Specific interactions of serpins in their native forms attenuate their conformational transitions

YU-RAN NA AND HANA IM

Department of Molecular Biology, Sejong University, Kwangjin-gu, Seoul 143-747, Korea

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Abstract

Plasminogen activator inhibitor-1 (PAI-1) belongs to the serine protease inhibitor (serpin) protein superfamily. Serpins are unique in that their native forms are not the most thermodynamically stable conformation; instead, a more stable, latent conformation exists. During the transition to the latent form, the first strand of β -sheet C (s1C) in the serpin is peeled away from the β -sheet, and the reactive center loop (RCL) is inserted into β -sheet A, rendering the serpin inactive. To elucidate the contribution of specific interactions in the metastable native form to the latency transition, we examined the effect of mutations at the s1C of PAI-1, specifically in positions P4' through P10'. Several mutations strengthened the interactions between these residues and the core protein, and slowed the transition of the protein from the metastable native form to the latent form. In particular, anchoring of the strand to the protein's hydrophobic core at the beginning (P4' site) and center of the strand (P8' site) greatly retarded the latency transition. Mutations that weakened the interactions at the s1C region facilitated the conformational conversion of the protein to the latent form. PAI-1's overall structural stability was largely unchanged by the mutations, as evaluated by urea-induced equilibrium unfolding monitored via fluorescence emission. Therefore, the mutations likely exerted their effects by modulating the height of the energy barrier from the native to the latent form. Our results show that interactions found only in the metastable native form of serpins are important structural features that attenuate folding of the proteins into their latent forms.

Keywords: kinetic trap; latency transition; plasminogen activator inhibitor-1; protein folding; serine protease inhibitors

The native form of most proteins is their most thermodynamically stable state; however, exceptions do exist, including serpins (serine protease inhibitors) (Ny et al. 1986; Pannekoek et al. 1986) and the viral membrane fusion proteins of influenza and HIV (Wiley and Skehel 1987). The serpin superfamily includes plasma protease inhibitors, such as α_1 -antitrypsin (α_1 AT), α_1 -antichymo-

trypsin, antithrombin III, plasminogen activator inhibitor-1 (PAI-1), C1-inhibitor, and α_2 -antiplasmin (Stein and Carrell 1995). PAI-1, a single-chain glycoprotein of 379 amino acids, is the primary inhibitor of both tissue- and urokinase-type plasminogen activator (tPA and uPA, respectively). Thus, PAI-1 regulates plasmin-catalyzed extracellular proteolysis, including fibrinolysis (Vaughan 1998) and turnover of the extracellular matrix (van Meijer and Pannekoek 1995). X-ray crystal structure analyses have shown that serpins share a common tertiary structure composed of three β -sheets and several α -helices (Huber and Carrell 1989). In the metastable native form, the reactive center loop (RCL) of the serpin, which contains the target protease-recognition site, is exposed (Stein and Carrell 1995). Upon cleavage of the RCL by the target protease, the RCL is inserted as a β -strand into its own

Reprint requests to: Hana Im, Department of Molecular Biology, Sejong University, 98 Gunja-dong, Kwangjin-gu, Seoul 143-747, Korea; e-mail: hanaim@sejong.ac.kr; fax: 82-2-3408-3661.

Abbreviations: α_1 AT, α_1 -antitrypsin; PAI-1, plasminogen activator inhibitor-1; serpin, serine protease inhibitor; RCL, reactive center loop; tPA, tissue-type plasminogen activator; uPA, urokinase-type plasminogen activator; sXY, the Xth strand of β -sheet Y.

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β -sheet (β -sheet A) with the protease attached in acyl-form. The first strand of β -sheet C (s1C) is still attached to β -sheet C in this complex form. Complex formation dramatically increases the stability of the serpin molecule (Bruch et al. 1988), and the energy stored in the metastable native form may facilitate RCL insertion during the formation of the inhibitory complex with the target protease (Lee et al. 2000).

The metastable native form of serpins can be converted into the “latent” form independent of interactions with target proteases. The X-ray structures of the latent forms of PAI-1 (Fig. 1; Mottonen et al. 1992) and antithrombin III (Carrell et al. 1994), as well as a mutant version of α_1 AT (Im et al. 2002), have been reported. In the latent form, the RCL is inserted into β -sheet A without cleavage by a target protease. Since the s1C is connected to the C-terminal portion of the RCL, it must be detached from β -sheet C to allow RCL insertion without cleavage (Fig. 1). The latent form can revert to the metastable native form upon denaturing and refolding (Hekman and Loskutoff 1985); therefore, the metastable native form can be considered as a kinetically trapped folding intermediate. Although the cleaved serpin in the acyl-serpin–protease complex is more stable than the latent form thermodynamically, the cleaved form is not considered to be the same polypeptide as the native serpin from the perspective of protein folding, since it is divided into two polypeptides. The cleaved form (and the acyl-serpin–

protease complex) cannot revert to the metastable native form upon denaturing and refolding.

