Polymerization of Plasminogen Activator Inhibitor-1*

Received for publication, November 26, 2000
Published, JBC Papers in Press, December 1, 2000, DOI 10.1074/jbc.M010631200

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The activity of the serine proteinase inhibitor (serpin) plasminogen activator inhibitor-1 (PAI-1) is controlled by the intramolecular incorporation of the reactive loop into β-sheet A with the generation of an inactive latent species. Other members of the serpin superfamily can be pathologically inactivated by intermolecular linkage between the reactive loop of one molecule and β-sheet A of a second to form chains of polymers associated with diverse diseases. It has long been believed that PAI-1 is unique among active serpins in that it does not form polymers. We show here that recombinant native and latent PAI-1 spontaneously form polymers in vitro at low pH although with distinctly different electrophoretic patterns of polymerization. The polymers of both the native and latent species differ from the typical loop-A-sheet polymers of other serpins in that they readily dissociate back to their original monomeric form. The findings with PAI-1 are compatible with different mechanisms of linkage, each involving β-strand addition of the reactive loop to s7A in native PAI-1 and to s1C in latent PAI-1. Glycosylated native and latent PAI-1 can also form polymers under similar conditions, which may be of in vivo importance in the low pH environment of the platelet.

Plasminogen activator inhibitor type 1 (PAI-1)1 is a member of the serine proteinase inhibitor or serpin superfamily (1, 2). Serpins play an important role in the control of proteinases involved in blood coagulation, complement activation, and inflammation and are distinguished functionally from other types of protein inhibitors by their ability to form SDS stable complexes with target proteinases. Crystal structures have shown that members of the family share a highly conserved tertiary fold consisting of three large β-sheets surrounded by nine α-helices. This scaffold presents the reactive loop as a pseudosubstrate that binds to and inhibits the target proteinase (3). The target proteinases of PAI-1 are urokinase-type plasminogen activator and tissue-type plasminogen activator (TPA) (4) and as such it is an important modulator of events of extracellular proteolysis in fibrinolysis and in the turnover of extracellular matrix (5).

One of the most striking features of serpins is their ability to undergo a dramatic conformational rearrangement with the N-terminal portion of the reactive loop inserting into β-sheet A (6). This transition with cleavage of the loop is central to the formation of a stable inhibitory complex (7–10), but it can also occur spontaneously in vivo, without cleavage of the loop, to form an inactive latent conformation (11–14). Moreover, antithrombin and α₁-antitrypsin can be induced to adopt a latent conformation by heating in stabilizing concentrations of sodium citrate (15–17). Serpins are also able to link their reactive loop to a β-sheet of another molecule to form loop-sheet polymers, one form of which (18) has recently been crystalized (19, 20). These polymers are of considerable importance because they underlie the deficiency of α₁-antitrypsin (3, 21–25), antithrombin (13, 26, 27), α₁-antichymotrypsin (28), and C1-inhibitor (29, 30) in association with liver cirrhosis, thromboembolism, emphysema, and angioedema, respectively. The process also underlies a novel early onset dementia characterized by inclusion bodies of neuroserpin polymers (31).

Plasminogen activator inhibitor-2 can undergo this noncovalent polymerization spontaneously (32), and most other inhibitory serpins will polymerize upon heating or treatment with mild denaturants (16, 33). This conformational change has not been described in PAI-1, which more readily adopts the inert latent configuration. However, the recent crystal structure of recombinant active PAI-1 revealed PAI-1 as a polymer in which the reactive loop anneals as strand 7A of β-sheet A (34). This is in contrast to other models of serpin polymers in which the loop anneals with either the A- or C-sheet of another molecule (Fig. 1). We have examined the behavior of PAI-1 under a variety of conditions and show here that both the native and latent species can form polymers at low pH.

