



# Local Transient Unfolding of Native State PAI-1 Associated with Serpin Metastability\*\*

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**Abstract:** The metastability of the native fold makes serpin (serine protease inhibitor) proteins prone to pathological conformational change, often by insertion of an extra  $\beta$ -strand into the central  $\beta$ -sheet A. How this insertion is made possible is a hitherto unresolved question. By the use of advanced hydrogen/deuterium-exchange mass spectrometry (HDX-MS) it is shown that the serpin plasminogen activator inhibitor 1 (PAI-1) transiently unfolds under native condition, on a second-to-minute time scale. The unfolding regions comprise  $\beta$ -strand 5A as well as the underlying hydrophobic core, including  $\beta$ -strand 6B and parts of helices A, B, and C. Based thereon, a mechanism is proposed by which PAI-1 makes transitions through progressively more unfolded states along the reaction coordinate to the inactive, so-called latent form. Our results highlight the profound utility of HDX-MS in detecting sparsely populated, transiently unfolded protein states.

Serpins natively fold to a kinetically trapped metastable conformation characterized by an incomplete five-stranded central  $\beta$ -sheet A (Figure 1a). Protease inhibition by serpins involves a profound 70 Å translocation of the protease, driven by the insertion of the reactive center loop (RCL) into the central  $\beta$ -sheet A of the serpin.<sup>[1]</sup> Loop insertion may also occur in the absence of protease, as in the case of the natural inactivation mechanism in PAI-1, and formation of the latent conformation. Moreover, serpin mutations with pathological outcome are associated with latency transition or formation of toxic,  $\beta$ -sheet A expanded, polymeric serpin species.<sup>[2]</sup>

Protease-independent loop insertion in serpins represents some of the largest known conformational changes without covalent modification. The minimum of structural events

which have to occur for a serpin to adopt the latent conformation is well defined by X-ray crystal structures of the native and latent conformation.<sup>[3]</sup> It includes opening of  $\beta$ -sheet A to allow insertion of the RCL as the sixth strand. However, the transition state(s) during latency transition are unknown. PAI-1 spontaneously adopts the latent conformation at physiological temperature and salt concentration<sup>[4]</sup> and therefore offers a unique opportunity for studying serpin dynamics and conformational change in a physiologically relevant setting. Furthermore, PAI-1 hypo- and hyperactivity is linked to disease development.<sup>[5]</sup> Development of pharmaceuticals which can effectively modulate the activity of PAI-1 is therefore desirable, possibly through acceleration or deceleration of latency transition.

The rate of PAI-1 latency transition is typically measured using an activity assay.<sup>[6]</sup> As a basis of comparison, we have devised a new method to measure the rate of latency transition under the same experimental conditions as used for investigation of local unfolding in PAI-1 (see Figure S1 in the Supporting Information). The half-life of the active form was found to be ~120 min in H<sub>2</sub>O and ~450 min in D<sub>2</sub>O at 37 °C and physiological salt concentration, in agreement with values obtained from PAI-1 activity assays.<sup>[7]</sup>

We have now investigated native state unfolding reactions in PAI-1 using state-of-the-art HDX-MS.<sup>[8]</sup> Cooperative unfolding events in proteins can be monitored using HDX-MS if exchange of the backbone amide hydrogens in the unfolding region occurs simultaneously when the region is unfolded. If this requirement is met, the unfolding yields a unique signature in the mass spectrum, namely, bimodal mass distributions. The high- and low-mass populations of this bimodal distribution correspond to molecules that have already and have not yet visited the unfolded state, respectively.<sup>[8a]</sup> Here, we report the first observation of bimodal mass distributions in a serpin under native conditions, that is, evidence of native state unfolding events. The observations were made in three PAI-1 peptides covering residues 46–63 (helices B and C), 16–40 (helix A,  $\beta$ -strand 6B and helix B) and 307–340 ( $\beta$ -strand 5A and the RCL, Figure 1a,b). The unfolding reaction pertaining to peptide 46–63 was investigated by incubation of active PAI-1 in 90% D<sub>2</sub>O for 5–400 seconds at 15 °C (Figure 1c). After 5 seconds, about 1.5 and 8.5 deuterons were incorporated into the low and high mass populations, respectively, corresponding to correlated exchange of at least nine backbone amides out of the total 17 in the peptide, when correcting for the 90% D<sub>2</sub>O concentration and 14.4% back-exchange (see Figure S2). These more than 9 amide hydrogen atoms must be simultaneously separated from previously hydrogen-bonded acceptor oxygen atoms by at least 3 Å, to allow access of the catalyst anion

