Kinetically Controlled Folding of the Serpin Plasminogen Activator Inhibitor 1[†]

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Received May 21, 1996; Revised Manuscript Received September 18, 1996[⊗]

ABSTRACT: The serpin plasminogen activator inhibitor 1 (PAI-1) folds into an active structure and then converts slowly to a more stable, but low-activity, "latent" conformation [Hekman, C. M., & Loskutoff, D. J. (1985) *J. Biol. Chem. 260*, 11581−11587]. Thus, the folding of PAI-1 is apparently under kinetic control. We have determined the urea denaturation and refolding transitions of both latent and active PAI-1 proteins by using intrinsic tryptophan fluorescence. While folding of active PAI-1 is reversible, the denaturation and refolding of latent PAI-1 are not. Instead, denatured latent PAI-1 refolds in lower concentrations of urea to give the active protein. Thus, the high-stability latent conformation is kinetically inaccessible over a range of urea concentrations. Complete denaturation of latent PAI-1 occurs at 5.5 M urea [$\Delta G(H_2O) \sim 21$ kcal] whereas active PAI-1 denatures in only 3.8 M urea [$\Delta G(H_2O) \sim 12$ kcal]. The fluorescence emission profile, as a function of urea of both the active and latent forms of the protein, reveals intermediates with partial structure. Circular dichroism measurements and limited protease digestion with Lys-C suggest that the intermediate in the denaturation of latent PAI-1 retains most of the secondary structure of the fully folded protein, whereas the intermediate in the denaturation of active PAI-1 exhibits significant loss of secondary structure. The Lys-C digestion patterns show that the active protein is more susceptible to proteolysis near sheet A than is the latent form. The studies suggest a model for the kinetically controlled folding pathway of PAI-1.

The serpin family of protease inhibitors offers an important system in which to understand the protein folding problem because the folding of these molecules is under kinetic control. This is particularly evident from the properties of the serpin plasminogen activator inhibitor 1 (PAI-1),[1] the major physiological inhibitor of tissue-type plasminogen activator (tPA), and urokinase plasminogen activator (uPA) (Loskutoff et al., 1989). PAI-1 folds into an active conformation on secretion *in vivo* or refolding *in vitro* (Hekman & Loskutoff, 1985) and then inactivates in a spontaneous unimolecular process with a half-life of 2 h at 37 °C (Lindahl et al., 1989; Levin, 1986). The inactive conformation has been termed "latent" because inhibitory activity can be recovered through a cycle of complete denaturation of the latent form followed by refolding. The structure of latent PAI-1 (Mottonen et al., 1992) resembles cleaved serpins (Loebermann et al., 1984) which are formed on denaturation of serpin−protease complexes or through the action of noncognate proteases (Mast et al., 1991). Like cleaved serpins, latent PAI-1 is more stable to heat-induced denaturation than active PAI-1 (Munch et al., 1991). Thus, PAI-1, and presumably other serpins as well, is metastable and folded by kinetically determined events. While some progress has been made in understanding the structural basis of the latency transition (Berkenpas et al., 1995; Tucker et al., 1995), no data are available on the folding steps that account for the formation of a metastable structure.

Serpins are α/β proteins possessing three β-sheets; a large sheet (A, purple in Figure 1a), and sheets B and C that form a barrel. Distance analysis (unpublished data) suggests that the molecule consists of a single domain with two subdomains. The latent form (Figure 1b), like the cleaved form (Figure 1c), has one more strand in sheet A (s4A) than does the active form (Figure 1a). Assuming that active PAI-1 resembles its homologs, antithrombin (Carrell et al., 1994; Schreuder et al., 1994) or antichymotrypsin (Wei et al., 1994), the latency transition involves insertion of a surface loop, including the reactive center, as s4A of sheet A and displacement of the gate (s3C, s4C, Figure 1b).