Although the recent accumulation of genomic data has increased interest in connecting the gap between amino acid sequences and their tertiary structures, protein folding remains an unsolved enigma. The identification of folding intermediates would contribute to our understanding of protein folding, but, unfortunately, such intermediates are usually short-lived unstable structures, thereby hampering detailed studies. According to the theory of protein folding under kinetic control, the native state of metastable proteins exists at a local energy minimum, and conversion to the lower-energy state is blocked by a remarkably high folding barrier (Baker and Agard 1994). Therefore, the metastable native form of serpins is analogous to a kinetically trapped folding intermediate. While the transition to the latent form cannot be observed under physiological conditions for most serpins, PAI-1 converts from its metastable native form to the latent conformation on an easily measurable timescale, with a functional half-life ($t_{1/2}$) of 1–2 h at 37°C (Levin and Santell 1987). Thus, PAI-1 is a good model for studying protein folding intermediates, and for elucidating the structural determinants that affect the conversion of proteins into their fully folded forms. Nonnative stable interactions during protein folding may produce kinetic traps that result in long-lived intermediates. Similarly, specific interactions within the metastable native forms may somehow prevent their conversion into a more stable structure (Carr et al. 1997). Since the s1C must be detached from β -sheet C for conformational conversion to the latent form to occur, interactions with the s1C exist only in the metastable native form (i.e., a folding intermediate) and not in the latent form (i.e., the final product of folding). In this study, we examined the specific factors that affect the conversion of PAI-1 into its latent form, including whether stabilization or destabilization of interactions at the s1C effects the kinetic barrier that prevents folding of the protein into a more stable state.

Results

Design of the s1C mutations

To understand how specific interactions within the native form of PAI-1 affect its transition to the latent form, various substitutions were introduced into the s1C (Table 1). To evaluate the contribution of each residue within the s1C, alanine-scanning mutagenesis was performed from the P4' (Glu350; the fourth residue C-terminal to the scissile peptide bond at the RCL) to the P10' site (Arg356; the tenth residue C-terminal to the cleavage site). Residues found in the functionally more stable

Figure 1. The native structure (1B3K.pdb) (Sharp et al. 1999) and the latent structure (1DVN.pdb) (Mottonen et al. 1992) of PAI-1, showing the s1C region. (Blue strands) β -sheet C; (orange) the RCL. (Ball and stick model) The residues of the s1C region, color-coded by atoms. (Pink) Leu272 on s2C and Arg30 on s6B, described in the text. The native structure of a quadruple mutant PAI-1 has Ile at the Met354 site. In the latent form, the N-terminal portion of the scissile peptide bond is inserted into β -sheet A without cleavage, forming the fourth strand of β -sheet A (s4A; indicated in orange) and s1C is peeled away from β -sheet C. These figures were prepared using the ViewerLite program (Accelrys Inc.).

Table 1. Designing scheme of PAI-1 mutations

Locus	P4' (Glu350)	P5' (Glu351)	P6' (Ile352)	P7' (Ile353)	P8' (Met354)	P9' (Asp355)	P10' (Arg356)
Ala-scanning	Ala	Ala	Ala	Ala	Ala	Ala	Ala
Homology	Pro, Val, Leu		Phe	Lys, Glu	Phe, Val	Asn	
Charge (exposed)/Size (buried)	Lys, Gln	Lys, Gln	Leu	Lys, Glu	Ile, Val, Leu	Asn	Glu

serpins α_1 AT, antithrombin III, and c1-inhibitor, were also introduced into PAI-1. To evaluate charge effects, the negatively charged residues Glu350, Glu351, and Asp355 were replaced with either the uncharged residues Gln or Asn, or with the positively charged amino acid Lys. Meanwhile, the positively charged residue Arg356 was substituted by the negatively charged residue Glu. The residues pointing toward the hydrophobic protein core, Ile352 (P6') and Met354 (P8'), were substituted by several hydrophobic residues of various sizes. Using these altered versions of PAI-1, the residues that affect the protein's transition to the latent form and its overall structural stability were determined.