EXPERIMENTAL PROCEDURES

Materials—Unless otherwise stated, all reagents were obtained from BDH Chemicals Ltd., Subtilisin Carlsberg, and riboflavin were purchased from Sigma, and phenyl-Sepharose (low substituted), CM-Sepharose FF, and Superdex S200 resins were from Amersham Pharmacia Biotech. Human tPA was from Roche Molecular Biochemicals. Active glycosylated PAI-1 (from HT1080 cells) was a generous gift from Dr. Peter Andreasen (Department of Molecular and Structural Biology, Aarhus University, Aarhus, Denmark), or prepared by refolding latent glycosylated PAI-1 (from Alpha Laboratories, Hants, UK) as described previously (35). The PAI-1 expression plasmid pET11c-PAI-1 was a kind gift from Prof. Xianxiu Xu (Nanjing University) and the peptide Ac-TVASSSTA (encoding P14-P7 for PAI-1) was synthesized by MWG-Biotech UK Ltd.

Preparation of Mutant PAI-1 and Purification of Recombinant Protein—The construction of pET11c-PAI-1 and expression of recombinant
PAI-1 have been described previously (36). The stable PAI-1 mutant (N150H, K154T, GQ19L, and M354I) (37) was prepared by polymerase chain reaction mutagenesis and confirmed by DNA sequencing. The purification protocol was a modification of published methods. Briefly the cell lysate was applied to a CM-Sepharose column (2.5 × 40 cm), and then, after washing to base line with buffer A (20 mM NaOAc, 0.2 mM NaCl, 0.1 mM EDTA, pH 5.6), the bound proteins were eluted with a linear gradient of buffer A containing 0.2–1.0 mM NaCl. PAI-1 eluted at 0.6 mM NaCl, and PAI-1 containing fractions were pooled and mixed with ½ volume of buffer B (5 mM (NH₄)₂SO₄, 20 mM NaOAc, 0.1 mM EDTA, pH 5.6). The solution was then loaded onto a phenyl-Sepharose column (1.6 × 10 cm) that had been equilibrated with buffer C (1 mM (NH₄)₂SO₄, 20 mM NaOAc, 0.1 mM EDTA, pH 5.6), and the protein was eluted with a 400 ml gradient of 1.0–0 mM (NH₄)₂SO₄ in 20 mM NaOAc, 0.1 mM EDTA, pH 5.6. Native and latent PAI-1 were collected separately, concentrated to 1 mg/ml in 5 mM NaOAc, 0.1 mM EDTA, 0.1 mM NaCl, pH 5.6, and stored at −70 °C. The purity of PAI-1 in each peak was characterized by SDS-PAGE.

Preparation of Reactive Loop Cleaved PAI-1—Native PAI-1 (200 µg/ml) was incubated with subtilisin (4 µg/ml) at a final ratio of 100:1 (w/w) in 5 mM NaOAc, 0.1 mM EDTA, 0.1 mM NaCl, pH 5.6, at room temperature for 45 min, and then the reaction was stopped by the addition of phenylmethylsulfonfluoride to a final concentration of 1 mM. A ½ volume of buffer C was added and reactive loop cleaved PAI-1 was separated from the native and latent form on the phenyl-Sepharose column as described above. The cleaved form of PAI-1 was characterized by SDS-PAGE, mass spectroscopy, and N-terminal sequencing.

Nondenaturing Polyacrylamide Gel Electrophoresis with Low pH Discontinuous Buffer System—This system was first described by Jovin in 1973 (38) and modified to assess the conformers of PAI-1. The separating gel contained 12% (w/v) acrylamide, 0.4% (w/v) bisacrylamide, and 260 mM acetic acid adjusted to pH 4 with KOH. The stacking gel contained 2.5% (w/v) acrylamide, 0.625% (w/v) bisacrylamide, and 90 mM acetic acid adjusted to pH 5.0 with KOH. The pH of both were varied to assess conformations of PAI-1. A mixture of riboflavin and TEMED (with final concentrations of 5 mM/gel and 1.25 ml/liter, respectively) was used to initiate polymerization of the acrylamide. The electrode buffer contained 40 mM β-alanine adjusted to pH 4 with acetic acid, and methyl green was used as the tracking dye. The proteins migrated toward the negative electrode. All the gel electrophoresis was performed at room temperature unless otherwise stated.