Previously, Robert Gerard and we designed and synthesized mutant PAI-1 molecules with slowed kinetics of the latency transition based, in part, on the model that latency involves formation of s4A (Tucker et al., 1995). The mutant EP8 (T339 → E) was designed to prevent or slow the latency transition by introducing a charged residue that should become buried in a hydrophobic environment in the latent conformation, as part of s4A. EP8 has a much longer lifetime in the active conformation than wild type and is the longest lived of the mutants we synthesized. Here we use wild-type PAI-1 and the mutant EP8 to study the denaturation and folding of latent and active PAI-1, with the goal to understand the energy differences between active and latent PAI-1, respectively, and to better understand the folding events that give rise to the active form of serpins. The results described below show that the folding into the active form is preferred over the entire range of urea concentrations. Further, we observe that the intermediate in the denaturation profile of latent PAI-1 retains secondary structure both in

[†] This research is supported by the Robert A. Welch Foundation (I-1128 to E.J.G.).

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[⊗] Abstract published in *Advance ACS Abstracts,* December 1, 1996.

[1] Abbreviations: serpin, serine protease inhibitor; PAI-1, plasminogen activator inhibitor 1; tPA, tissue plasminogen activator; uPA, urokinase plasminogen activator; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; CD, circular dichroism; BCA, bicinchoninic acid.

S0006-2960(96)01214-7 CCC: $12.00

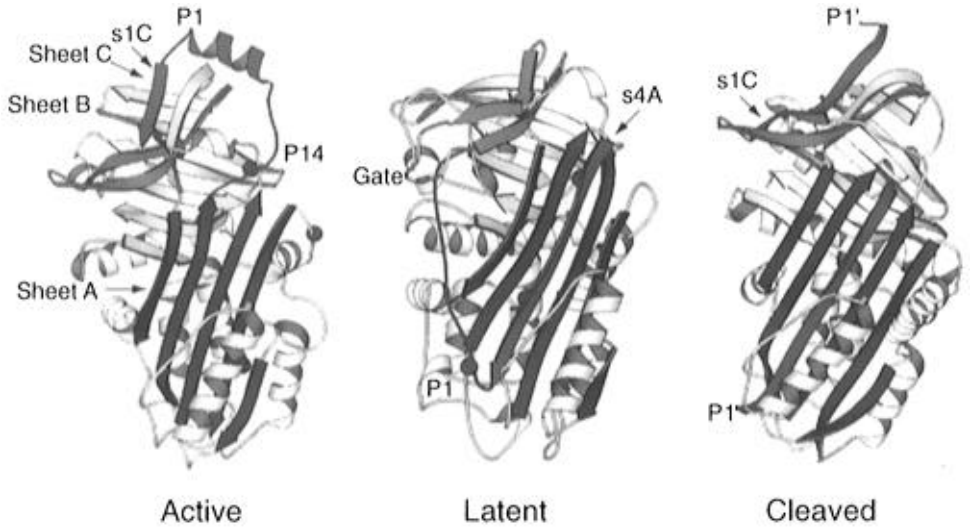

FIGURE 1: Intact serpin conformations. (a, left) Ovalbumin (Stein et al., 1990) serves as a generic model for active serpins. (b, middle) Latent PAI-1 (Mottonen et al., 1992). The reactive center loop, referring to the 14 residues N-terminal to the recognition/cleavage site through 10 residues C-terminal (P14−P10′) (orange), is differently situated in the two proteins. The positions of the tryptophan residues in PAI-1 are shown as brown spheres in (a). The PAI-1 EP8 mutation is shown as a red sphere (middle one in b). (c, right) Cleaved α1-proteinase inhibitor (Loebermann et al., 1984).

sheet A and in the B−C barrel, whereas the intermediate in the denaturation of active PAI-1 exhibits significant losses in secondary structure and a greater degree of susceptibility to proteolysis near sheet A. The results suggest a model in which the B−C barrel is more stable than sheet A in the active conformation of the protein and thus perhaps folded first to form the metastable active structure.