Transition rates of the PAI-1 mutants

The recombinant PAI-1 proteins were expressed in *Escherichia coli* and purified as described previously (Lee and Im 2003). All mutant proteins possessed inhibitory activity, as indicated by the formation of SDS-stable inhibitory complexes with tPA. The kinetics of the latency transition was visually monitored by electrophoresis on gels containing 4 M urea (Fig. 2A). By this method, the metastable native PAI-1 unfolds to yield a single-protein band of low electrophoretic mobility, while the latent form remains intact and yields a high-mobility species (Lee and Im 2003). During incubation at 37°C, wild-type PAI-1 gradually transitioned from the metastable native conformation into the urea-stable latent form with an approximate $t_{1/2}$ of 2 h (Fig. 2A). Given that the RCL of the latent form is inserted into β -sheet A and is not available for protease binding, it does not form an inhibitory complex with target proteases (Gils and Declerck 1997). The conformational change at 37°C was accompanied by a decrease in the formation of SDS-stable inhibitory complexes with tPA (Fig. 2B). The rate of latency conversion was quantified as the loss of inhibitory activity against uPA during the incubation at 37°C (Fig. 2C). Transition rates for representative PAI-1 mutants are shown in Figure 2, and the results for all mutant proteins are summarized in Table 2.

The P4' site (Glu350) is where the RCL connects to the s1C, and proline is found at this position in α_1 AT. An E350P substitution in PAI-1 increased its $t_{1/2}$ from 92 min (wild type) to 140 min (Fig. 2). The effect of mutations at this site seems to depend on the size of the residues

introduced. Substitution by small residues (e.g., Ala and Val) facilitated the transition of PAI-1, while residues of similar size (e.g., Leu and Gln) did not greatly affect the transition rate compared to wild type; substitution with the large, positively charged amino acid Lys slowed the transition by ~60%.

On the other hand, compared to substitutions at Glu350, substitutions at the P5' site (Glu351) had the opposite effect on the transition from the metastable native to the latent form. Substitution by Ala retarded the transition, while the similarly sized amino acid Gln had little effect; Lys decreased the functional half-life of the protein by ~60%. In the native structure of PAI-1 (Fig. 1A; Sharp et al. 1999), Glu351 is squeezed by the turn between the RCL and the s1C, and O $_{\delta 1}$ of Glu351 has an unfavorable polar–nonpolar interaction with the C $_{\delta 1}$ atom of Leu272 in s2C at a distance of 3.7 Å.

Substitution of Ile352 at the P6' position by a small residue such as Ala dramatically increased the conversion to the latent form ($t_{1/2} < 10$ min), possibly by introducing a cavity inside the PAI-1 molecule. Meanwhile, substitution by Ile or Phe had only minor effects on the transition. Substitutions at the P7' site (Ile353) produced similar results: PAI-1 with I353A rapidly converted to the latent form (Fig. 2), while substitution by a charged residue (e.g., Glu or Lys) had negligible effects at this exposed site.

Met354 at the P8' position points inward toward the hydrophobic core, and various hydrophobic residues were introduced at this site. Some of these substitutions produced the most functionally stabilizing effects observed in this study, based on the negligible level of urea-stable forms detected during the 4-h incubation at 37°C (Fig. 3A). As expected, M354I, which was included in the stable quadruple PAI-1 mutant described by Berkenpas et al. (1995), increased the functional half-life of the protein 3.9-fold (Fig. 3; Table 2). Substitutions by Val and Phe, which are smaller and larger, respectively, than Ile also increased the $t_{1/2}$ about twofold. Meanwhile, Ala, Leu, and Met (the wild-type residue) with no branched β -carbon produced a similar level of functional stability (1.20, 0.91, and 1.0, respectively). The conversion rates obtained by monitoring the loss of inhibitory activity were consistent with those obtained from the 4 M urea gels for all PAI-1 proteins, except for the version containing M354F. During its 4-h incubation at 37°C,

Figure 2. Latency transition rates of representative PAI-1 mutants. (A) Conversion rates were determined by following the appearance of the urea-stable form on polyacrylamide gels containing 4 M urea. The wild-type (WT) and mutant PAI-1 proteins were incubated in latency conversion buffer (45 mM phosphate, 70 mM NaCl, 0.01% Tween 80 at pH 7.4) for the indicated lengths of time at 37°C. (Arrowheads) The positions of the native and latent forms on the gel. (B) Latency transition rates of representative PAI-1 mutants were determined by following the ability to form an SDS-stable inhibitory complex formation. After PAI-1 proteins were incubated in latency conversion buffer for various lengths of time at 37°C, PAI-1 was incubated with tPA. The reaction products were analyzed on 10% SDS-PAGE. (Lane U) Unreacted PAI-1 protein. The positions of the inhibitory complexes (Cp), tPA, intact PAI-1 (I), and cleaved PAI-1 (Cl) are labeled accordingly. (C) Latency transition rates of representative PAI-1 mutants were quantified by following the loss of inhibitory activity. After PAI-1 proteins were incubated in latency conversion buffer for various lengths of time at 37°C, the remaining inhibitory activity against uPA was measured. (○) Wild type; (●) E350P; (■) E350K; (▲) I353A; (◆) D355N.