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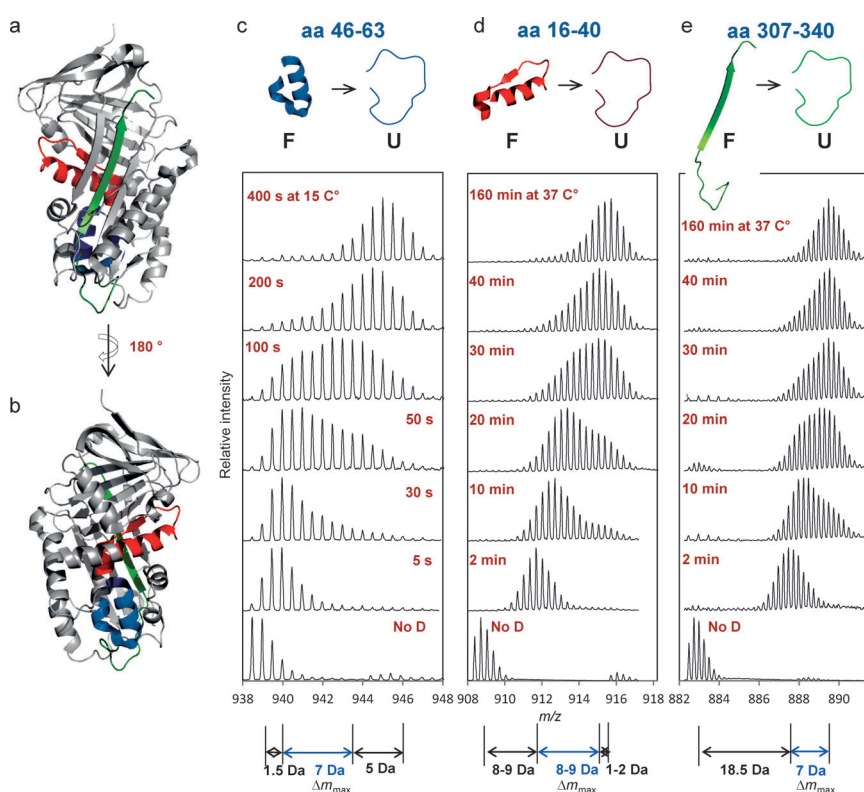
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OD- as well as D<sub>2</sub>O for completion of the HDX reaction.<sup>[9]</sup> Moreover, the unfolded state must have a residence time of hundreds of milliseconds or more, since the half-life of intrinsic chemical exchange for backbone amides in the peptide are in the 90–900 millisecond range at pD 7.4 and 15 °C<sup>[10]</sup> (Figure S2). Whether unfolding occurs only in helix C or in both helices C and B is not known. A 5 seconds incubation at 37 °C also resulted in bimodal mass distributions, confirming that the unfolding reaction also occurs at physiological temperature (Figure S2).

The unfolding reactions pertaining to peptides 16–40 and peptide 307–340 were investigated by incubation of active PAI-1 in 90% D<sub>2</sub>O for 2–160 minutes at 37 °C. Peptide 16–40 incorporates 8–9 deuterons within the first 2 minutes, followed by a slower correlated exchange of another 8–9 deuterons, corresponding to at least 11 out of 23 amides (Figure 1d and Figure S3). Electron transfer dissociation of the peptide,<sup>[8b]</sup> following 5 minutes of exposure to D<sub>2</sub>O, revealed high deuterium uptake in residues 22–30 and hence the 11 amides exchanging in a correlated fashion must reside within residues 16–21 and 31–40 (Figure 2a and Figure S4). These residues have a large contact surface in the PAI-1 structure, enabling correlated exchange through an