MATERIALS AND METHODS

Expression and Purification of Proteins. Wild-type and EP8 PAI-1 were expressed from a plasmid containing the T7 gene 10 promoter (Studier & Moffatt, 1986) followed by the sequence Met-$(His)_6$-Ala-Ser-Glu-Asn-Leu-Tyr-Phe-Gln-Gly coding for a Ni^{2+} affinity tag and the tobacco etch viral protease cleavage site (Dougherty et al., 1988) and the mature PAI-1 sequence (Franke et al., 1990) as described in Tucker et al. (1995). *Escherichia coli* strain BL21[DE3]-$pLys^s$ was used as the expression host. The proteins were purified with a procedure modified from our previous studies (Tucker et al., 1995). Bacterial cell extract (25 mL) was loaded onto 2.5 mL of Ni^{2+}−NTA−agarose (Qiagen, CA) in 0.2 M NaCl, 10% glycerol, and 50 mM sodium phosphate, pH 8.0. PAI-1 eluted between 0.25 and 0.5 M imidazole, in a 100 mL gradient, and was dialyzed into 0.2 M NaCl, 10% glycerol, and 50 mM sodium phosphate, pH 6.0 (buffer B). The 20 mL protein sample, approximately 0.5 mg/mL, was further purified on a 5 mL HiTrap heparin−Sepharose column (Pharmacia, Sweden) in a 100 mL linear gradient of 0.2−1.0 M NaCl in 10% glycerol and 50 mM sodium phosphate, pH 7.0. The protein was dialyzed into buffer B, snap frozen in liquid nitrogen, and stored in −80 °C. All the purification procedures were performed at 4 °C. The protein was 98−99% pure as assessed by SDS−PAGE followed by silver staining. The expression yield was 4−8 mg of pure soluble protein/L of cell culture.

Assay and Latency. The protein activity was assayed routinely by complex formation with tPA or uPA, by gel shift in SDS−PAGE. The results agreed well with the spectrophotometric assay carried out with tPA (Genentech, San Francisco, CA) and substrate Spec-PA (American Diagnostica, CT) (Tucker et al., 1995). The purified wild-type PAI-1 was only ≤50% active, whereas mutant EP8 was 90−95% active. The time dependence of activity was monitored by the gel-shift assay and fluorescence intensity change. There is no evident activity loss for EP8 at room temperature in 1 day and at 4 °C in 3 days under our experimental conditions (data not shown). To obtain latent PAI-1, rather than separating the latent and active forms (Kvassman & Shore, 1995), partially active wild-type PAI-1 protein was left at room temperature for 2 days to convert to the latent form (>95%). The rate of the transition to the latent state for this recombinant His-tagged PAI-1 is similar to the plasma-purified (untagged) inhibitor (Tucker et al., 1995; Hekman & Loskutoff, 1985).

Denaturation and Refolding. Denaturation studies were carried out in urea. An 8.0 M urea (ultrapure grade, ICN, OH) stock solution was freshly prepared for each experiment. In the denaturation studies, 0.05 mg/mL solutions of PAI-1 in varying concentrations of urea were allowed to stand for ∼14 h. In the folding experiments, PAI-1 samples were allowed to stand for ∼14 h in 7.5 M urea, diluted into buffer or lower concentrations of urea, and allowed to stand for ∼20 h before the fluorescence spectra were recorded. Both wild-type and EP8 were used in the refolding study.

Fluorescence and Circular Dichroism (CD). Fluorescence emission spectra of active, latent, and denatured PAI-1 were recorded from 300 to 380 nm with an excitation wavelength of 288 nm, on a Photon Technology International (PTI, Canada) spectrofluorometer with temperature controlled to 8 °C. A step size of 0.5 nm was used with an average scan time of 2 s/nm. Spectra were background corrected. The protein concentration was typically ∼0.05 mg/mL in 0.1 M NaCl, 1% glycerol, and 50 mM sodium phosphate, pH 6.5. CD measurements were conducted on an Aviv Model 62DS spectrophotometer (Aviv, NJ) and temperature controlled to 25 °C. A quartz cell of 0.1 cm path length was used for the measurement in far-UV region of 190−260 nm. Only the region from 210 to 260 nm was measured for samples in 3 M urea. The spectra are the average of two scans. An integration window of 3 s was used. CD spectra were