negligible conversion of M354F was observed using 4 M urea gels (Fig. 3A); however, the activity loss measured by the uPA assay was faster than that observed from the gel (Fig. 3C). When the tPA reaction products were analyzed by SDS-PAGE, a greater proportion of the M354F molecules had converted to the substrate form, rather than to the latent form (Fig. 3B). This result suggests that P8' influences the configuration of the RCL. In the native structure of PAI-1, Asp355 (P9') and Arg356 (P10') form a salt bridge with a length of 3.59 Å. Disruption of this favorable interaction by mutation decreased the $t_{1/2}$ of the protein. In fact, D355A and

Table 2. Latency transition rates and structural stability of PAI-1 mutants

Mutant ^a	Relative half-life ^b	$\Delta\Delta G$ (kcal mol ⁻¹) ^c
(1) P4' site		
E350A	0.59 ± 0.11	-0.22 ± 0.06
E350V	0.84 ± 0.08	-0.10 ± 0.08
E350Q	0.92 ± 0.12	0.12 ± 0.06
E350L	0.94 ± 0.11	0.06 ± 0.08
E350P	1.52 ± 0.09	0.22 ± 0.06
E350K	1.57 ± 0.09	0.48 ± 0.06 ^d
(2) P5' site		
E351A	1.28 ± 0.03	0.14 ± 0.08
E351Q	0.92 ± 0.07	-0.16 ± 0.06
E351K	0.39 ± 0.06	0.04 ± 0.04
(3) P6' site		
I352A	0.07 ± 0.03	ND ^e
I352L	1.06 ± 0.09	0 ± 0.09
I352F	0.99 ± 0.08	0.12 ± 0.03
(4) P7' site		
I353A	0.28 ± 0.06	0.30 ± 0.07 ^d
I353K	1.02 ± 0.11	0 ± 0.10
I353E	1.02 ± 0.03	0.08 ± 0.07
(5) P8' site		
M354A	1.20 ± 0.15	-0.14 ± 0.07
M354V	1.92 ± 0.03	0.20 ± 0.05
M354L	0.91 ± 0.14	ND ^e
M354I	3.90 ± 0.20	0.42 ± 0.03 ^d
M354F	2.06 ± 0.08	0.36 ± 0.08 ^d
(6) P9' site		
D355A	0.70 ± 0.09	ND ^e
D355N	0.40 ± 0.05	0.08 ± 0.12
(7) P10' site		
R356A	0.19 ± 0.06	0.18 ± 0.07
R356E	0.05 ± 0.03	ND ^e

^aOne-letter codes for amino acids are used for mutations, which are designated by the code for the wild-type residue followed by the residue number and the code for the replacement residue.
^bHalf-life of mutant PAI-1 relative to that of the wild-type protein (designated as 1.0) during incubation at 37°C in the latency conversion buffer. The standard error ranges are shown.
^cFrom $\Delta C_m \times 2.0$ (average m -value). ΔC_m is the difference between the transition midpoints of the wild-type and mutant PAI-1 proteins in urea-induced equilibrium-unfolding experiments. The standard error ranges are shown.
^dThe value is statistically different from that of the wild-type PAI-1, as judged by t -test ($p < 0.05$).
^eNot determined.

Figure 3. Latency transition rates of various Met354 substitution mutants. Latency transition rates were measured as described in Figure 2. (A) Conversion rates of Met354 mutants were followed by the appearance of the urea-stable form on polyacrylamide gels containing 4 M urea. (B) Latency transition rates were determined by following the ability to form an SDS-stable inhibitory complex formation with tPA. (C) The latency transition rates were quantified by following the loss of inhibitory activity against uPA. (○) Wild type; (●) M354I; (▼) M354F; (▲) M354V; (■) M354A; (◆) M354L PAI-1.

D355N decreased the $t_{1/2}$ to 63 min and 37 min, respectively, compared to wild type (92 min). Similarly, R356A and R356E drastically reduced the $t_{1/2}$ to <20 min.

PAI-1 structural stability was not significantly altered by mutation

The structural stability of the native mutant proteins was quantified by urea-dependent equilibrium-unfolding experiments, which monitor the intrinsic fluorescence changes that occur upon protein unfolding. The unfolding transition midpoint of wild-type PAI-1 was 2.0 ± 0.1 M urea, as previously reported (Na and Im 2005). The equilibrium-unfolding results for representative mutants are shown in Figure 4, and the changes in structural stability for all mutant PAI-1 proteins are summarized in Table 2. Most mutations had minor effects on the structural stability of the protein; transition midpoints were shifted by <0.2 M urea, corresponding to a free energy change ($\Delta\Delta G$) of <0.4 kcal mol⁻¹. Only two mutations, E350K and M354I, increased the structural stability of the protein by >0.4 kcal mol⁻¹ (0.48 and 0.42 kcal mol⁻¹, respectively).