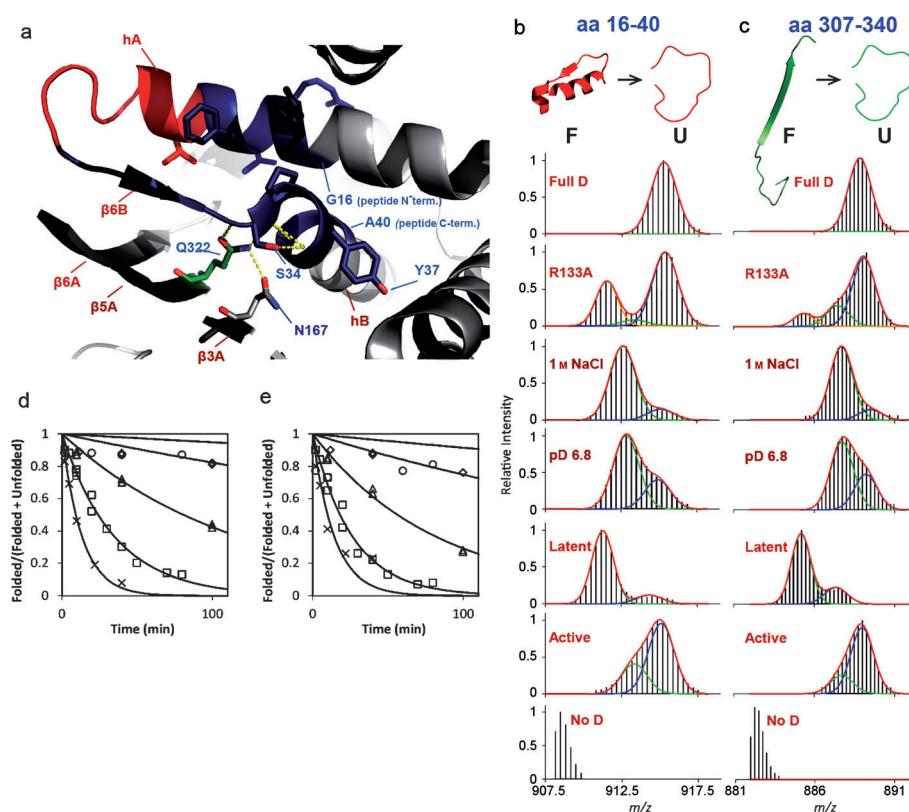
unfolding event. Peptide 307–340 incorporates 18.5 deuterons within the first two minutes of exchange, followed by slow correlated exchange of 7 deuterons, corresponding to at least 9.6 amides (Figure 1e and Figure S5). The 9–10 amides exchanging in a correlated manner must include the 9 amides of residues 319–327 composing  $\beta$ -strand 5A, since residues 306–319 (preceding  $\beta$ -strand 5A) exchange completely within 10 minutes at 25 °C<sup>[7]</sup> and the flexibility and solvent exposure of the RCL precludes slow correlated exchange. Of the backbone amide hydrogen atoms in peptide 16–40 and 307–340 involved in the correlated exchange, the half-life of chemical exchange are generally in the order of 10–100 milliseconds at pD 7.4 and 37 °C<sup>[10]</sup> (Figures S3 and S5). This implies a residence time in the unfolded states of several hundreds of milliseconds. These observations indicate complete unfolding of, at least, the secondary structural elements covered by the two peptides. While most PAI-1 peptides reach full deuteration within 160 minutes of exchange at 37 °C, a number of peptides from  $\beta$ -sheet B remain substantially protected against exchange (Figure S6) and no bimodal mass distributions were observed for those. The unfolding events detected here do therefore not extend to all of  $\beta$ -sheet B.



**Figure 1.** Cooperative unfolding in active PAI-1 revealed by hydrogen/deuterium-exchange mass spectrometry. Structure of PAI-1 (3Q02, W175F mutant<sup>[22]</sup>) a) front and b) back view. Peptide 16–40 is shown in red, peptide 46–63 in blue and peptide 307–340 in green. Mass spectra of peptide c) 46–63, d) 16–40 and e) 307–340 following incubation of 40 pmol (0.2  $\mu$ M) active PAI-1 in 90% D<sub>2</sub>O containing buffer (10 mM phosphate, 137 mM NaCl, pD 7.4) at c) 15 °C or d) and e) 37 °C for the indicated periods of time. Based on Gaussian fits to the interchanging low- and high-mass populations approximate mass differences between unlabeled and the low-mass populations, maximum mass differences between the low-mass and high-mass populations ( $\Delta m_{\max}$ ), and mass differences between the high-mass populations and the fully deuterated control are indicated.

Next, the rate of unfolding was evaluated. The low-mass fraction of peptide 46–63 displayed a half-life of 80 seconds at 15 °C, whereas the low-mass fractions of peptides 16–40 and 307–340 displayed similar half-lives of 24 and 18 minutes at 37 °C, respectively (Figures S2, S3, and S5). Similar experiments on purified latent PAI-1 revealed no correlated unfolding in peptide 16–40 and 307–340 at 37 °C, whereas slow unfolding was observed in peptide 46–63 at 37 °C (Figures S2, S3, and S5). The unfolding events occurring in the native ensemble of active PAI-1 are thus effectively quenched in the latent form. Control experiments confirmed that unfolding of the active form also occurs in protiated solvent (Figure S7) and that the unfolding reactions were reversible (Figure S8).