Transverse Urea Gradient Gel Electrophoresis—12% (w/v) polyacrylamide gels were cast with a double lumen tube and a peristaltic pump to give a linear gradient from 0 to 8 M urea using the low pH nondenaturing PAGE buffer system. The gels were rotated through 90°, the stacking gel was poured, and the gels were run using the same electrode buffer as above. The proteins were visualized by staining with Coomassie Blue or by silver staining.

Fluorescence Measurements—Fluorescence measurements were made using a PerkinElmer Life Sciences 50B spectrofluorimeter as detailed previously (39). Intrinsic tryptophan fluorescence of PAI-1 was measured using an excitation wavelength of 295 nm and detecting photons with a wavelength of 340 nm emitted at 90° to the excitation beam. Wherever possible the slits controlling the intensity of the excitation light source were kept at the minimum machine-permissible limit of 2.5 nm. Emission slit widths were varied between 2.5 and 15 nm depending on the experimental conditions to give the optimal emission signal. The experiments were performed in a 0.5-ml cuvette with a path length of 1 cm on the excitation axis and 0.2 cm on the emission axis. Light scattering experiments were performed under the same conditions but at an excitation wavelength of 400 nm and an emission wavelength of 405 nm.

Circular Dichroism—CD experiments were undertaken in a 0.5 mm path length quartz cell using a JASCO J-810 spectropolarimeter. Samples of PAI-1 were prepared in 0.1 mM NaOAc for pH values between 4 and 5.5, 0.1 mM sodium phosphate for pH values between 6 and 8, and 100 mM Tris for pH 8.9. The protein was examined at 0.28 mg/ml with 25 mM NaCl from pH 4 to 8.9. All buffers were filtered, and samples were centrifuged before the experiment. Changes in secondary structure of PAI-1 with temperature were measured by monitoring the CD signal at 222 nm or 216 nm between 25 and 95 °C with a heating rate of 2 °C/min. The second derivative of the resulting data was then used to calculate the inflection point of the transition and hence the Tm (39). Measurement of the far UV CD spectra of native and polymerized PAI-1 at pH 4 was complicated by the high absorbance of the buffer (0.1 M NaOAc). This was reduced by dialyzing PAI-1 against water at 4 °C and then diluting into 5 mM NaOAc, pH 4 (0.48 mg/ml). The spectra were recorded at 20 °C in 5 mM NaOAc, pH 4, and averaged for 20 runs to give the profile for the native protein. PAI-1 was then incubated at 37 °C for 2 h before recording the spectrum of the polymerized protein.

N-terminal Amino Acid Sequencing and Mass Spectrometry—Purified PAI-1 or PAI-1 electrotransferred to Problot (40) was N-terminally sequenced by the Department of Biochemistry, University of Cambridge. The subtilisin cleavage of PAI-1 was also analyzed by matrix-assisted laser desorption ionization-time-of-flight mass spectrometry. Cleaved PAI-1 was exchanged with distilled water by Microcon Ultrafiltration. Sinapinic acid was used as the matrix. The Kratos Kompakt matrix-assisted laser desorption ionization IV (Kratos Analytical Ltd.,

**Fig. 1.** Schematic representation of serpin polymers. The loop-A-sheet interaction (left panel) is believed to underlie the long chain polymers that result from naturally occurring mutants of α₁-antitrypsin (57), but the C-sheet (middle panel) and s7A linkages (right panel) have been described in crystal structures of antithrombin and PAI-1, respectively (15, 34). In each of these models, the acceptor molecule is shown in red, and the linking reactive loop center loop is in cyan.
Manchester, UK) was operated in positive ion linear mode with an acceleration voltage of \(120 \text{ kV}\). The results were the averages of over 100 shots.