Discussion

Interactions at the S1C region in the metastable native form regulate the latency transition of PAI-1 by modulating the energy barrier

In this study, we showed that substitutions within the s1C selectively affect the transition of PAI-1 from the metastable native to the latent form. Among the substitutions, those at P4' (Glu350; at the beginning of the s1C, where the RCL is connected) and P8' (Met354; at the center of the s1C, where the residue is anchored to the body of the molecule) had the largest effect on the functional stability of PAI-1. It has been suggested that the disruption of a salt bridge, which exists only in the latent form, between Glu350 and Arg30 by an E350P substitution increases the functional half-life of the protein by selectively destabilizing its latent form (Lawrence et al. 1994). Consistent with this result, E350P increased the functional half-life of PAI-1 by >50% in our study (Table 2). However, our results show that the effect of the substitution at Glu350 is probably not due to perturbation of a salt bridge in the final latent form, since the removal of a negative charge by substitution with Gln or Leu (i.e., residues of similar size) had only minor effects on the transition rate (i.e., <10%) (Table 2). In contrast, substitutions by smaller, nonpolar residues facilitated the transition. Based on the native structure of PAI-1 (Fig. 1A), it appears that having a side chain of sufficient length at the Glu350 site is important for attaching the s1C to the protein body.

Figure 4. Structural stability of representative PAI-1 mutants. Urea-induced equilibrium-unfolding transitions were followed by measuring the change in fluorescence emission intensity at 333 nm ($\lambda_{\text{ex}} = 295$ nm). (○) Wild type; (●) E350P; (■) E350K; (▲) I353A; (◆) D355N.

Owing to the ease of latency conversion of wild-type PAI-1, the X-ray crystal structure of the native form was obtained for a very stable quadruple mutant version of PAI-1 (N150H, K154T, Q319L, and M354I) (Sharp et al. 1999). Figure 1A shows that an Ile residue at the Met354 site, indeed, adequately fits into the cavity, thus anchoring the s1C to the protein core.

Meanwhile, the effects of the mutations on the overall structural stability of the protein were trivial. Only E350K (at P4') and M354I (P8') slightly increased the structural stability (0.48 and 0.42 kcal mol⁻¹, respectively). The results of our substitutions in the s1C contrast to that observed for T93I (at s2A), which had a negligible effect on the transition of PAI-1, although its structural stability was increased by >2 kcal mol⁻¹, corresponding to a 25-fold delay in the conformational change (Yi and Im 2007). Therefore, the conversion rate is not simply affected by the thermodynamic stability of the native form. Instead, it is regulated mainly through stabilization/destabilization of the transition state, thus affecting the height of the energy barrier. Releasing the constraint on the RCL by extending the loop facilitates the transition of PAI-1 by bypassing the kinetic trap (Na and Im 2005).

A high frequency of functionally stabilizing substitutions was obtained at this strategic region. Seven of the 24 substitutions tested significantly increased the $t_{1/2}$. Property-improving mutations are rarely obtained; mutations are more likely to either damage a protein or to have negligible effects. In our previous study of the archetypal serpin α_1 -antitrypsin (Seo et al. 2000), stabilizing mutations were obtained at a frequency of only 1%–2% throughout the molecule. The high frequency of function-stabilizing mutations in the s1C region of PAI-1

suggests that interactions in the s1C region are particularly important for regulating the transition from the metastable native form to the latent form. It has been reported recently that a monoclonal antibody that binds to the cleft revealed by s1C detachment facilitates the transition of native PAI-1 to the latent form, possibly by stabilizing a pre-latent intermediate (Dupont et al. 2006). That result also suggests that the s1C region is critical for controlling the transition of PAI-1 to its latent form. Since interactions with the s1C do not occur in the latent form, specific interactions in the metastable native form impede the transition to the latent form. If our results are extended to general protein folding, they favor the hypothesis that nonnative interactions in folding intermediates attenuate the conversion to the final form.

Deficient s1C variants of serpins

Many disease-associated natural variants of serpins have mutations within the s1C (Stein and Carrell 1995), suggesting that this region plays a critical role in the conformational transition of serpins. At P8' (corresponding to Met354 in PAI-1), an antithrombin III mutation (A404T) (Lane et al. 1992) and a C1 inhibitor mutation (V451M) (Verpy et al. 1995) have been reported to cause severe deficiency leading to thrombosis and angioedema, respectively. Mutations at the P6' site of antithrombin III (F402L, F402C, and F402S) (Lane et al. 1992) may destabilize the s1C by introducing a cavity. Indeed, introduction of a cavity by a V364A substitution at the P6' site of α_1 AT allows conversion of this functionally stable serpin to the latent form (Im et al. 2002). These variants demonstrate the importance of anchoring the s1C to the main body of the protein via residues at buried sites. Deficient variants at the P9' site (e.g., antithrombin N405K) (Lane et al. 1992) or at the P10' site (e.g., antithrombin R406M) (Nakagawa et al. 1991) have been also reported. The conserved proline at P11', located at the end of the s1C, plays a structural role in making the turn to s4B, and Thr (in antithrombin) (Lane et al. 1992) or Leu (in antithrombin and α_1 AT) (Hofker et al. 1989; Millar et al. 1993) variants cause deficiency. Antithrombin III is the only serpin besides PAI-1 for which the latent structure of the wild-type molecule has been reported (Carrell et al. 1994). This fact may be in line with the frequent occurrence of s1C mutations in deficient antithrombin III natural variants, although whether these pathological variants convert into the latent form remains to be elucidated.

