The length of the reactive center loop modulates the latency transition of plasminogen activator inhibitor-1

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Abstract

Plasminogen activator inhibitor-1 (PAI-1) belongs to the serine protease inhibitor (serpin) protein family, which has a common tertiary structure consisting of three β -sheets and several α -helices. Despite the similarity of its structure with those of other serpins, PAI-1 is unique in its conformational lability, which allows the conversion of the metastable active form to a more stable latent conformation under physiological conditions. For the conformational conversion to occur, the reactive center loop (RCL) of PAI-1 must be mobilized and inserted into the major β -sheet, A sheet. In an effort to understand how the structural conversion is regulated in this conformationally labile serpin, we modulated the length of the RCL of PAI-1. We show that releasing the constraint on the RCL by extension of the loop facilitates a conformational transition of PAI-1 to a stable state. Biochemical data strongly suggest that the stabilization of the transformed conformation is owing to the insertion of the RCL into A β -sheet, as in the known latent form. In contrast, reducing the loop length drastically retards the conformational change. The results clearly show that the constraint on the RCL is a factor that regulates the conformational transition of PAI-1.

Keywords: conformational transition; kinetic trap; latency transition; loop length; plasminogen activator inhibitor-1; protein folding; serpin

Plasminogen activator inhibitor-1 (PAI-1) acts as the major inhibitor of fibrinolysis by inhibiting tissue-type and urokinase-type plasminogen activators (tPA and uPA, respectively) in vivo (Vaughan 1998). These plasminogen activators cleave plasminogen to generate plasmin, a broad-specificity protease. Balances between plasminogen activators and their inhibitors are important in maintaining hemostasis and in the turnover of the extracellular matrix (van Meijer and Pannekoek 1995). A deficiency in PAI-1 may cause unopposed attacks by target proteases against normal tissues, which may result in hemorrhagic episodes in humans (Loskutoff et al. 1989). Elevated levels of plasma PAI-1 are

associated with a number of diseases, including deep-vein thrombosis (Juhan-Vague et al. 1987), atherosclerosis (Schneiderman et al. 1992), and noninsulin-dependent diabetes mellitus (Juhan-Vague and Alessi 1997). PAI-1 also controls cell migration, through binding to vitronectin (Stefansson and Lawrence 1996) and urokinase receptor-mediated cell adhesion (Deng et al. 1996). PAI-1 activity is regulated at several levels. Although the transcriptional level of the *PAI-1* gene is high in hepatocytes and endothelial cells, PAI-1 activity is usually low in plasma, because the protein spontaneously undergoes a conformational conversion from the active native state to an inactive latent conformation, with a functional half-life of 1–2 h at 37°C (Levin and Santell 1987). Binding to vitronectin stabilizes the active conformation of PAI-1 about twofold (Levin and Santell 1987). Another level of regulation is achieved by uPA receptors through endocytosis and degradation of PAI-1/uPA complexes (Heegaard et al. 1995; Rodenburg et al. 1998).

PAI-1 belongs to the serine protease inhibitor (serpin) superfamily, which has a common tertiary structure com-

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Abbreviations: PAI-1, plasminogen activator inhibitor-1; serpin, serine protease inhibitor; RCL, reactive center loop; tPA, tissue-type plasminogen activator; uPA, urokinase-type plasminogen activator.

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posed of three β -sheets and several α -helices (Huber and Carrell 1989). The serpins include protease inhibitors present in blood plasma such as α_1 -antitrypsin, α_1 -antichymotrypsin, antithrombin III, PAI-1, C1-inhibitor, and α_2 -antiplasmin (Huber and Carrell 1989; Stein and Carrell 1995). Two salient structural features of inhibitory serpins are their native metastability and the mobility of the reactive center loop (RCL). The most stable state of the majority of proteins is the biologically active native form (Anfinsen 1973), but the native form of serpins is a metastable state. In the native conformation of serpins (Fig. 1A), the RCL is presented as a pseudo-substrate for binding to the target protease (Elliott et al. 1996). Target proteases cleave the RCL of serpins, allowing a portion of the RCL to insert into the serpin's A β -sheet (Wright and Scarsdale 1995), and the protease tethered to the inserted RCL is structurally distorted, resulting in the formation of an SDS stable protease-serpin complex (Huntington et al. 2000). In the latent form of serpins (Fig. 1B), the first strand of C β -sheet (s1C) is peeled off from the sheet, and the RCL is inserted into A β -sheet, without the RCL cleavage (Mottonen et al. 1992). Therefore, the latent form is more stable than the native form (Hekman and Loskutoff 1985; Wang et al. 1996) but is inactive. Usually, the spontaneous conformational conversion of the metastable native state into the stable latent form is kinetically blocked by a high energy barrier. PAI-1 is unique among inhibitory serpins in that the latent form can be produced within hours under physiological conditions (Lawrence et al. 1989), whereas for other serpins, mild denaturing or pathological conditions are necessary to induce the conver-