RESULTS AND DISCUSSION

Preparation of Native, Latent, and Cleaved PAI-1—Glycosylated recombinant PAI-1 spontaneously converts to the latent conformation with a half-life of 3 h under physiological conditions (4, 11), but this may be increased to 100 h if it is stored at high salt concentration, low pH, and room temperature (41). Our purification protocol of PAI-1 was a modification of existing methods (42, 43), and all the steps were carried out at room temperature. Following expression of PAI-1 and cell lysis, the supernatant was directly loaded onto a CM-Sepharose column and eluted with a NaCl gradient that gave \(\sim 90\%\) pure PAI-1 with a small fraction of latent protein (data not shown). The eluted PAI-1 was then salted to \(1 \text{ M} (\text{NH}_4)_2\text{SO}_4\) and further purified by phenyl-Sepharose chromatography, which resulted in complete separation of active PAI-1 from its latent conformation (Fig. 2a). The active peak (hereafter referred to as the native component) was confirmed to be PAI-1 by N-terminal sequencing (1VHHPP), and typically 1 g of wet Escherichia coli cell pellet yielded 5 mg of active and 0.2 mg of latent PAI-1.

Native, but not latent, PAI-1 was able to form SDS stable complexes with tPA (Fig. 2b) and could be cleaved at the P1-P1' bond of the reactive loop by subtilisin (Fig. 2c). Reactive loop cleaved PAI-1 was separated from native and latent protein by phenyl-Sepharose chromatography as the cleaved protein eluted before the other conformations (Fig. 2a); cleaved PAI-1 eluted at 0.6 \text{ M} (\text{NH}_4)_2\text{SO}_4, native PAI-1 at 0.4 \text{ M}, and the latent species eluted at 0.2 \text{ M} (\text{NH}_4)_2\text{SO}_4. The purity of all the PAI-1 species was assessed by SDS-PAGE, and the cleavage sites of subtilisin were analyzed by N-terminal sequencing and confirmed by matrix-assisted laser desorption ionization-time of flight mass spectrometry. The primary cleavage site was at P1-P1' (\text{Arg}^{346-\text{Met}^{347}}), which gives an N-terminal sequence of 1VHHPP, and typically 1 g of wet Escherichia coli cell pellet yielded 5 mg of active and 0.2 mg of latent PAI-1.

Native PAI-1 was easily distinguished from the reactive loop cleaved protein by SDS-PAGE (Fig. 2c) but migrated with the same electrophoretic mobility as latent PAI-1. Native PAI-1 migrated as a smear or remained in the sample well on a pH 7.8 nondenaturing gel. Because native PAI-1 is more stable at low pH, it was characterized on a pH 4 nondenaturing discontinuous buffer system (acid-PAGE). This differentiated between native, cleaved, and latent PAI-1 (Fig. 3a), and to our surprise both native and latent PAI-1 formed discrete high molecular mass ladders (Fig. 3a, lanes 1 and 2) characterized in other