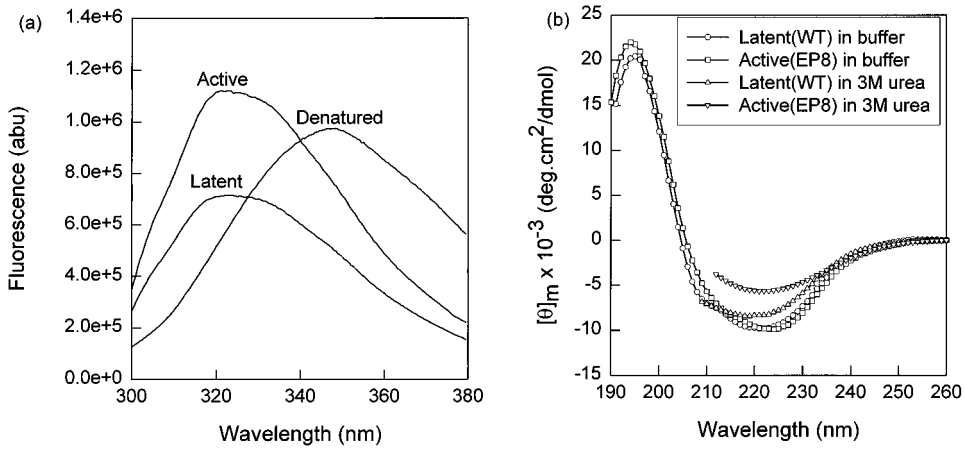

FIGURE 2: (a) Fluorescence emission spectra of active, latent, and denatured PAI-1 with an excitation wavelength of 288 nm at 8 °C. (b) CD spectra of active and latent PAI-1 proteins in buffer and in 3 M urea at 25 °C. All the CD spectra were background corrected and converted to mean residue ellipticity by using the protein concentration calibrated by micro-BCA assay (Pierce).

background corrected, converted to mean residue ellipticity, and smoothed by a polynomial fitting function (Aviv Associates, Inc.). The protein concentration was typically ~0.1 mg/mL in 15 mM NaCl, 0.7% glycerol, and 15 mM sodium phosphate, pH 6.5.

Lys-C. Limited proteolysis with Lys-C endopeptidase (Promega, WI) was carried out on ice over a time course of 0−120 min. The ratio of PAI-1 to Lys-C was 50:1 with 0.35 mg/mL PAI-1 in pure buffer B or 3 M urea in buffer B. The proteolytic protein fragments were separated on SDS−PAGE (10−20% gradient gel, Bio-Rad). N-Terminal sequences were obtained for major protein fragments released in 20 min incubation.

RESULTS

Wild-Type PAI-1 and EP8. Wild-type human PAI-1 and EP8 were expressed in *E. coli* with non-native sequences at the N-termini (described in Materials and Methods). Purified wild-type PAI-1 is about 50% active, while EP8 is about 90−95% active. Further, EP8 has a much longer lifetime in the active state compared with wild type (Tucker et al., 1995). Here we use EP8 to analyze the denaturation of active PAI-1 and wild-type PAI-1, which has been converted to >95% latent form, to analyze denaturation of latent PAI-1. Both wild type and EP8 were used to study refolding.

Fluorescence Spectra of PAI-1. Active, latent, and denatured PAI-1 have different fluorescence spectra. When excited at 288 nm, where absorption is due predominantly to tryptophan residues, the fluorescence emission intensity of active PAI-1 is greater than that of latent PAI-1, and the maximum is 322 nm, compared with the maximum at 324 nm for latent PAI-1 (Figure 2a). The fluorescence emission spectrum of the unfolded molecule is distinct as well, with a maximum at 348 nm. The spectra are similar to those reported by Boström et al. (1990) and Dwivedi et al. (1991) with respect to the relative fluorescence intensities of active and latent PAI-1. However, slight differences in the position of the emission maxima are present, which could be due to differences in the excitation wavelength and buffer conditions or differences in the protein sample, whether purified from mammalian cell culture or expressed in bacteria.