sion to the latent form (Carrell et al. 1991, 2001; Lomas et al. 1995; Chang and Lomas 1998; Gooptu et al. 2000; Jung and Im 2003). Latent forms of antithrombin III (Carrell et al. 1994), α_1 -antitrypsin (Lomas et al. 1995; Im et al. 2002), and α_1 -antichymotrypsin (Chang and Lomas 1998) have been obtained, but none of these serpins undergoes conformational conversion as readily as does PAI-1.

Although both the native (Sharp et al. 1999) and latent structures (Mottonen et al. 1992) of PAI-1 are known (Fig. 1), the factors that cause this molecule to rapidly undergo a conformational switch to the latent form are not clear. The identification of the factors that are responsible for the unique conformational lability of PAI-1 is of great scientific and clinical interest. The examination of the structure of the latent form suggests that the turn (the so-called "gate" region) between the third and fourth strands of C β -sheet (s3C and s4C, respectively) must be lifted for the conformational switch to occur (Mottonen et al. 1992). Protein engineering of the gate region has increased the functional half-life of active PAI-1 about two- to threefold (Tucker et al. 1995). As the RCL needs to be inserted into A β -sheet during the latency transition, the incorporation of charged residues into the proximal RCL region also retards the conformational conversion (Tucker et al. 1995). Helix F and the subsequent turn must be mobilized during the insertion of the RCL, and variations in these regions also affect the conformational transition rate (Wind et al. 2003). The wild-type residues in the PAI-1 shutter domain (the region underlying the opening of β -sheet A, which is mobilized upon formation of the inhibitory complex) are not likely to be responsible for the conformational lability of PAI-1, as the introduction of mutations that encode sequences conserved among the serpins accelerate the conformational conversion rather than retard it, as in other serpins (Hansen et al. 2001). However, no single mutation in PAI-1 has efficiently reduced the latency conversion to a rate comparable to those of other serpins.

Given that the RCL must be mobilized for the conformational transition to occur, the constraint in the RCL polypeptide connection is likely to impede the conversion of the native serpins into the RCL-inserted stable form. In this study, we modulated the length of the RCL of PAI-1 to determine if the length of the loop is critical in regulating the conformational conversion in this uniquely labile serpin, or if other factors are more important in the facile latency transition of this protein.

Results

Extension of the RCL facilitated conformational conversion of PAI-1 into a urea-stable form, and reduction of the RCL retarded the transition

We examined whether there is a relationship between the length of the RCL and the rate of the conformational con-

Figure 1. A schematic diagram of PAI-1. (A) The native structure (Sharp et al. 1999; 1B3K.pdb). The β -sheets A is indicated as black strands, and the RCL and the first strand of β -sheet C (s1C) is indicated in light gray. The scissile peptide bond, recognized by tPA and uPA, is indicated with an arrow. Glu 350, where insertion or deletion of a few amino acids was introduced, is shown as an inverted triangle. (B) The structure of the latent form (Mottonen et al. 1992; 1DVN.pdb). The β -sheets A, the RCL, and the first strand of β -sheet C (s1C) are colored as in the native form. The N-terminal portion of the scissile peptide bond is inserted into β -sheet A without cleavage, forming the fourth strand of the β -sheet A (s4A; indicated in light gray). These figures were prepared using ViewerLite (Accelrys Inc.).

Figure 2. Conformational conversion of PAI-1 mutants. The wild-type (Wt) and mutant PAI-1 proteins were incubated in a buffer (45 mM phosphate, 70 mM NaCl, 0.01% Tween 80, pH 7.4) at 37°C for 1 h. Conformational conversion was monitored on transverse urea gradient gels, containing a gradient of 0–8 M urea perpendicular to the direction of electrophoresis. The migration positions of the native and the urea-stable forms are indicated with arrows.