![Fig. 2. Purification of recombinant PAI-1 from cell pellets. a, separation of active (peak I) from latent (peak II) PAI-1 by phenyl-Sepharose chromatography by elution with a 1.0–0 \text{ M} (\text{NH}_4)_2\text{SO}_4\) gradient. The dashed line shows the purification profile of cleaved PAI-1. b, 10\% w/v SDS-PAGE to show the purity and activity of native and latent PAI-1. Each lane contains 2.5 \text{ \mu g} of PAI-1 and, where indicated, 5 \text{ \mu g} of tPA. PAI-1-tPA complexes were formed by incubating enzyme and inhibitor at room temperature for 30 min in PAI-1 storage buffer. Lane M, molecular mass markers (78, 66, 45, and 32 kDa); lane 1, native PAI-1 from peak I; lane 2, native PAI-1 with tPA; lane 3, latent PAI-1 from peak II; lane 4, latent PAI-1 with tPA; lane 5, PAI-1 with subtilisin; lane 6, PAI-1 incubated with subtilisin in a 100:1 (w/w) ratio for different time intervals at room temperature before being separated by 10\% w/v SDS-PAGE. Each lane contains 2.5 \text{ \mu g} of PAI-1. Lane M, molecular mass markers (78, 66, 45, and 32 kDa); lanes 2–6, native PAI-1 incubated with subtilisin for 0, 5, 10, 20, and 30 min, respectively; lanes 7–11, latent PAI-1 incubated with subtilisin for 0, 5, 10, 20, and 30 min, respectively. d, cleavage sites of native PAI-1 by subtilisin. Cleaved PAI-1 samples were analyzed by N-terminal amino acid sequencing and mass spectrometry. The molecular masses of C-terminal fragments of cleaved PAI-1 are consistent with the calculated values.]}
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FIG. 3. Polymerization of native PAI-1. a, 12% (w/v) pH 4 non-denaturing PAGE. Lane N, native PAI-1; lane L, latent PAI-1; lane C, reactive loop cleaved PAI-1. Each lane contains 10 μg of PAI-1. The band marked with an asterisk is a dimer of cleaved PAI-1, which was confirmed by N-terminal amino acid sequencing and gel filtration chromatography. b, native PAI-1 was incubated at 0.1 mg/ml in 0.1 mM NaOAc, pH 4, at 37 °C for up to 12 h. Samples were withdrawn, snap frozen, and then analyzed on a pH 4 acid PAGE at 4 °C. Each lane contained 10 μg of protein. Lane L represents latent PAI-1 that was also incubated in 0.1 mM NaOAc, pH 4, for 12 h at 37 °C. c, assessment of the polymerization of PAI-1 using light scattering (dashed line) and intrinsic tryptophan fluorescence (continuous line). PAI-1 was incubated at 0.1 mg/ml in 0.1 mM NaOAc, pH 4, at 37 °C, and light scattering was assessed at an excitation wavelength of 400 nm and a detection wavelength of 405 nm. The data were fitted to a single exponential curve to give a rate constant (k1). Intrinsic tryptophan fluorescence was assessed at excitation and emission wavelengths of 295 and 340 nm, respectively. The data were fitted with a double exponential decay to give a fast initial rate (k1) and a slow second rate (k2). The value of k2 was similar to that obtained for the rate of increase in light scattering (k1) and comparable with the rate of polymerization observed in b. The rate of polymerization was assessed at different protein concentrations using intrinsic tryptophan fluorescence (d). The fast rate (k1) was independent of protein concentration, but the slow rate (k2) shown in the figure increased with increasing protein concentration.

The data were fitted with a double exponential decay to give a fast rate (k1) and a slow second rate (k2). The rate of polymerization was independent of protein concentration (1.24 ± 0.09 × 10⁻³ s⁻¹) over a range of concentrations of PAI-1 from 0.025 to 0.4 mg/ml. In contrast the slow rate increased with protein concentration (Fig. 3d), indicating a bimolecular process in keeping with polymer formation. The lack of concentration dependence of the fast rate implies that it is a unimolecular process in keeping with polymer formation. The kinetics of the Polymerization of PAI-1—To determine the kinetics of polymerization, native PAI-1 was incubated at pH 4 prior to separation on an acid-PAGE. Native PAI-1 completely polymerized after incubation at pH 4 and 37 °C for 3 h (Fig. 3b). The rate of polymerization of PAI-1 at pH 4 was assessed using light scattering at 0.1 mg/ml and 37 °C (Fig. 3c). The data were fitted to a single exponential that gave a rate of polymerization (k1) of 3.03 SD ± 0.49 × 10⁻⁵ s⁻¹ (n = 4). This rate corresponded well to the rate of polymer formation when assessed by acid-PAGE (Fig. 3b). The polymerization of PAI-1 was further examined using intrinsic tryptophan fluorescence. Incubation at 0.1 mg/ml, pH 4, and 37 °C resulted in a reduction in the fluorescence signal (Fig. 3c). This was fitted to a double exponential decay to generate an initial fast rate (k1) and then a slow rate (k2). The slow rate (2.93 ± 0.19 × 10⁻⁵ s⁻¹; n = 4) was comparable with the rate obtained from the light scattering and from the acid-PAGE.