Denaturation of Active EP8. We used EP8 to study the denaturation and refolding of active PAI-1. The denaturation was monitored by the change in wavelength of the fluorescence emission maximum (Figure 2a). EP8 exhibits two transitions when denatured in urea, one at 0.6 M urea and a second near 3.8 M urea at 8 °C, pH 6.5 (Figure 3). An intermediate has the same fluorescence spectrum from 1.0 to 3.2 M urea.

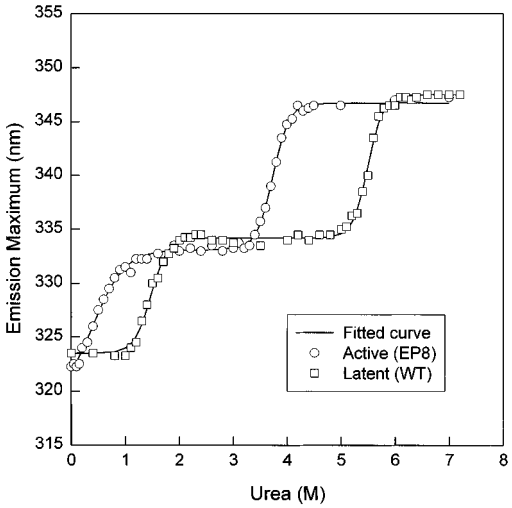

FIGURE 3: Denaturation of active EP8 (circles) and latent wild-type PAI-1 (squares) in urea. The fluorescence emission spectra were scanned from 300 to 380 nm with an excitation wavelength of 288 nm.

Denaturation of Latent Wild-Type PAI-1. As with the denaturation of active EP8, two transitions are also observed in the fluorescence emission profile (Figure 3), one at 1.5 M urea and the second at 5.8 M urea. The two-transition profile is similar to those observed by Bruch et al. (1988) in the guanidine hydrochloride induced denaturation of active and cleaved serpins measured by CD at 220 nm.

The position of the fluorescence emission maximum of the intermediates suggests that the environment of one or more of the four tryptophan residues in PAI-1 is changed, the red shift indicating a greater solvent exposure in the intermediate (Figure 3). The four tryptophan residues are disposed two each to the two subdomains of the molecule: W86 and W139 are near sheet A, while W175 and W262 are in the B−C barrel (Figure 1a). The two tryptophan residues near sheet A are close to the surface of the molecule, while the two in the B−C barrel are completely buried. The questions arise then, what is the nature of the intermediate

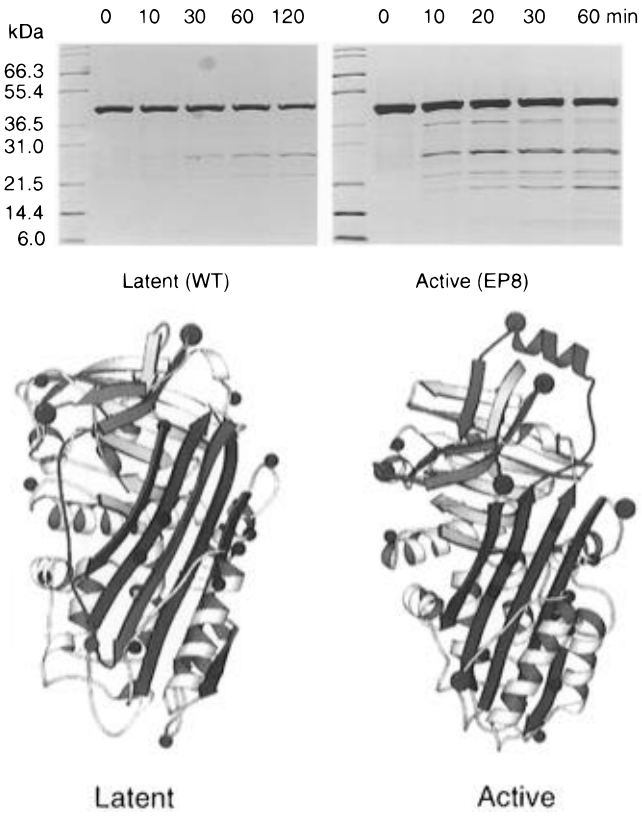

FIGURE 4: (a, top) SDS−PAGE gels of the Lys-C digests of active and latent PAI-1 (0.35 mg/mL) denatured in 3 M urea and treated with Lys-C for the times indicated. (b, c) Potential (small dark blue spheres) and actual (large blue spheres) lysine residue cleavage sites mapped (b, bottom left) onto the structure of latent PAI-1 and (c, bottom right) onto a model of the active structure based on ovalbumin. The red sphere is the cleavage site for its target protease. It was also cleaved in the active form.