version to the latent form in the conformationally labile serpin, PAI-1. We first generated several PAI-1 variants with various lengths in the RCL region. One or two additional glycine(s) were inserted between P3' (Pro 349; the third residue C-terminal to the scissile peptide bond) and P4' (Glu 350; the fourth residue C-terminal to the scissile peptide bond) positions in the RCL of PAI-1 (Fig. 1) by cassette mutagenesis. The insertion mutants containing one and two extra glycine residues were designated Ins1 and Ins2, respectively. In other variants, one glutamate residue at P4' or two glutamates at P4' and P5' positions were deleted, and the mutant proteins were designated Del1 and Del2, respectively. After expression and purification of the variant PAI-1 proteins, the conformation of the mutant serpins was analyzed by transverse urea gradient gel electrophoresis (Fig. 2). In this gel system, the electrophoretic mobility of proteins depends on the hydrodynamic volumes of different conformational states, induced by urea-dependent denaturation. With gradually increasing urea concentrations, the native wild-type PAI-1 exhibited a cooperative unfolding transition. The latent PAI-1 was more stable, remaining intact in higher concentrations of urea than the native form. Initially, the mutant PAI-1 proteins folded into the native form with stabilities comparable to that of the wild-type PAI-1 (Fig. 2, top panel). However, the insertion mutants were most easily converted into a urea-stable form when incubated at 37°C at pH 7.4. Most of the Ins1 molecules were converted into the urea-stable conformation during incubation for 1 h, whereas only 40% of the wild-type molecules were transformed into the urea-stable form under the same conditions (Fig. 2, bottom panel). The conformational transition rate of Ins2 was even more rapid, and a portion of Ins2 was converted to the urea-stable form even during incubation at 4°C in a storage buffer (20 mM sodium acetate, pH 5.6, 0.5 M NaCl), under which conditions the wild-type PAI-1 was stable for at least several months (data

not shown). The deletion mutants also folded into the native form with stabilities similar to that of the native wild-type PAI-1 (Fig. 2, top panel). However, the reduction of the length of the RCL greatly retarded the conformational transition into a urea-stable form. During incubation at 37°C for 1 h, most of the Del1 molecules remained in the native conformation, whereas about half of the wild-type molecules were transformed to the urea-stable form (Fig. 2, bottom panel). Del2 was even more resistant to the conformational transition. These results clearly show that the extension of the RCL promoted the structural conversion of PAI-1 to a urea-stable form, and reducing the length of the loop drastically retarded the structural conversion of PAI-1.

Stability was drastically increased by conformational transition

The conformational stability of the native mutant proteins was quantitatively measured by urea-dependent equilibrium unfolding experiments, which monitor intrinsic changes in fluorescence upon the unfolding of proteins. Consistent with the above results, that the native form of PAI-1 mutants showed conformational stabilities similar to that of the wild-type PAI-1 (Fig. 3). The unfolding transition midpoints were 2.0 ± 0.1 M urea in all cases except Del2, in which the transition midpoint was slightly shifted to 1.8 ± 0.1 M urea. The results correspond to a free energy change ($\Delta\Delta G$) of less than 0.4 kcal mol⁻¹.

The wild-type, Ins1, and Ins2 proteins were allowed to convert into the urea-stable form, and the stable form was

Figure 3. Conformational stability of native PAI-1 variants. Urea-induced equilibrium unfolding transitions were measured by changes in fluorescence emission intensity at 333 nm ($\lambda_{\text{ex}} = 295$ nm). (●) The native wild-type; (▼) the native Ins1; (■) the native Ins2; (○) the native Del1; (▽) the native Del2.

purified using an anhydrotypsin column as described in Materials and Methods. As only very small amounts of the deletion PAI-1 mutants are converted to urea-stable forms, even after prolonged incubation, the urea-stable forms of these mutants were not purified. The stabilizing effects of the conformational switch induced by incubation at 37°C were drastic, in that the equilibrium unfolding transition midpoints were shifted from 0.8 ± 0.1 M guanidine for the native form to 3.2 ± 0.1 M guanidine for the urea-stable forms of both Ins1 and Ins2, conditions that are very similar to that of the wild-type latent form (3.2 M).

