.3 ± 0.08
Phe ¹²⁶ $\rightarrow$ Ser	n.d.	n.d.
Glu ¹²⁸ $\rightarrow$ Lys	1.7 ± 0.10	2.2 ± 0.12
Glu ¹³⁰ $\rightarrow$ Lys	4.3 ± 0.30	6.8 ± 0.38
Arg ¹³¹ $\rightarrow$ Asp	2.8 ± 0.14	3.9 ± 0.24
Arg ¹³³ $\rightarrow$ Asp*	0.52 ± 0.08	2.3 ± 0.14
Phe ¹³⁴ $\rightarrow$ Lys	1.8 ± 0.09	2.0 ± 0.04
Asp ¹³⁸ $\rightarrow$ Lys	1.5 ± 0.10	1.9 ± 0.07

* Reactivated material.

Figure 4 Binding of some of the PAI-1 variants to vitronectin-coated microtitre plates as a function of PAI-1 activity

The following variants are shown: $\bullet$, wtPAI-1; $\blacktriangle$, Asp¹²⁵ $\rightarrow$ Lys; $\square$, Glu¹²⁸ $\rightarrow$ Lys; ∇ , Arg¹³¹ $\rightarrow$ Asp; $\diamond$, Arg¹³³ $\rightarrow$ Asp.

degradation of the complex with time but is something occurring rapidly in the initial phase of the reaction.

Stability of PAI-1 variants

The conversion of functionally active PAI-1 to the latent form was studied by incubating the PAI-1 variants at pH 7.3 and 37 °C for different periods in the absence or in the presence of vitronectin and subsequently measuring the residual PAI-1 activity with the Spectrolyse PL PAI kit. The decline in activity followed first-order kinetics for all variants tested. The calculated half-lives are summarized in Table 3. In the absence of vitronectin many of the variants, including wtPAI-1, had a half-life of approx. 2 h, in agreement with published data for PAI-1 from HT1080 cells [11]. Mutant Glu¹³⁰ $\rightarrow$ Lys was, however, more stable, with a half-life of 4.3 h. The three variants with very low activity could not be tested in this system. These were instead tested after reactivation by guanidinium chloride (see below). In the presence of vitronectin the half-life was prolonged to approx. 3 h for most mutants, which is quite similar to the values obtained with human PAI-1 from plasma or from HT1080 cells

[11]. However, mutant Glu¹³⁰ $\rightarrow$ Lys was more stable than wtPAI-1 also in the presence of vitronectin ($t_{1/2}$ approx. 7 h).

Binding of PAI-1 variants to vitronectin

The binding of PAI-1 variants to vitronectin was studied by using vitronectin-coated microtitre plates. The binding is demonstrated in Figure 4 as a function of PAI-1 activity. All of the functionally active PAI-1 mutants displayed similar binding curves to those obtained with wtPAI-1, but only the results for some of the more interesting variants are shown. The activity of mutant Phe¹²⁶ $\rightarrow$ Ser was too low to obtain meaningful results in this analysis. For comparison, latent wtPAI, obtained by incubating wtPAI-1 at 37 °C for 24 h at neutral pH (containing approx. 0.2 % active PAI-1), was also tested in the same system. A decrease in affinity for vitronectin to approx. 1/50 was observed for this material (results not shown).

Studies on reactivated PAI-1 variants

For most PAI-1 variants, including wtPAI-1, the specific activity did not change significantly as a result of the reactivation process. However, regarding mutants Asp¹²⁵ $\rightarrow$ Lys, Phe¹²⁶ $\rightarrow$ Ser and Arg¹³³ $\rightarrow$ Asp, which all were at the most only a few per cent

active, Asp¹²⁵ → Lys and Arg¹³³ → Asp gained considerably in specific activity during reactivation, but not Phe¹²⁶ → Ser. After reactivation and chromatographic purification on anhydrotrypsin-agarose, mutant Asp¹²⁵ → Lys had a specific activity of 450 000 units/mg (43 % as active as wtPAI-1), whereas the value for mutant Arg¹³³ → Asp was 330 000 units/mg (31 % as active as wtPAI-1). Furthermore, stability experiments with these reactivated variants (Asp¹²⁵ → Lys and Arg¹³³ → Asp) indicated a pronounced decrease in stability for both variants with half-lives of 22 and 31 min respectively (Table 3). This is the reason that these variants lost most of their activity during the production and purification procedures. However, in the presence of vitronectin these variants were almost as stable ($t_{1/2}$ 2.3 h) as wtPAI-1 ($t_{1/2}$ 3.0 h). Before treatment with guanidinium chloride, the two mutants Asp¹²⁵ → Lys and Arg¹³³ → Asp acted largely as substrates for tPA (Figure 3). However, as mentioned above, after reactivation they became 30–50 % active, owing to partial transformation into the 'active' conformation. Unfortunately, the concentrations of the mutants obtained after reactivation and subsequent stability test were too low to allow investigation with SDS/PAGE to settle the question of whether they were transformed into latent PAI-1 or substrate PAI-1 during the inactivation process.