and does it involve loss of structure of a subdomain or local loss of surface secondary structure?

Characterization of Denaturation Intermediates by Circular Dichroism. The circular dichroism (CD) spectra of active (EP8) and latent (wild-type) PAI-1 are shown in Figure 2b. The spectra are consistent with the α/β content of the protein and similar to other serpins (Powell & Pain, 1992). We accurately determined the protein concentration in our samples using BCA assay and observed only very small differences between the spectra of active and latent PAI-1. We conclude that previous reports of large CD differences between active and latent PAI-1 (Boström et al., 1990) and antithrombin (Carrell et al., 1991) could be due to scaling errors. The CD spectrum of latent PAI-1 denatured in 3.0 M urea also differs to a slight extent from the undenatured protein, suggesting that it retains much of the secondary structure of the native protein. However, the CD of 3.0 M urea-denatured active EP8 indicates significant loss of secondary structure, particularly, helical structure (note the loss of signal at 222 nm).

Proteolysis of Active, Latent, and the 3.5 M Urea Intermediates. To further analyze the nature of the intermediate in the denaturation profile of PAI-1, we carried out limited proteolysis of PAI-1 with the endopeptidase Lys-C, which is specific for lysine residues (in the P1 position) (Elliott & Cohen, 1986). Undenatured active EP8 and latent wild-type PAI-1 were highly resistant to proteolysis by Lys-C, showing no evident proteolysis in 24 h on ice. Lys-C is

Table 1: Lys-C Cleavage Sites

peptide	N-terminus	molecular mass (kDa)	probable cleavage sites
latent PAI-1 in 3.0 M urea			
band 1	^{1}MHHH	27	K207
band 2	^{192}SDGS		K191
active EP8 in 3.0 M urea			
band 1	^{1}MHHH	42	K323, K325, *R346*
band 2	^{1}MHHH	36	K277
band 3 (mixture)	(a) ^{1}MHHH	27	K207
	(b) ^{89}DEIS		K88
	(c) ^{192}SDGS		K191
	(d) ^{278}FSLE		K277
band 4	^{192}SDGS		K191
band 5 (19.4)	^{1}MHHH	19	K141, K145, *K154*
band 6	^{1}MHHH	12	K88

stable in urea up to 5 M (Elliott & Cohen, 1986). Therefore, we treated active EP8 and latent wild-type PAI-1 with Lys-C in 3 M urea to determine which lysine residues are protected from proteolysis in the denaturation intermediates. Peptides in the digest were separated by SDS−PAGE electrophoresis (Figure 4a). Only two major bands were found in the digest of latent PAI-1 treated with 3 M urea. By contrast, the digest of active PAI-1 (EP8) in 3 M urea gave five major bands and one minor band; one of the major bands contained four peptides. Sequence analysis allows unambiguous identification of the N-terminus of six bands (Table 1). The peptide C-termini were estimated by molecular weight on the basis of migration in SDS−PAGE gels and the positions of lysine residues in the sequence of PAI-1 (Franke et al., 1990).

The two bands in the latent PAI-1 digest were found to map to K191 in the gate and K207 also in the B−C barrel (Figure 4b). Lys-C also cleaves the 3 M urea-denatured active EP8 at K191 and K207. In addition, several other cleavages occur at R346 in the reactive center, at K277 (between s2C and s6A), at K154 (in the loop following helix F), and at K88 at the end of helix D (Figure 4c). The identity of several of the cleavage sites is verified by the presence of two peptides in the digest corresponding to that cleavage. The results suggest that sheet A is less stable and/or more exposed to proteolysis in active EP8 than in latent wild-type PAI-1.