Proteolytic susceptibility of the urea-stable form

The increase in conformational stability of the converted PAI-1 proteins may be attributable to the insertion of the RCL into A β -sheet, as occurs in the latent form. This possibility was investigated by probing the accessibility of the RCL to proteases that cleave the exposed RCL of the native PAI-1 (Kjøller et al. 1996). The incubation of the native forms of the wild-type and Ins1 proteins with either porcine pancreatic elastase or *Staphylococcus aureus* V8 protease generated species with molecular masses of about 38 kDa in all cases (Fig. 4). Under the same cleavage conditions, smaller amounts of Ins2 protein were cleaved by these proteases. Likewise, Ins2 formed very little amounts of inhibitory complex with the target protease, tPA, whereas the native wild-type and Ins1 proteins were able to form an SDS-resistant inhibitory complex with the protease (Figs. 4, 5B). It appears that the configuration of the RCL in Ins2 is not optimal for protease recognition. The incubation of the native wild-type and Ins1 proteins with trypsin produced small amounts of an SDS-stable complex with a molecular mass of about 63 kDa and a cleavage product with a molecular mass of about 38 kDa, as expected, but only a small amount of cleavage product was produced from Ins2 by this protease.

When the purified urea-stable forms of insertion mutants were treated with porcine pancreatic elastase or *S. aureus* V8 protease, the RCLs of the proteins were resistant to proteolysis. These results suggest that the protease recognition sequences on the RCLs of the urea-stable forms are inaccessible, just as in the latent form (Fig. 4). The urea-stable form of the wild-type and insertion mutants also failed to react with tPA, the target protease (Fig. 4), further supporting the idea that in the urea-stable form the RCL has inserted into the β -sheet, where it is inaccessible to proteolytic attack. The latent form exhibits a distinct pattern upon digestion by trypsin, which specifically hydrolyzes the K193-S194 peptide bond in the gate region (Egelund et al. 1997), whereas the native form is cleaved by this enzyme at the P1-P1' bond (Kjøller et al. 1996). The trypsin digestion pattern of the urea-stable form was similar that of the latent form (Fig. 4). This result suggests that the conformation of

Figure 4. Limited proteolysis of PAI-1 to probe the conformation of the RCL in the urea-stable form. The native wild-type (Wt), native Ins1, native Del1, and the latent wild-type, Ins1, and Ins2 proteins were treated with trypsin (T), porcine pancreatic elastase (PE), *Staphylococcus aureus* V8 protease (V8), or tPA. Digestion patterns were analyzed by 12% SDS-polyacrylamide gel electrophoresis. The migration positions of molecular mass standards (lane M; Bio-Rad Co., low range) are indicated on the left of the gels. U is the untreated PAI-1 protein. The locations of inhibitory complexes with tPA (Cp), tPA, un-reacted PAI-1 (PAI-1), the RCL-cleaved PAI-1 (Cl_{RCL}), and two cleaved latent PAI-1 fragments by trypsin (Cl_T) are marked accordingly.

the urea-stable form is similar to that of the latent form, exposing a protease-susceptible site in the gate region. Protease accessibility experiments indicate that the stability increase following the conformational change of the PAI-1 mutants is owing to the insertion of the RCL into A β -sheet as the fourth strand, as in the latent form.

Measurement of conversion rates

The kinetics of the latency transition were visually monitored by urea-containing gel electrophoresis (Fig. 5A). In the transverse urea gradient gels, the native and latent PAI-1 forms exhibited unfolding transitions with midpoints at ~ 2 and ~ 6 M urea, respectively (Fig. 2). This result suggests that in the presence of 4 M urea, the active conformation is unfolded, but the latent form remains intact. Therefore, we included 4 M urea in the acidic nondenaturing polyacrylamide gel system described by Jovin (1973). In this gel system, latent PAI-1 yielded a single band of high electrophoretic mobility as expected, and the native PAI-1 yielded almost exclusively a low-mobility species, as expected for the unfolded form (Fig. 5A). During incubation at 37°C, the

Figure 5. Conformational conversion rates of PAI-1 variant proteins. (A) Conversion rates were followed by appearance of the urea-stable form on polyacrylamide gels containing 4 M urea. The migration positions of the native form and the latent form are indicated with arrow heads. (B) Inhibitory complex formation of the native PAI-1 variants with tPA. One microgram of native PAI-1 proteins was incubated with (+) or without (–) 3 μ g of tPA, and the formation of the SDS-resistant PAI-1-tPA complex was analyzed by 10% SDS-polyacrylamide gel electrophoresis. The locations of inhibitory complexes (Cp), tPA, un-reacted PAI-1 (PAI-1) and cleaved PAI-1 (CI) are marked accordingly. (Lane M) Molecular mass standards (Bio-Rad Co., low range). (C) The conversion rates of the wild-type, Ins1, and Del1 proteins were followed by inhibitory activity loss. After PAI-1 proteins were incubated at 37°C for various times, the remaining inhibitory activity was measured with urokinase using Spectrozyme UK as a substrate. (●) The wild-type; (▼) Ins1; (○) Del1 PAI-1.

native wild-type PAI-1 gradually transformed into the urea-stable form. The latency transition was facilitated in Ins1, and the conversion rate of Ins2 was even more rapid than that of Ins1, with most of the Ins2 molecules rapidly converting into the urea-stable species within 10 min. In contrast, the conformational transitions of the Del1 and Del2 proteins were drastically retarded, with negligible formation of the urea-stable forms during incubation for 4 h at 37°C.