Native and latent PAI-1 polymers were analyzed by gel filtration on a Superdex S200 column in 20 mM NaOAc, pH 4, or 20 mM Tris-HCl, pH 8.0, with 0.15 mM NaCl, 1 mM EDTA at room temperature. PAI-1 was incubated at 37 °C for 30 min at pH 4 and centrifuged to remove aggregates before loading serpins as loop-sheet polymers (16, 18, 33). Varying the pH confirmed that both native and latent PAI-1 formed polymers when assessed on gels between pH 3.5 and 5, but these were most marked at lower pH values. Reactive loop cleaved PAI-1 did not form polymers even after assessment on gels at pH 3.5. The resistance of the reactive loop cleaved PAI-1 to pH-induced polymerization may result from either inaccessibility of the reactive loop to act as a donor for polymerization or from the filling of the β-sheet A binding site by the cleaved reactive loop. These alternatives were assessed using PAI-1 that had been stabilized by incubation with an exogenous peptide corresponding to P14-P7 that binds to β-sheet A but leaves the reactive loop in its external exposed position (44, 45). The binary complex was formed by incubating PAI-1 with 100-fold molar excess of P14-P7 reactive loop peptide at 37 °C in 10 mM NaOAc, 50 mM NaCl, pH 5.6, for 24 h. Formation of the complex was confirmed by a loss of inhibitory activity with PAI-1 becoming a substrate for tPA. The peptide-PAI-1 complex failed to form polymers on an acid-PAGE with either the pattern seen with native PAI-1 or with latent PAI-1. Structurally the significant difference between the peptide-complexed and native PAI-1 is that of a six-stranded versus a five-stranded A-sheet, and the significant difference between latent PAI-1 and both the peptide-complexed and cleaved form PAI-1 is the presence of a vacant strand 1C position in the latent form. Thus, the results suggest that the polymerization of native PAI-1 requires a five-stranded A-sheet compatible with s7A linkage observed in the crystal structure (34) and that the different polymerization observed with latent PAI-1 may require a vacated s1C position, as in the C-sheet model (Fig. 1). Support for these conclusions of separate mechanisms of loop linkage is provided by the absence of either of the patterns of polymerization in reactive loop cleaved PAI-1, which has both a six-stranded A-sheet and an intact s1C.
onto the column. In both cases only a monomeric peak was apparent (data not shown). Thus, both polymers of native and latent PAI-1 were dissociable. These data are similar to the results obtained for the dimer of antithrombin which was crystallographically shown to be due to reactive loop-C-sheet linkage (15). This similarly dissociates to monomeric protein on gel filtration (46, 47). The inhibitory activity of the pH 4 treated native PAI-1 could not be assessed against tPA directly because tPA loses its enzymatic activity at this pH. However, after polymerization at pH 3.5–5 for 30 min, native PAI-1 was shown to regain almost full inhibitory activity against tPA when the pH was raised above 5. Conversely, no inhibitory activity was detected after treating latent PAI-1 at low pH for 2 h at 37 °C and then raising the protein to a neutral pH. Thus, in the latent PAI-1 polymers, the reactive loop must remain at least partially inserted into β-sheet A.

Characterization of the Unfolding of PAI-1 on pH 4 Transverse Urea Gradient Gels—The demonstration that conformations of PAI-1 could be distinguished on low pH nondenaturating gels prompted their examination by transverse urea gradient gels with low pH buffers. Active and cleaved PAI-1 exhibited similar profiles to the corresponding species of α1-antitrypsin or antithrombin (16, 48) but with an unfolding transition at ~3 M urea for the native species and resistance to unfolding in 8 M urea for reactive loop cleaved PAI-1 (Fig. 4, a and b). Latent PAI-1 had an unfolding transition on transverse urea gradient gels at ~6 M urea (Fig. 4c), which is unlike the latent conformations of α1-antitrypsin, antithrombin, and α1-antichymotrypsin, which are resistant to unfolding in 8 M urea (14, 16, 49). The binary complex of PAI-1 with the P14-P7 reactive loop peptide also starts to unfold at ~7 M urea (Fig. 4d), which again is different from those of α1-antitrypsin and antithrombin. These data demonstrate that incorporation of the reactive loop into the β-sheet of latent PAI-1 is not as stable as in other serpins and are consistent with the well recognized finding that latent PAI-1 can be reactivated by treatment with denaturants (4, 11) or by heating (50).