Biological implications in protein misfolding diseases

Our results show that mutations of the conformationally labile protein can accelerate or decelerate the transition to

a more stable form by affecting the energy barrier between two states, rather than by affecting the thermodynamic stability of the metastable native form. Our findings have significant implications for understanding the structural and biochemical basis of mutations associated with protein misfolding diseases. Some mutant proteins enhance the conformational transition to a more stable form without affecting the thermodynamic stability of the native state significantly. For example, the native form of the M_{malton} variant of human α_1 -antitrypsin has stability comparable to that of the wild-type molecule, but it spontaneously undergoes conformational conversion to the latent form and to polymers (Lomas et al. 1995; Jung and Im 2003). Mutational analysis of the activation domain of human procarboxypeptidase A2 has also shown that the rate at which the protein forms amyloid can be altered substantially without perturbing its overall stability or activity significantly (Villegas et al. 2000). The energy barrier to conformational transition is likely to be modulated in these mutations, and more of these types of kinetic mutation, associated with protein misfolding diseases, will be identified in the near future.

Materials and Methods

Materials

E. coli strain BL21(DE3) pLysS (Novagen, Inc.) was used for expression of recombinant PAI-1. Spectrozyme UK [carboxybenzoyl-L- γ -glutamyl (α -ot-but)-glycyl-arginine-p-nitroanilide] was purchased from American Diagnostica Inc. Urokinase (uPA) was purchased from Green Cross BioTech Co., and tPA from Genentech Inc. The Bio-Rad DC (detergent-compatible) protein assay kit was from Bio-Rad Laboratories, Inc. Ultrapure urea was purchased from ICN Biochemicals. All other chemicals were reagent-grade.

Mutagenesis and expression of PAI-1 in *E. coli*

The plasmid for human PAI-1 expression in *E. coli*, pRSET-PAI1, was described previously (Lee and Im 2003). Substitutions were introduced by oligonucleotide-directed mutagenesis (Kunkel et al. 1987), and confirmed by dideoxynucleotide DNA sequencing. Recombinant PAI-1 was expressed as inclusion bodies in *E. coli*, and refolded and purified as described previously (Lee and Im 2003). Concentrations of PAI-1 proteins were determined using the Bio-Rad DC protein assay kit, with bovine serum albumin as a standard.

Monitoring latency transition by gel electrophoresis

To follow latency transition of PAI-1 proteins, recombinant PAI-1 proteins were incubated at 37°C in latency conversion buffer (45 mM phosphate, 70 mM NaCl, 0.01% Tween 80 at pH 7.4) for various lengths of time. The kinetics of the latency transition was visually monitored by electrophoresis on a gel containing 4 M urea. Nondenaturing acidic gel 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 protein bands were visualized by Coomassie Brilliant Blue staining.

Complex formation with the target protease

One microgram of PAI-1 protein was incubated with 3 μ g of tPA in a buffer (30 mM phosphate at pH 7.4, 160 mM NaCl, 0.1% PEG, 0.1% Triton X-100) for 10 min at 37°C. Formation of the inhibitory complex of PAI-1 with tPA was monitored by the appearance of SDS-resistant covalent complexes on 10% SDS-polyacrylamide gels. The protein bands were visualized by Coomassie Brilliant Blue staining. All mutant proteins possessed inhibitory activity, as indicated by the formation of SDS-stable inhibitory complexes with tPA. There were small amounts of unreactive proteins in each preparation of PAI-1, as previously reported (Taeye et al. 2003).

Measurement of latency conversion rates as the loss of inhibitory activity

PAI-1 proteins were taken at various time points during incubation at 37°C in latency conversion buffer, and the remaining inhibitory activity was determined. PAI-1 proteins were incubated with 20 units of uPA in 50 μ L of uPA assay buffer (0.15 M NaCl, 50 mM Tris-Cl, 0.01% Tween 80, and 100 μ g/mL BSA at pH 7.5) for 10 min at 37°C. The reaction mixture was diluted 20-fold with the assay buffer, and the residual proteolytic activity of uPA was measured with 50 μ M Spectrozyme UK. The amounts of products were measured at 410 nm using a Beckman DU-650 spectrophotometer. Each experiment was performed in duplicate and repeated three times. The experimental data were fitted to a single exponential decay.