The inhibitory activity of the mutant PAI-1 was examined by monitoring the formation of an SDS-stable complex with the target protease, tPA (Fig. 5B). The deletion or insertion of one amino acid in the RCL did not abolish the inhibitory activity of the PAI-1 proteins. However, altering the RCL length by more than one residue seriously hampered the ability of the protein to form an inhibitory complex, probably as a result of changes in the configuration of the RCL (Fig. 5B, Ins2 and Del2). These proteins were barely recognized by tPA, as judged by their remaining mostly intact during incubation with the protease.

Given that the RCL of the latent form is inserted into A β -sheet and is not available for protease binding, it does not form an inhibitory complex with target proteases (Gils and Declercq 1997; Fig. 4). For the wild-type, Del1, and Ins1 proteins, whose native forms retained inhibitory activity, the latency conversion was followed by a loss of inhibitory activity during incubation at 37°C (Fig. 5C). Consistent with the conversion rates monitored by gel electrophoresis (Figs. 2, 5A), the extension of the RCL by one residue promoted the conformational switch of the PAI-1 protein, shifting the functional half-life of Ins1 to 26 min from the 80 min of the wild-type PAI-1. However, most of the Del1 molecules remained active throughout the experiment, with an increase in the functional half-life to more than 24 h. As the Ins2 and Del2 mutants did not exhibit inhibitory activity toward target proteases (Fig. 5B), the conversion rates could not be measured by the loss of inhibitory activity. However, the conversion rates obtained by inhibitory activity assays were compatible with those from the gels containing 4 M urea shown in Fig. 5A, at least for the wild-type, Ins1, and Del1 proteins.

Discussion

The kinetic trap in the latency transition

Our results show that the extension of the RCL of PAI-1 facilitated its conformational conversion to a stable form. The increase in stability and proteolytic resistance suggest that this stable form adopts a conformation similar to that of the latent form, in which the RCL is inserted into A β -sheet. The facile latent transition may be the result of either destabilization of the native state or lowering of the kinetic barrier per se. Therefore, we analyzed the effects of the mutations on the native stability and conversion rate. In the

insertion mutants, the stability of the native form changed only negligibly, within the experimental error ranges, as measured by equilibrium unfolding in the presence of guanidine (Fig. 3). If the effects of the mutations are limited to destabilization of the native state, simply destabilizing the native state ($\Delta\Delta G < 0.2 \text{ kcal mol}^{-1}$) should facilitate the conversion of no more than 40% more of the protein, as occurs for the wild-type PAI-1. However, the functional half-lives of the mutants were drastically decreased, and the measured conversion rates were at least three and 20-fold that of the wild-type PAI-1 for Ins1 and Ins2, respectively (Fig. 5). Meanwhile, the latency transition of Del1 was reduced by over 20-fold, but the native stability of the protein was comparable to that of the wild-type molecule. The native form of Del2 was only slightly destabilized compared with the wild-type PAI-1 ($\Delta\Delta G$ of $0.4 \text{ kcal mol}^{-1}$), but the effect on the latency transition was drastic; no formation of any latent form was observed. Therefore, changes in the conversion rates can be reasonably explained by modification of the kinetic barrier. These results suggest that the constraint on the RCL owing to a polypeptide connection is a critical factor that prevents the conversion of PAI-1 into the lowest energy state and that the extension of the RCL allows the native PAI-1 to bypass the kinetic trap. Meanwhile, reducing the length of the loop arrests PAI-1 in the kinetic trap, and only negligible amounts of latent form were detected during incubation at 37°C for 4 h in deletion mutants (Fig. 5C).