The cleavage at K277 in active EP8 is also surprising. This residue is totally conserved, forms an ion pair with E332 at the C-terminus of s5A, and is partially buried by the gate in cleaved and latent PAI-1 (Figure 4b). Most potential Lys-C sites are not cut in either intermediate (Figure 4b,c), suggesting that even in 3 M urea a proportion of the secondary structure of PAI-1 is left intact, consistent with the CD and fluorescence measurements.

Folding of Active and Latent PAI-1 and EP8. To study the reversibility of the denaturation and folding of PAI-1, active and latent PAI-1 were denatured in 7.2 M urea and then diluted into lower concentrations of urea. The folding curves were similar, irrespective of the origin of the denatured protein, either active EP8 or latent wild-type PAI-1. While the refolding curves do not superimpose perfectly with either denaturation curve, they resemble most closely the denaturation curve for active PAI-1 (Figure 5). Apparently, active PAI-1 denatures and folds reversibly whereas latent PAI-1 does not. Dilution into pure buffer, in the absence of urea, yields approximately 95% active protein

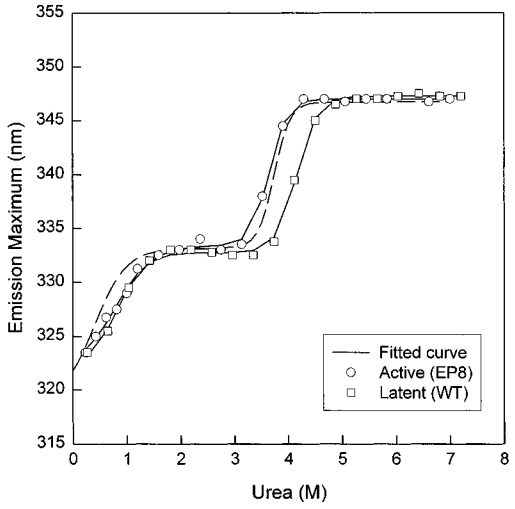

FIGURE 5: Folding of active EP8 (circles) on dilution from 7.2 M urea to the urea concentration indicated on the ordinate. Folding (squares) of latent wild-type PAI-1 on dilution from 7.2 M urea to the concentrations indicated. Note that the refolding of active PAI-1 (EP8) recapitulates the denaturation curve (dotted line), whereas the refolding of latent PAI-1 also approximately recapitulates the denaturation curve for the active form.

as assessed by gel-shift assay with uPA. Dilution of latent PAI-1 partially denatured in 3 M urea to lower concentrations of urea does not yield active protein (data not shown).

Conformational Energies. We used the perturbation method and fit the denaturation data to a two-transition model (Horowitz & Criscimagna, 1990). Assuming that the second transition in the urea denaturation profile corresponds to the final unfolding of the structure, active PAI-1 has a stabilization of $\Delta G(H_2O) \sim 12$ kcal while latent PAI-1 has a stabilization of $\Delta G(H_2O) \sim 21$ kcal.

DISCUSSION

The serpins are "spring-loaded" and capable of cleavage-induced or spontaneous inactivation. Kinetically driven folding events form a metastable active structure that converts with time to the more stable latent conformation. We show here that there are substantial energy differences between the active and latent state; the latent state is more stable by 9 kcal. The greater stability of the latent conformation may derive from differences in secondary structure, namely, the formation of strand s4A with the loss of strand 1C and a surface helix, in the latency transition.