Denaturant-induced equilibrium-unfolding transition

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

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References

Baker, D. and Agard, D.A. 1994. Influenza hemagglutinin: Kinetic control of protein function. *Structure* **2**: 907–910.

- Berkenpas, M.B., Lawrence, D.A., and Ginsburg, D. 1995. Molecular evolution of plasminogen activator inhibitor-1 functional stability. *EMBO J.* **14**: 2969–2977.
- Bruch, M., Weiss, V., and Engel, J. 1988. Plasma serine proteinase inhibitors (serpins) exhibit major conformational changes and a large increase in conformational stability upon cleavage at their reactive sites. *J. Biol. Chem.* **263**: 16626–16630.
- Carr, C.M., Chaudhry, C., and Kim, P.S. 1997. Influenza hemagglutinin is spring-loaded by a metastable native conformation. *Proc. Natl. Acad. Sci.* **94**: 14306–14313.
- Carrell, R.W., Stein, P.E., Fermi, G., and Wardell, M.R. 1994. Biological implication of 3 Å structure of dimeric antithrombin. *Structure* **2**: 257–270.
- Dupont, D.M., Blouse, G.E., Hansen, M., Mathiasen, L., Kjelgaard, S., Jensen, J.K., Christensen, A., Gils, A., Declerck, P.J., Andreasen, P.A., et al. 2006. Evidence for a pre-latent form of the serpin plasminogen activator inhibitor-1 with a detached β -strand 1C. *J. Biol. Chem.* **281**: 36071–36081.
- Gils, A. and Declerck, P.J. 1997. Proteinase specificity and functional diversity in point mutants of plasminogen activator inhibitor-1. *J. Biol. Chem.* **272**: 12662–12666.
- Hekman, C.M. and Loskutoff, D.J. 1985. Endothelial cells produce a latent inhibitor of plasminogen activators that can be activated by denaturants. *J. Biol. Chem.* **260**: 11581–11587.
- Hofker, M.H., Nukiwa, T., van Paassen, H.M., Nelen, M., Kramps, J.A., Klasen, E.C., Frants, R.R., and Crystal, R.G. 1989. A Pro $\rightarrow$ Leu substitution in codon 369 of the α_1 -antitrypsin deficiency variant PI M^{Heerlen}. *Hum. Genet.* **81**: 264–268.
- Huber, R. and Carrell, R.W. 1989. Implications of the three-dimensional structure of α_1 -antitrypsin for structure and function of serpins. *Biochemistry* **28**: 8951–8966.
- Im, H., Woo, M.-S., Hwang, K.Y., and Yu, M.-H. 2002. Interactions causing the kinetic trap in serpin protein folding. *J. Biol. Chem.* **277**: 46347–46354.
- Jovin, T.M. 1973. Multiphasic zone electrophoresis, IV: Design and analysis of discontinuous buffer systems with a digital computer. *Ann. N.Y. Acad. Sci.* **209**: 477–496.
- Jung, C.-H. and Im, H. 2003. A recombinant human α_1 -antitrypsin variant, M^{malton}, undergoes a spontaneous conformational conversion into the latent form. *J. Microbiol.* **41**: 335–339.
- Kunkel, T.A., Roberts, J.D., and Zakour, R.A. 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol.* **154**: 367–382.
- Lane, D.A., Olds, R.J., Conard, J., Boisclair, M., Bock, S.C., Hultin, M., Abildgaard, U., Ireland, H., Thompson, E., Sas, G., et al. 1992. Pleiotropic effects of antithrombin strand 1C substitution mutations. *J. Clin. Invest.* **90**: 2422–2433.
- Lawrence, D.A., Olson, S.T., Palaniappan, S., and Ginsburg, D. 1994. Engineering plasminogen activator inhibitor 1 mutants with increased functional stability. *Biochemistry* **33**: 3643–3648.
- Lee, H.J. and Im, H. 2003. Purification of recombinant plasminogen activator inhibitor-1 in the active conformation by refolding from inclusion bodies. *Protein Expr. Purif.* **31**: 99–107.
- Lee, C., Park, S.-H., Lee, M.-Y., and Yu, M.-H. 2000. Regulation of protein function by native metastability. *Proc. Natl. Acad. Sci.* **97**: 7727–7731.
- Levin, E.G. and Santell, L. 1987. Conversion of the active to latent plasminogen activator inhibitor from human endothelial cells. *Blood* **70**: 1090–1098.