The Effect of pH on Reactive Loop Peptide Induced Inactivation of Native PAI-1—External peptides corresponding to the N-terminal portion of the reactive center loop of serpins form stable binary complexes, converting each inhibitor to a substate for target proteinases. Recent crystal structures of such binary complexes confirm that as assumed previously, the peptides bind to the vacant strand 4A position (45, 51). Thus, a measure of the readiness and accessibility of the opening of the A-sheet at s4A is indicated both by the rate of self-insertion to give the latent form and the rate of external peptide insertion to give the binary complex. To assess this, native PAI-1 was incubated with or without peptide (Ac-TVASSSTA, P14-P7) under different pH conditions for 20 h at room temperature. The mixtures were adjusted to neutral pH, excess tPA was added, and the samples were analyzed by SDS-PAGE (Fig. 5). The result indicates the proportion of active PAI-1 represented by the SDS stable complex with tPA and the proportion of binary complex represented by the cleaved band, as well as the proportion of the latent form represented by the intact band. At pH 8, after 20 h of incubation, most of the active PAI-1 was converted to the latent form when incubated alone (Fig. 5, lane 6), and almost all PAI-1 formed binary complex in the presence of peptide (Fig. 5, lane 7). However, when incubated at pH 4 (Fig. 5, lane 2), PAI-1 remains almost fully active and comparable with that of the starting material (Fig. 5, lane 1). Less than 5% of PAI-1 forms binary complex in the presence of peptide (Fig. 5, lane 3). Thus, at low pH the central β-sheet A of native PAI-1 is tightly closed, which inhibits self-loop insertion to form the latent form or the insertion of an external peptide from another molecule to form s4A loop-sheet polymers.

Circular Dichroism Signal Changes during the Polymerization of Native and Latent PAI-1—The effect of pH on the stability of native and latent PAI-1 was then assessed by monitoring the circular dichroic signal at 222 nm with increasing temperature. The conformers were incubated at pH 4–8.9 for 5 min at room temperature before assessing stability (Fig. 6a). Reducing the pH resulted in a progressive increase in stability of native PAI-1 such that the melting point of native PAI-1 was over 90 °C at pH 4.5. The protein showed no marked increase in ellipticity at temperatures of up to
98 °C at pH 4 (data not shown). The melting points ($T_m$) of latent PAI-1 were less sensitive to the change in pH (Fig. 6a). The CD signal at 222 nm indicates the $\alpha$-helical content of a protein; to assess changes in $\beta$-sheet content, the CD signal of native PAI-1 was monitored at 216 nm. Following incubation at pH 4.0 (but not at pH 8.0), there was a negative increase in ellipticity in keeping with an increase in $\beta$-sheet structure (Fig. 6b). Because the melting point of native PAI-1 incubated at pH 4 is much higher than that of latent form of PAI-1 (approx. 72 °C), the stable species formed at higher temperature cannot be ascribed to a transition to the latent conformation. Moreover, pH 4 acid-PAGE confirmed that the protein was in the polymeric conformation, and a direct comparison between the far UV CD profiles of native and latent PAI-1 showed less than 1% difference at 216 nm in keeping with the results of others (52).

The changes in secondary structure of native PAI-1 during polymerization were examined in more detail by circular dichroic spectra (Fig. 6c). The spectra were deconvoluted using the program Selcon3 (53) and a basis set containing 29 proteins with spectra between 178 and 260 nm. Monomeric PAI-1 contained 35% $\alpha$-helix, 19% $\beta$-strand, 20% $\beta$-turn, and 23% random coil. By contrast the polymeric protein contained 29% $\alpha$-helix, 19% $\beta$-strand, 24% $\beta$-turn, and 27% random coil. This indicates that polymerization at low pH results in a conformational change that includes an overall loss of $\alpha$-helical structure but no increase in $\beta$-sheet structure. The loss of $\alpha$-helix is consistent with a partial unfolding of the protein, an event often observed at extremes of pH. The lack of change of $\beta$-sheet content agrees well with the secondary structural changes that occur during the polymerization of $\alpha_1$-antitrypsin (39).