Stable intermediates are found in the denaturation profiles of both active and latent PAI-1. The position of the fluorescence maximum suggests that some of the four tryptophan residues have become exposed to solvent in the intermediate. Two possible models for the structure of this intermediate are that much of one subdomain is disordered, exposing tryptophan residues (Matthews & Crisanti, 1981), and the second one is that tryptophan residues near the surface are exposed (Smith et al., 1991) without significant losses in secondary structure. The CD measurements show that the intermediate in the denaturation profiles of latent PAI-1 possesses significant amounts of secondary structure. Therefore, it is likely that the intermediates are the result of local losses of structure rather than loss of a subdomain. The Lys-C proteolysis data (Figure 4) confirm that the intermediate in the denaturation profile of latent PAI-1 contains significant secondary structure: many potential proteolysis sites in both of the subdomains of PAI-1 have not been accessed by Lys-C enzyme in the denaturation intermediate. Thus, the model that tryptophans near the surface and partially exposed have become fully exposed, similar to the findings for the two-stage denaturation profiles of glyceraldehyde 3-phosphate (Smith et al., 1991), is likely correct for the intermediate in latent PAI-1.

The Lys-C cleavage pattern of the 3 M urea intermediates reveals greater accessibility of lysine residues or flexibility in active versus latent PAI-1. The added flexibility is near sheet A, in the loop following helix F (thFs3A) and at the end of helix D. It is interesting that both of these regions are known to change conformation in the latency transition (Stein & Chothia, 1991). The data are consistent with the Ginsburg results where random mutagenesis and screening were used to identify PAI-1 molecules with slowed latency kinetics (Berkenpas et al., 1995). Three of the Ginsburg mutations (L91I, N150H, and K154T) are close to these cleavage sites.

The cleavage of K277 in the 3 M urea intermediate of active PAI-1 is also intriguing because it is likely to be buried by interactions with the gate, as described above. This observation suggests the gate is perhaps poorly tethered in the active structure intermediate (Figure 4). The gate is disordered in latent PAI-1 (Mottonen et al., 1992) crystals, and well fixed in the cleaved form (Aertgeerts et al., 1995). We used the hypothesis that the gate is not well ordered in the active structure successfully to make site-directed mutants to stabilize active PAI-1 (Tucker et al., 1995).

Previous experimentation has shown that latent PAI-1 folds into the active structure on dilution into buffer from high concentrations of denaturant (Hekman & Loskutoff, 1985). Our major finding here is that latent PAI-1, denatured in urea, folds into the active conformation over the full range of urea concentrations. What do these folding profiles tell us about the apparent kinetic control of the folding of this protein? Two well-studied metastable proteins are examples of opposing models for origin of the kinetic control of folding. Subtilisin refolded in the absence of its propeptide will remain indefinitely in a "molten globule" state but in the presence of propeptide folds into an active well-defined structure (Bryan et al., 1995). A similar phenomenon has been observed for α-lytic protease (Baker & Agard, 1994). These molecules continue to move, presumably sampling conformational space in the molten globule state, but never find the energetically favored conformation. Consideration of the refolding curve for latent PAI-1 near 5 M urea reveals that the latent structure is stable but apparently is kinetically inaccessible: the fluorescence measurements suggest that there is little structure under these conditions. At high urea concentrations then, the polypeptide does not find the stable structure available to it in a fashion reminiscent of the subtilisin molten globule. As discussed by Bryan et al. (1995), it may be that in the "energy landscape" around the stable conformation are high energy intermediates.

An alternative model is that a fast folding event traps the structure in the active conformation. Considering the folding of PAI-1 in buffer, and at low urea concentrations, the latter model appears appropriate: the stable latent conformation is not formed because it is the active structure that forms more rapidly, creating a kinetic trap. Sali et al. (1994) have suggested that the speed with which a structure folds is related to its stability, since the retention of a particular

substructure on folding is dominated by the off-rate. The limited proteolysis of active PAI-1 suggests that the sheet A subdomain is less stable in the active conformation than in the latent. Perhaps the B−C barrel is rapidly formed in active serpins, driving the folding of the active conformation. These questions will be addressed with further experimentation.

Carrell has suggested that latency transition is promoted by denaturants (Carrell et al., 1991). We have no evidence for this in the present study. If some fraction of the active protein were becoming latent during the 24 h incubation used in these denaturation studies, this would be reflected in some intermediate shift in the fluorescence maximum, and the presence of three transitions.

ACKNOWLEDGMENT

We are grateful to Dr. Phil Thomas for the use of his PTI instrument.

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BI961214P