- Lomas, D.A., Elliott, P.R., Sidhar, S.K., Forman, R.C., Finch, J.T., Cox, D.W., Whisstock, J.C., and Carrell, R.W. 1995. α_1 -Antitrypsin M^{malton} (Phe52-deleted) forms loop-sheet polymers in vivo: Evidence for the C sheet mechanism of polymerization. *J. Biol. Chem.* **270**: 16864–16870.
- Millar, D.S., Lopez, A., White, D., Abraham, G., Laursen, B., Holding, S., Reverter, J.C., Reynaud, J., Martinowitz, U., Hayes, J.P., et al. 1993. Screening for mutations in the antithrombin III gene causing recurrent venous thrombosis by single-strand conformation polymorphism analysis. *Hum. Mutat.* **2**: 324–326.
- Mottonen, J., Strand, A., Symersky, J., Sweet, R.M., Danley, D.E., Geoghegan, K.F., and Goldsmith, E.J. 1992. Structural basis of latency in plasminogen activator inhibitor-1. *Nature* **355**: 270–273.
- Na, Y.-R. and Im, H. 2005. The length of the reactive center loop modulates the latency transition of plasminogen activator inhibitor-1. *Protein Sci.* **14**: 55–63.
- Nakagawa, M., Tanaka, S., Tsuji, H., Takada, O., Uno, M., Hashimoto-Gotoh, T., and Wagatsuma, M. 1991. Congenital antithrombin III deficiency (AT-III Kyoto): Identification of a point mutation altering arginine-406 to methionine behind the reactive site. *Thromb. Res.* **64**: 101–108.
- Ny, T., Sawdey, M., Lawrence, D., Millan, J.L., and Loskutoff, D.J. 1986. Cloning and sequence of a cDNA coding for the human β -migrating endothelial-cell-type plasminogen activator inhibitor. *Proc. Natl. Acad. Sci.* **83**: 6776–6780.
- Pace, C.N., Shirley, B.A., and Thomson, J.A. 1989. Measuring the conformational stability of a protein. In *Protein structure: A practical approach*. (ed. T.E. Creghton), pp. 311–330. IRL Press at Oxford University Press, Oxford, UK.
- Pannekoek, H., Veerman, H., Lambers, H., Diegaarde, P., Verweij, C.L., van Zonneveld, A.J., and van Mourik, J.A. 1986. Endothelial plasminogen activator inhibitor (PAI): A new member of the serpin gene family. *EMBO J.* **5**: 2539–2544.
- Seo, E.J., Im, H., Maeng, J.-S., Kim, K.E., and Yu, M.-H. 2000. Distribution of the native strain in human α_1 -antitrypsin and its association with protease inhibitor function. *J. Biol. Chem.* **275**: 16904–16909.
- Sharp, A.M., Stein, P.E., Pannu, N.S., Carrell, R.W., Berkenpas, M.B., Ginsburg, D., Lawrence, D.A., and Read, R.J. 1999. The active conformation of plasminogen activator inhibitor 1, a target for drugs to control fibrinolysis and cell adhesion. *Struct. Fold. Des.* **7**: 111–118.
- Stein, P.E. and Carrell, R.W. 1995. What do dysfunctional serpins tell us about molecular mobility and disease? *Nat. Struct. Biol.* **2**: 96–113.
- Taeye, B.D., Gompennolle, G., Dewilde, M., Biesemans, W., and Declerck, P. 2003. Immobilization of the distal hinge in the labile serpin plasminogen activator inhibitor 1. *J. Biol. Chem.* **278**: 23899–23905.
- van Meijer, M. and Pannekoek, H. 1995. Structure of plasminogen activator inhibitor 1 (PAI-1) and its function in fibrinolysis: An update. *Fibrinolysis* **9**: 263–276.
- Vaughan, D.E. 1998. Plasminogen activator inhibitor-1: A common denominator in cardiovascular disease. *J. Invest. Med.* **46**: 370–376.
- Verpy, E., Couture-Tosi, E., Eldering, E., Lopez-Trascasa, M., Spath, P., Meo, T., and Tosi, M. 1995. Crucial residues in the carboxy-terminal end of C1 inhibitor revealed by pathogenic mutants impaired in secretion or function. *J. Clin. Invest.* **95**: 350–359.
- Villegas, V., Zurdo, J., Filimonov, V.V., Aviles, F.X., Dobson, C.M., and Serrano, L. 2000. Protein engineering as a strategy to avoid formation of amyloid fibrils. *Protein Sci.* **9**: 1700–1708.
- Wiley, D.C. and Skehel, J.J. 1987. The structure and function of the hemagglutinin membrane glycoprotein of influenza virus. *Annu. Rev. Biochem.* **56**: 365–394.
- Yi, J.-Y. and Im, H. 2007. Structural factors affecting the choice between latency transition and polymerization in inhibitory serpins. *Protein Sci.* **16**: 833–841.