Residues 31–40 comprise part of the hydrophobic core of PAI-1 and participate in a hydrogen-bonding network important for  $\beta$ -sheet A stability<sup>[11]</sup> (Figure 2a). Unfolding in this region as well as in  $\beta$ -sheet A, at comparable rates, is intriguing considering the requirement for  $\beta$ -sheet A opening during protease inhibition and latency transition. The rate of latency transition is very sensitive to salt concentration and pH and here we show that the observed unfolding reactions are as well. Decreasing the pD from 7.4 to 6.8 decreased the rate of unfolding in peptides 16–40 and 307–340



**Figure 2.** Transient unfolding of  $\beta$ -strand 5A and the underlying hydrophobic core of PAI-1 suggest a role in latency transition. a) Cross-sectional view of helix A,  $\beta$ -strand 6B, helix B and  $\beta$ -strands 3, 5 and 6A in active PAI-1 (3Q02, W175F mutant).<sup>[22]</sup> Peptide 16–40 residues containing backbone amide hydrogens exchanging in a slow/correlated and fast/uncorrelated manner are colored blue and red, respectively. Residues S34, Y37, N167 and Q322 are involved in a conserved hydrogen bonding network shown as yellow dashed lines. Mass spectra of residue 16–40 and 307–340 peptide ions are shown in b) and c), respectively. From the bottom and to the top the spectra are measured for an undeuterated control (No D), active wild-type (active) or latent wild-type (latent) PAI-1 incubated for 40 min in 90%  $D_2O$ , 10 mM phosphate, pD 7.4 or pD 6.8 (active wild-type, pD 6.8), 137 mM NaCl or 1 M NaCl (active wild-type, 1 M NaCl) at 37°C, R133A mutant PAI-1 (R133A) incubated for 40 min in 90%  $D_2O$ , 10 mM phosphate pD 7.4, 137 mM NaCl at 37°C, and finally active wild-type PAI-1 incubated for 20 min in 4 M guanidine-HCl at 50°C to achieve full deuteration (Full D). A pre-existing latent fraction of the R133A mutant was present and hence three mass-populations were observed following deuteration of this mutant. The mass populations were quantified using Gaussian fits and the relative quantity of the low-mass fraction (green line) is plotted as a function of time for d) peptide 16–40 and e) peptide 307–340. Squares represent active PAI-1, circles represent latent PAI-1, triangles represent pD 6.8, diamonds represent 1 M NaCl, crosses represent R133A mutant PAI-1. The data were fitted to exponential decay functions (black lines) to obtain the rate constants of unfolding ( $k_u$ ) and the half-life ( $t_{1/2}$ ) of the folded state.

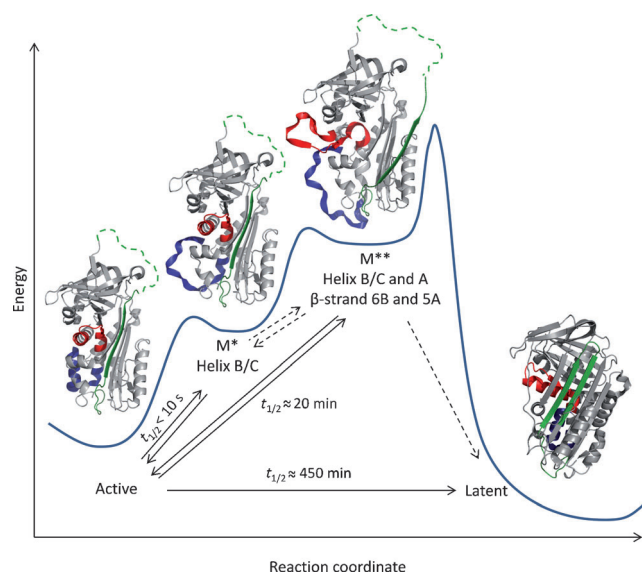
approximately 3-fold (Figure 2 and Figures S9 and S10). Increasing the NaCl concentration from 137 mM to 1000 mM decreased the rate of unfolding in peptides 16–40 and 307–340 approximately 14-fold (Figure 2 and Figures S9 and S10). Similar delays of latency transition as a result of decreasing pH or increasing salt was previously reported.<sup>[12]</sup> Finally, analysis of the unfolding rate in a PAI-1 R133A mutant, exhibiting a 2-fold increase in rate of latency transition over wild-type,<sup>[13]</sup> also showed approximately 2-fold increased rates of unfolding in peptide 16–40 and 307–340 (Figure 2 and Figure S11).