**Polymerization of Glycosylated PAI-1 and Stable PAI-1 Mutant**—Active and latent glycosylated PAI-1 were assessed on a pH 4 nondenaturing PAGE after incubation at pH 4 at 37 °C for 10 min. Both of them formed high molecular mass polymers similar to those of the recombinant protein (Fig. 7, lanes 6 and 7), respectively. PAI-1 can be rendered stable by four point mutations, N150H, K154T, Q319L, and M354I, that reduce the rate of intramolecular loop incorporation and hence transition to a latent conformation (37). This stable variant was prepared and assessed at low pH. The active conformation also formed high molecular mass species that were similar to those of native PAI-1 (Fig. 7, lane 1).

**Mechanism of PAI-1 Polymerization**—Native serpins are in a metastable conformation, and under mild denaturing conditions they adopt an intermediate species that may progress to a latent or polymeric conformation. The pathway may be described by the following equation (39).

![Fig. 6. Thermal stability and circular dichroic profile of native and latent PAI-1.](https://example.com/fig6.png)

![Fig. 7. 10% (w/v) pH 4 nondenaturing PAGE of recombinant and glycosylated PAI-1.](https://example.com/fig7.png)
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1

2

\[ M \rightarrow M^* \rightarrow P \]  

(Eq. 1)

where step 1 represents the conformational change of the serpin to a polymerogenic monomeric form (M*), step 2 represents the formation of polymers (P), and step 3 represents a side pathway that leads to the formation of the latent conformation (L). Although little is known about the exact structure of M*, it is obvious that the loop should be converted to or stay in β-strand conformation, which is necessary for polymerization.

Generation of the unstable intermediate M* occurs spontaneously in some serpins (28) in association with clinically relevant point mutations that facilitate the conformational transition. In serpins such as PAI-1, the protein scaffold favors conversion of the intermediate conformation to a latent species (54), whereas in others, such as α1-antitrypsin and C1-inhibitor, the serpin polymerizes (21, 33). Clearly such transitions are dependent upon environment, and this is best illustrated by α1-antitrypsin and antithrombin, which can be induced to adopt a latent configuration by heating in stabilizing concentrations of sodium citrate (15–17). It is perhaps not surprising that under specific conditions PAI-1 can also form the discrete polymers of latent PAI-1 might favor, in this case, the alternative loop-C-sheet (s1C) linkage (56). Our data suggest that the increase in stability at pH 4 for native PAI-1 is due to polymerization rather than transition to the latent con-

former. The mechanism of polymerization of native PAI-1 is similar to that of α1-antitrypsin (39) in that there is no increase in overall β-sheet structure despite being characterized by an aberrant β-strand linkage. The reactive loop can simply anneal as an extra strand of β-sheet A, as s7A for PAI-1 (Fig. 1c), and as s4A for α1-antitrypsin (Fig. 1c). Similarly there is no increase in overall β-sheet structure in the s4A or s1C polymerization models, in which s1C release is necessary (Fig. 1b).

In conclusion, this study shows that both native and latent PAI-1 can reversibly polymerize by mechanisms that are compatible with previously structurally-demonstrated s7A and s1C β-linkages. Physiologically it may be of significance that this polymerization occurs at low pH such as that found in platelets or at foci of inflammation.

Acknowledgments—We are grateful to Prof. Randy Read (Cambridge Institute for Medical Research, Cambridge, UK) for helpful discussions. We thank Dr. Peter Andreassen (Department of Molecular and Structural Biology, Aarhus University, Denmark) for providing active glycosylated PAI-1 and Dr. Hui Hong (Department of Chemistry, University of Cambridge, UK) for helping with mass spectrometry analysis.

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