On reactivation, the binding to vitronectin increased proportionally with PAI-1 activity for the variants Asp¹²⁵ → Lys and Arg¹³³ → Asp. On an activity basis, the binding curves were still very similar to those obtained with wtPAI-1 (results not shown).

DISCUSSION

Site-directed mutagenesis and subsequent functional investigations of the mutated proteins are powerful tools in the study of structure–function relations in proteins. With respect to PAI-1, such studies have been performed, mainly in the vicinity of the 'bait' peptide bond, Arg³⁴⁶-Met³⁴⁷ [34–38]. Some other regions of PAI-1 have also been studied as a result of randomized mutagenesis and identification of amino acid residues of importance for the interaction with vitronectin [23]. In addition, results obtained with monoclonal antibodies have suggested that the region between residues 110 and 145 in PAI-1 is involved in vitronectin binding [24]. In the present study we therefore decided to examine in more detail the functional role of this part of the PAI-1 molecule, which contains one of the two clusters of negatively charged residues (on a sequence basis). This was performed by site-directed mutagenesis in the cDNA region coding for the Phe¹¹³ to Asp¹³⁸ portion of the PAI-1 molecule. Within this stretch of residues in PAI-1, single-site mutants were produced where all of the acidic residues (Asp/Glu) were mutated towards a basic residue (Lys), all different phenylalanine residues were mutated to a serine residue and all basic residues (only Arg) were mutated to an acidic residue (Asp). To obtain this goal we basically followed the strategy of synthesizing DNA primers containing the desired mutations and then producing PCR products with these primers in combination with a common downstream primer. The PCR products containing the mutations were subsequently allowed to replace the corresponding stretch in the wild-type PAI-1 gene. After transfer to an expression system in *E. coli* and purification of the different PAI-1 mutants, these were characterized for their reactions with tPA, interactions with vitronectin or heparin, and stability.

Although one of our primary goals with this work was the identification and characterization of the vitronectin-binding site in the PAI-1 molecule, the results obtained from this point of view were somewhat disappointing because only minor effects could be detected unless the activity of the PAI-1 variants was

decreased, as with the mutants Asp¹²⁵ → Lys, Phe¹²⁶ → Ser and Arg¹³³ → Asp. The decrease in affinity for vitronectin seemed to parallel the decrease in activity. It is already known from earlier studies that the latent form of PAI-1 interacts with vitronectin with a much lower affinity [26], probably involving other sites, distant from or in close vicinity to the high-affinity sites. Previous results have also suggested that the high-affinity interaction between the N-terminal part of vitronectin and PAI-1 is stabilized by a combination of hydrophobic interactions and ionic interactions, involving one or several guanidino groups, either in the vitronectin molecule or in the PAI-1 molecule [30]. Therefore it is likely that the amino acid substitutions described here would, at least to some extent, affect the PAI-1–vitronectin interaction if the high-affinity binding site for vitronectin in PAI-1 were located in this part of the molecule. Previous work, employing mutagenesis [23] and with the aid of monoclonal antibodies [24], has indicated that this region might contain the 'high-affinity' binding site interacting with the N-terminal portion of vitronectin. As discussed below, our results are somewhat in conflict with this theory. As already mentioned, three mutants had a very low activity, even after purification on anhydrotrypsin-agarose (Asp¹²⁵ → Lys, Phe¹²⁶ → Ser and Arg¹³³ → Asp). Furthermore SDS/PAGE analysis after incubation with tPA revealed that these mutants consisted to a very large extent of the substrate form of the inhibitor (Figure 3). Thus the results on adsorption on anhydrotrypsin-agarose and cleavage by tPA show that the reactive site loop of PAI-1 is exposed in all three of these mutants. The major portions of these mutants, existing in the substrate form, did not bind to vitronectin with a high affinity; only the portions of active PAI-1 showed this property. After treatment with guanidinium chloride and subsequent dialysis, causing regeneration of PAI-1 activity (only with mutants Asp¹²⁵ → Lys and Arg¹³³ → Asp), the interaction with vitronectin increased in proportion to the increase in PAI-1 activity. Thus it seems that modifications in this part of the molecule by itself do not prohibit the interaction with vitronectin but rather that they cause conformational changes elsewhere in the molecule, increasing the likelihood of the substrate conformation. Therefore none of the mutated amino acids in this work seem to be involved in the binding of vitronectin.