Conformational lability of PAI-1

We searched for a correlation between the length of the RCL and the latency transition rates among inhibitory serpins. Surprisingly, the RCL of PAI-1, a labile serpin, contained the same number of residues as that of α_1 -antitrypsin, a conformationally stable serpin. The RCL region of α_1 -antichymotrypsin, another serpin that does not spontaneously convert into the latent form, contains four more residues than that of PAI-1 (Huber and Carrell 1989). Therefore, the difference in the lengths of the RCLs of inhibitory serpins is not correlated with an intrinsic tendency to convert into the latent form. Our previous studies of α_1 -antitrypsin, a prototype serpin, show that releasing the constraint by extension (Im et al. 2000) or cleavage of the RCL is sufficient to bypass the barrier to folding to the lowest energy state (Im and Yu 2000). However, changing the length of the loop affected the conformational conversion of PAI-1 more than that of α_1 -antitrypsin: The addition of one residue was effective in facilitating the conformational conversion of PAI-1, whereas the insertion of more than three residues was required to promote the structural conversion of α_1 -antitrypsin (Im et al. 2000). Also, reducing the loop length by one residue was sufficient to significantly retard the conversion rate of PAI-1, and removing two residues

from the loop almost prevented the latency conversion. These results indicate that other factors also contribute to the intrinsic conformational lability of the PAI-1 molecule to induce a weak conformational trigger. Single mutations in PAI-1 that stabilize the native conformation and modestly decrease the latency transition rate have been reported. The stabilization of the gate region (Tucker et al. 1995), helix F (Wind et al. 2003), or s2B (Sui and Wiman 1998; Vleugels et al. 2000) retarded the latency transition by several fold. The destabilization of the latent form by introducing negative charges into the inserted RCL also moderately increased the functional half-life of the native form (Tucker et al. 1995). The effects of Del1 and Del2 on the latency transition were the greatest thus far obtained among the PAI-1 variants carrying single mutations. Multiple mutations scattered at several locations in the PAI-1 molecule were necessary to delay the latency conversion to degrees comparable to those of other serpins (Berkenpas et al. 1995; Stoop et al. 2000). PAI-1 appears to have evolved a unique conformational lability, possibly for the fine-tuning of its physiological activity, which utilizes several devices throughout the molecule, especially at strategic points such as the gate region and the helix F.

Inhibitory activity of the mutant PAI-1 proteins and biological implications

It was surprising that the insertion or deletion of one residue in the RCL did not abolish the inhibitory activity of these proteins toward target proteases. A naturally occurring mutant serpin, α_2 -antiplasmin Enschede, which contains an insertion of an alanine between residues P8 and P12 of the RCL, acts as a substrate of plasmin rather than an inhibitor (Holmes et al. 1987a). The N-terminal portion of the RCL must be inserted into A β -sheet to form a stable acyl-enzyme complex, and the length of this portion is appropriate to cause a distortion in the active site of the target protease. It is reasonable to assume that shortening this portion of the RCL prevents the full insertion of the RCL and thus stable inhibitory complex formation, resulting in hydrolysis of the acyl-enzyme complex, and that extension of this portion would not cause distortion of the active site. As our PAI-1 mutants contain changes only at positions C-terminal to the cleavage site, their RCLs may have altered configurations that affect recognition by proteases. However, the length of the inserted RCL of acyl-enzyme complexes, once formed, would be the same as in the wild-type PAI-1, providing sufficient torsion on the enzyme active site. Similarly, it has been reported that the deletion of the P1' Met of α_2 -antiplasmin has little effect on its activity (Holmes et al. 1987b). Indeed, our preliminary measurement of reaction constants by surface plasmon resonance using a BiaCore 2000 (D. I. Biotech) showed that the insertion and deletion of one residue at P4' site decreased the association rate constant about

100-fold as compared with wild-type PAI-1, but had no detectable effect on the dissociation rate constant of the inhibitory complex.

Other proteins also fold into metastable states, and the cleavage of an internal loop induces a conformational switch into a presumably more stable form, similar to serpins. The cleavages of the precursors of influenza hemagglutinin (Chen et al. 1998), α -lytic protease (Sauter et al. 1998), and subtilisin (Gallagher et al. 1995) induce conformational rearrangements that separate the residues adjacent to the scissile bond by over 20 Å. The design of these proteins is such that they only fold into a metastable state when they are in appropriate conditions, after which they induce a conformational rearrangement into a more stable form through internal cleavage. It will be interesting to examine if the extension of the loop at the cleavage site in these proteins also releases the strain present in the precursor forms, allowing a conformational switch into a more stable form.

Materials and methods

Materials

E. coli strain BL21(DE3) pLysS (Novagen, Inc.) is used for expression of recombinant PAI-1. Spectrozyme UK and Spectrozyme TPA were purchased from American Diagnostica, Inc. Urokinase was purchased from Green Cross BioTech Co. and tPA was from Genentech Inc. SP-sepharose fast flow was purchased from Amersham Bioscience Co., and an anhydrotypsin-agarose affinity column was from Panvera. Bio-Rad DC (detergent compatible) protein assay kit was from Bio-Rad Laboratories, Inc.. Ultrapure urea and guanidine hydrochloride were purchased from ICN Biochemicals. *S. aureus* V8 protease, porcine pancreatic elastase, and human trypsin were purchased from Sigma. All other chemicals were reagent grade.