The observation of transiently unfolded structures being visited in the native ensemble of PAI-1 conflicts with “the minimalistic model” of latency transition previously de-

scribed.<sup>[7]</sup> Based on the observations reported here, we propose that the unfolding of helices B/C,  $\beta$ -strand 5A, and the underlying hydrophobic core in PAI-1 populates structural intermediates on the reaction trajectory to the latent form (Figure 3). We have previously shown that deuterium uptake in helices B/C is greatly reduced upon binding of ligands which function as inhibitors of latency transition.<sup>[7,14]</sup> Taken together with the observations reported here, these data suggest that the investigated ligands delay latency transition by prohibiting otherwise frequent and destabilizing unfolding events in helices B/C. The faster rate of unfolding in helices B/C ( $t_{1/2}$  is about 80 s at 15°C and < 10 s at 37°C) compared to the unfolding in helix A,  $\beta$ -strand 6B, top of helix B, and  $\beta$ -strand 5A ( $t_{1/2}$  is about 20 minutes at 37°C) is in agreement with a model of sequential unfolding.<sup>[15]</sup> According to this model, unfolding of helices B/C populates intermediates ( $M^*$ ) from which unfolding of the top of helix B,  $\beta$ -strand 6B, helix A, and  $\beta$ -strand 5A ( $M^{**}$ ) is facilitated (Figure 3). Because  $\beta$ -strand 5A unfolds at a similar rate as the underlying hydrophobic core it seems reasonable that these events are coupled. Latency transition is substantially slower ( $t_{1/2}$  is about 450 minutes at 37°C) than the unfolding of  $\beta$ -strand 5A and the underlying hydrophobic core, implying that another barrier exists along the reaction coordinate to the latent form. Although, the model of sequential unfolding en route to the latent conformation is in-line with

the experimental data obtained here the transitions from  $M^*$  to  $M^{**}$  and from  $M^{**}$  to the latent form remains to be proven. The existence of a “pre-latent” state of PAI-1 was previously proposed,<sup>[16]</sup> however, further investigations are required to establish the connection between the proposed “pre-latent” state and the unfolded states observed here.

Studies on  $\alpha$ 1-antitrypsin indicate that  $\beta$ -strand 5A is one of the final structural elements to fold<sup>[17]</sup> and one of the first to unfold under denaturing conditions,<sup>[18]</sup> suggesting that  $\beta$ -strand 5A may also visit unfolded states under native conditions in  $\alpha$ 1-antitrypsin. Furthermore, partial unfolding of  $\alpha$ 1-antitrypsin during complex formation with protease has been reported, including helix A,  $\beta$ -strand 6B and parts of  $\beta$ -sheet A.<sup>[19]</sup> Transient unfolding of  $\beta$ -sheet A as well as the



**Figure 3.** A model of PAI-1 latency transition involving transiently unfolded states. Potential-energy diagram of the conversion of PAI-1 from the active (3Q02, W175F mutant<sup>[22]</sup>) to the latent form (1DVN<sup>[13a]</sup>). The dashed green line indicates the hypothetical location of the RCL. Models of activated/unfolded structures M\* and M\*\* are created based on the HDXMS evidence of cooperative unfolding presented here. Full and dashed reaction arrows indicate measured and hypothetical transitions, respectively. Half-life's ( $t_{1/2}$ ) of unfolding and latency transition measured in 10 mM phosphate, 137 mM NaCl, pH 7.4 at 37°C are indicated.

underlying hydrophobic core may also be important for pathogenic latency transition of serpin mutants which are first secreted into blood in a actively folded conformation, as exemplified by antithrombin Rouen-VI<sup>[20]</sup> and antithrombin Wibble.<sup>[21]</sup> Our observations of transient unfolding from the native state in wild-type PAI-1, without the use of elevated temperature or denaturant, provides new insight to the intrinsic dynamics of the native serpin conformation. The observations reported here will thus have important ramifications for future studies of serpin structural dynamics in general and may prove instrumental in future drug design against serpin-related diseases.

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