The three mutants primarily existing in the substrate conformation are also interesting in that they do not form stable complexes with tPA. The initial attack of tPA to the 'bait' peptide bond seems to be fairly normal, but for some reasons the inactive stable protease–inhibitor complex is not formed in these cases. Instead, the 'bait' peptide bond is rapidly hydrolysed, as has been described for other PAI-1 variants [12]. An explanation might be that mutagenesis in the Asp¹²⁵ to Arg¹³³ part of PAI-1 induces conformational changes, affecting also other and distant parts in the vicinity of the reactive site of the molecule.

After reactivation, the two mutants Asp¹²⁵ → Lys and Arg¹³³ → Asp were found to have a faster than normal turnover rate in the transition from the active to the latent form of the inhibitor. Thus it seems that this part of the molecule is of importance in maintaining the active conformation of PAI-1, perhaps in prohibiting the insertion of the 'bait' peptide bond loop into the A β -pleated sheet structure in this inhibitor [39–42]. It is possible that the two residues Asp¹²⁵ and Arg¹³³ in the functional PAI-1 molecule interact with other charged residues via ionic interactions, and that disruption of these interactions causes conformational changes, facilitating the 'bait' peptide loop insertion. In contrast, mutant Glu¹³⁰ → Lys displayed an increased stability in the absence of vitronectin, in contrast with all other PAI-1 variants studied. In the presence of vitronectin, however, mutants Asp¹²⁵ → Lys and Arg¹³³ → Asp had half-lives

of 2.3 h, which is not too different from that of wtPAI-1 (3.0 h), suggesting that vitronectin can compensate at least in part for the conformational change obtained with these mutations. Mutant Glu¹³⁰ → Lys also displays a comparable increase in stability in the presence of vitronectin as in its absence. The Asp¹²⁵ to Arg¹³³ portion of the molecule therefore seems to be of importance in stabilization of the active PAI-1 conformation, perhaps by preventing, in one way or another, the insertion of the 'bait' peptide loop in the A β -pleated sheet structure.

It is well known from previous studies that PAI-1 interacts with heparin [20,21], favouring its complex formation with thrombin [22]. In this respect, our results suggest that Arg¹¹⁵ and Arg¹¹⁸, but not Arg¹³¹ or Arg¹³³, are involved in the heparin binding. Previous results by Ehrlich et al. [43] have suggested that residues Arg⁷⁶, Lys⁸⁰ and Lys⁸⁸ are also important for heparin binding. Interestingly, from the proposed three-dimensional structure of PAI-1 [41], these two stretches are close to each other. Also this seems to correspond, at least partly, to the region that binds heparin in the antithrombin molecule [19]. The reaction between the different PAI-1 variants described here and thrombin has not been investigated.

We conclude that the Phe¹¹³ to Asp¹³⁸ region in the PAI-1 molecule is an important part of the molecule, mainly in regulating the conformational state regarding stability and the transition to the substrate form.