Mutagenesis and expression of PAI-1 in *Escherichia coli*

The cDNA encoding human PAI-1 was amplified by the polymerase chain reaction (PCR), and cloned into the expression vector, pRSET B (Invitrogen Co.), between *Bgl*II and *Eco*RI sites, as described (Lee and Im 2003). The resulting plasmid for human PAI-1 expression in *E. coli* was named as pRSET-PAI1. Unique *Bss*HII and *Eco*RV sites were introduced on pRSET-PAI1 at P2 (Ala 345) and P5' (Glu 351) positions on the PAI-1-coding sequence, respectively, and the resulting plasmid is named as pPAI1-BE. To modulate the RCL length of PAI-1, each pair of complementary oligonucleotides, inserting one or two extra glycine(s) or removing one or two glutamate(s) at P4' site were synthesized: 5'-CGCGTATGGCCCCCGGTGAGGAG-3' and 5'-CTCCTCACCGGGGGCCATA-3' 5'-CGCGTATGGCCCCCGGTGAGGAG-3' and 5'-CTCCTCGCCACCGGGGGCCATA-3' 5'-CGCGTATGGCCCCCGAG-3' and 5'-CTCGGGGGCCATA-3' 5'-CGCGTATGGCCCCCGAG-3' and 5'-GGGGGGCCATA-3', respectively. Oligonucleotides were phosphorylated at 5'-termini, mutually annealed, and then replaced the *Bss*HII-*Eco*RV fragment of pPAI1-BE. The mutated sequences were confirmed by dideoxynucleotide

DNA sequencing. Recombinant PAI-1 was expressed as inclusion bodies in *E. coli*, and refolded as described previously (Lee and Im 2003). Refolded protein was applied to an SP-sepharose column, which is pre-equilibrated with 20 mM sodium acetate, 0.5 M NaCl, 0.01% Tween 80 (pH 5.6), and PAI-1 protein was eluted using a 0.5–1.5 M NaCl gradient in the buffer. To obtain a stable form of PAI-1 proteins, recombinant PAI-1 proteins were incubated at 37°C in 45 mM phosphate, 70 mM NaCl, 0.01% Tween 80 (pH 7.4). The latent form was purified as described previously (Gils et al. 1996), using an anhydrotypsin-agarose column equilibrated with 50 mM sodium phosphate, 50 mM NaCl (pH 7.4). The RCL-inserted latent form does not bind to anhydrotypsin, and thus flows through the column, while the native form binds to the column and is eluted with 0.3 M arginine in the buffer (Sui et al. 1999). Concentrations of PAI-1 were determined using Bio-Rad DC protein assay kit, using bovine serum albumin as a standard.

Denaturant-induced equilibrium unfolding transition

Unfolding of the native forms as a function of urea (ICN Biomedicals, Inc.) was monitored by fluorescence spectroscopy (λ_{ex} = 295 nm and λ_{em} = 333 nm, excitation and emission slit widths = 5 nm for both). The protein concentration was 20 $\mu\text{g ml}^{-1}$, and the buffer was 20 mM sodium acetate (pH 5.6), 0.5 M NaCl, 0.01% Tween 80. The native protein was incubated in the buffer containing various concentrations of urea at 25°C for 2 h. The PAI-1 protein was stable in this acidic, high salt buffer (Lee and Im 2003), retaining full activity at 25°C for more than a week. Experimental data were fitted to a two-state unfolding model (Pace et al. 1989).

To quantitatively measure the conformational stability of the urea-stable forms, guanidine hydrochloride (ICN Biomedicals, Inc.) was used to unfold the proteins. Unfolding was monitored by changes in circular dichroism (CD) signals at 222 nm. The protein concentration was 4 μM in a buffer (20 mM sodium acetate, pH 5.6, 0.5 M NaCl, 0.01% Tween 80). The protein was incubated in the buffer containing various concentrations of guanidine at 25°C for 2 h. CD spectra were measured on a Jasco-720 spectropolarimeter in a 1-mm path-length cell at 25°C.