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REFERENCES

- Hamsten, A., de Faire, U., Walldius, G., Dahlen, G., Szamosi, A. X., Landou, C., Blombäck, M. and Wiman, B. (1987) *Lancet* **ii**, 3–9
- Cortellaro, M., Cofrancesco, E., Boschetti, C., Mussoni, L., Donati, M. B., Cardillo, M., Catalano, M., Gabrielli, L., Lombardi, B., Specchia, G. et al. (1993) *Arterioscler. Thromb.* **13**, 1412–1417
- Paramo, J. A., Alfaro, M. J. and Rocha, E. (1985) *Thromb. Haemost.* **54**, 713–716
- Eriksson, B. I., Eriksson, E., Gyzander, E., Teger-Nilsson, A. C. and Risberg, B. (1989) *Acta Orthop. Scand.* **60**, 159–163
- Ginsburg, D., Zeheb, R., Yang, A. Y., Rafferty, U. M., Andreasen, P. A., Nielsen, L., Dano, K., Lebo, R. V. and Gelehrter, T. D. (1986) *J. Clin. Invest.* **78**, 1673–1680
- Ny, T., Sawdey, M., Lawrence, D., Millan, J. L. and Loskutoff, D. J. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 6776–6780
- Pannekoek, H., Veerman, H., Lambers, H., Diergaarde, P., Verweij, C. L., van Zonneveld, A. J. and van Mourik, J. A. (1986) *EMBO J.* **5**, 2539–2544
- Lindahl, T. L., Ohlsson, P. I. and Wiman, B. (1990) *Biochem. J.* **265**, 109–113
- Hekman, C. M. and Loskutoff, D. J. (1985) *J. Biol. Chem.* **260**, 11581–11587
- Levin, E. G. and Santell, L. (1987) *Blood* **70**, 1090–1098
- Lindahl, T. L., Sigurdardottir, O. and Wiman, B. (1989) *Thromb. Haemost.* **62**, 748–751
- Declerck, P. J., De Mol, M., Vaughan, D. E. and Collen, D. (1992) *J. Biol. Chem.* **267**, 11693–11696
- Urano, T., Strandberg, L., Johansson, L. B. and Ny, T. (1992) *Eur. J. Biochem.* **209**, 985–992
- Audenaert, A. M., Knockaert, I., Collen, D. and Declerck, P. J. (1994) *J. Biol. Chem.* **269**, 19559–19564
- Wiman, B., Almqvist, A., Sigurdardottir, O. and Lindahl, T. (1988) *FEBS Lett.* **242**, 125–128
- Declerck, P. J., De Mol, M., Alessi, M. C., Baudner, S., Paques, E. P., Preissner, K. T., Muller-Berghaus, G. and Collen, D. (1988) *J. Biol. Chem.* **263**, 15454–15461
- Chmielewska, J., Rånby, M. and Wiman, B. (1988) *Biochem. J.* **251**, 327–332
- Madison, E. L., Goldsmith, E. J., Gerard, R. D., Gething, M. J., Sambrook, J. F. and Bassel-Duby, R. S. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 3530–3533
- Carrell, R. W., Evans, D. L. and Stein, P. E. (1991) *Nature (London)* **353**, 576–578
- Lindahl, T. and Wiman, B. (1989) *Biochim. Biophys. Acta* **994**, 253–257
- Ehrlich, H. J., Keijer, J., Preissner, K. T., Gebbink, R. K. and Pannekoek, H. (1991) *Biochemistry* **30**, 1021–1028
- Keijer, J., Linders, M., Wegman, J. J., Ehrlich, H. J., Mertens, K. and Pannekoek, H. (1991) *Blood* **78**, 1254–1261
- Lawrence, D. A., Berkenpas, M. B., Palaniappan, S. and Ginsburg, D. (1994) *J. Biol. Chem.* **269**, 15223–15228
- van Meijer, M., Gebbink, R. K., Preissner, K. T. and Pannekoek, H. (1994) *FEBS Lett.* **352**, 342–346
- Wang, X. M., Li, Y. Y., Jin, Q., Zhang, Z. Q. and Hou, Y. D. (1992) *Sci. China B* **35**, 84–91
- Sigurdardottir, O. and Wiman, B. (1990) *Biochim. Biophys. Acta* **1035**, 56–61
- Sui, G. C., Sun, H., Zhang, M. and Hu, M. H. (1997) *Biochem. Mol. Biol. Int.* **42**, 621–629
- Fa, M., Karolin, J., Aleshkov, S., Strandberg, L., Johansson, L. B. and Ny, T. (1995) *Biochemistry* **34**, 13833–13840
- Vaughan, D. E., Declerck, P. J., Reilly, T. M., Park, K., Collen, D. and Fasman, G. D. (1993) *Biochim. Biophys. Acta* **1202**, 221–229
- Sigurdardottir, O. and Wiman, B. (1992) *Fibrinolysis* **6**, 27–32
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Tindall, K. R. and Kunkel, T. A. (1988) *Biochemistry* **27**, 6008–6013
- Mattila, P., Korpela, J., Tenkanen, T. and Pitkanen, K. (1991) *Nucleic Acids Res.* **19**, 4967–4973
- Lawrence, D. A., Strandberg, L., Ericson, J. and Ny, T. (1990) *J. Biol. Chem.* **265**, 20293–20301
- Shubeita, H. E., Cotter, T. L., Franke, A. E. and Gerard, R. D. (1990) *J. Biol. Chem.* **265**, 18379–18385
- York, J. D., Li, P. and Gardell, S. J. (1991) *J. Biol. Chem.* **266**, 8495–8500
- Sherman, P. M., Lawrence, D. A., Yang, A. Y., Vandenberg, E. T., Paielli, D., Olson, S. T., Shore, J. D. and Ginsburg, D. (1992) *J. Biol. Chem.* **267**, 7588–7595
- Tucker, H. M. and Gerard, R. D. (1996) *Eur. J. Biochem.* **237**, 180–187
- Mottonen, J., Strand, A., Symersky, J., Sweet, R. M., Danley, D. E., Geoghegan, K. F., Gerard, R. D. and Goldsmith, E. J. (1992) *Nature (London)* **355**, 270–273
- Aertgeerts, K., De Bondt, H. L., De Ranter, C. and Declerck, P. J. (1994) *J. Struct. Biol.* **113**, 239–245
- Aertgeerts, K., De Bondt, H. L., De Ranter, C. J. and Declerck, P. J. (1995) *Nature Struct. Biol.* **2**, 891–897
- Shore, J. D., Day, D. E., Francis-Chmura, A. M., Verhamme, I., Kvassman, J., Lawrence, D. A. and Ginsburg, D. (1995) *J. Biol. Chem.* **270**, 5395–5398
- Ehrlich, H. J., Gebbink, R. K., Keijer, J. and Pannekoek, H. (1992) *J. Biol. Chem.* **267**, 11606–11611