Conformational analysis by gel electrophoresis

Conformation of PAI-1 proteins was analyzed by transverse urea gradient gel electrophoresis (Goldenberg 1989) with slight modifications. Transverse urea gradient gels were prepared with a gradient of 0 ~ 8 M urea perpendicular to the direction of electrophoresis with an opposing gradient of acrylamide from 15% to 11% in 260 mM acetic acid, and pH was adjusted to pH 4.0 with KOH. A mixture of 5 mg/L riboflavin and 1.25 ml/L TEMED was used to initiate polymerization of acrylamide. Four slab gels (100 × 80 mm) were prepared simultaneously in a multigel caster (Hoefer Scientific Instruments) by using a gradient maker and a single-channel peristaltic pump. The PAI-1 protein (20 μg in 100 μL) was applied across top of the gel. The electrode buffer was 40 mM β -alanine, adjusted to pH 4.0 with acetic acid, and the tracking dye was methyl green. The gels were run at a constant current of 6 mA for 5 h at a controlled temperature of 25°C. PAI-1 protein migrated toward the anode. The protein bands were visualized by Coomassie Brilliant Blue staining.

To follow latency transition of PAI-1 proteins, recombinant PAI-1 proteins were incubated at 37°C in 45 mM phosphate, 70 mM NaCl, 0.01% Tween 80 (pH 7.4), for various times. Conformational transition of PAI-1 proteins was analyzed using a non-

denaturing gel system. Nondenaturing acidic gel system with a low pH discontinuous buffer system was first described by Jovin (1973) and modified by inclusion of 4 M urea (Lee and Im 2003). The latent form of PAI-1 migrated faster toward the anode than the native form. The protein bands were visualized by Coomassie Brilliant Blue staining.

Protease-accessibility of the RCL in the urea-stable conformation of PAI-1

The conformation of the stable form was investigated by probing the accessibility of the RCL to proteases, which are known to cleave the exposed RCL of the native PAI-1 (Kjøller et al. 1996). The native form of wild-type, Ins1, and Ins2 proteins, and the urea-stable form of wild-type, Ins1, and Ins2 proteins, were incubated with porcine pancreatic elastase at a molar ratio of 50:1 (PAI-1:protease) at 37°C for 30 min. The buffer was 30 mM phosphate, 160 mM NaCl, 0.1% PEG6000, and 0.1% Triton X-100 (pH 7.4). *S. aureus* V8 protease was incubated with the PAI-1 proteins at 37°C for 1 h, at a molar ratio of 1:0.2 in a buffer containing 0.1 M Tris-HCl (pH 7.8). The PAI-1 protein was 3 µg in total volume of 30 µL in all reactions. Trypsin was incubated with PAI-1 protein at a ratio of 1:15 at 37°C for 30 min. The buffer was 0.01 M Tris-HCl (pH 8.0). PAI-1 protein was incubated with 2 µg of tPA in a buffer (30 mM phosphate, pH 7.4, 160 mM NaCl, 0.1% PEG, 0.1% Triton X-100) at 37°C for 30 min. The reaction products were analyzed by 12% SDS-polyacrylamide gel electrophoresis and the protein bands were stained with Coomassie Brilliant Blue.

Complex formation with the target protease

Formation of inhibitory complex of PAI-1 with tPA was examined by monitoring the appearance of SDS-resistant covalent complexes on a SDS-polyacrylamide gel. One µg of PAI-1 protein was incubated with 3 µg of tPA in a buffer (30 mM phosphate, pH 7.4, 160 mM NaCl, 0.1% PEG, 0.1% Triton X-100) at 37°C for 30 min. Appearance of the SDS-resistant inhibitor-protease complex was monitored by 10% SDS-polyacrylamide gel electrophoresis, and the protein bands were visualized by Coomassie Brilliant Blue staining.

Measurement of conversion rates

The latency conversion rates of PAI-1 mutants were followed by the loss of inhibitory activity. PAI-1 proteins were taken at various time points during incubation at 37°C and the remaining inhibitory activity was determined. PAI-1 proteins were incubated with 20 units of uPA in 50 µL of uPA assay buffer (0.15 M NaCl, 50 mM Tris-Cl, 0.01% Tween 80, 100 µg/ml BSA, pH 7.5) at 37°C for 10 min. The reaction mixture was diluted 20-fold with the assay buffer and the residual proteolytic activity of uPA was measured with 50 µM Spectrozyme UK, according to the manufacturer's instruction (American Diagnostica, Inc.). The amounts of products were measured at 410 nm using a Beckman DU-650 spectrophotometer. The experimental data were fitted to a single exponential decay.

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