The Acid Stabilization of Plasminogen Activator Inhibitor-1 Depends on Protonation of a Single Group That Affects Loop Insertion into β-Sheet A*

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The serpin plasminogen activator inhibitor-1 (PAI-1) spontaneously adopts an inactive or latent conformation by inserting the N-terminal part of the reactive center loop as strand 4 into the major β-sheet (sheet A).

To examine factors that may regulate reactive loop insertion in PAI-1, we determined the inactivation rate of the inhibitor in the pH range 4.5–13. Below pH 9, inactivation led primarily to latent PAI-1, and one predominant effect of pH on the corresponding rate constant could be observed. Protonation of a group exhibiting a pK_a of 7.6 (25 °C, ionic strength = 0.15 M) reduced the rate of formation of latent PAI-1 by a factor of 35, from 0.17 h⁻¹ at pH 9 to about 0.005 h⁻¹ below pH 6. The ionization with a pK_a 7.6 was found to have no effect on the rate by which PAI-1 inhibits trypsin and is therefore unlikely to change the flexibility of the loop or the orientation of the reactive center. The peptides Ac-TEASSTTA and Ac-TVASSSTA (cf. P14-P7 in the reactive loop of PAI-1) formed stable complexes with PAI-1 and converted the inhibitor to a substrate for tissue type plasminogen activator.

We found that peptide binding and formation of latent PAI-1 are mutually exclusive events, similarly affected by the pK_a 7.6 ionization. This is direct evidence that external peptides can substitute for strand 4 in β-sheet A of PAI-1 and that the pK_a 7.6 ionization regulates insertion of complementary, internal or external, strands into this position. A model that accounts for the observed pH effects is presented, and the identity of the ionizing group is discussed based on the structure of latent PAI-1. The group is tentatively identified as His-143 in helix F, located on top of sheet A.

Plasminogen activator inhibitor, PAI-1, is a member of the serpin family of serine protease inhibitors and inhibits both tPA and urokinase plasminogen activator (1, 2) as well as trypsin (3). The serpins (4), which include most of the inhibitors that regulate blood coagulation and fibrinolysis, are structurally homogenous and are distinguished functionally from other types of proteinase inhibitors primarily by the ability to form SDS-stable complexes with target proteinases. The nature of these complexes and the mechanism by which they are formed are poorly understood.

Active serpins are metastable folding intermediates with considerable conformational strain (5). They can relax by inserting the N-terminal portion of the reactive center loop as strand 4 in the major β-sheet that faces one side of the protein (sheet A) and thereby adopt an inactive, or latent, conformation. This process occurs spontaneously in PAI-1 (6) and has been induced in antithrombin III (5) and α₁-proteinase inhibitor (7). Inhibitory serpins attain an even greater stability after cleavage of the reactive loop, near or at the susceptible bond, and insertion of the N-terminal portion into β-sheet A (8–10) or after forming complexes with peptides that mimic this part of the loop (11, 12). The fact that serpins with a completed six-stranded sheet A are inactive (13) clearly indicates a role for loop insertion in the inhibitory mechanism. Data obtained for α₁-Proteinase inhibitor in complex with N-terminal truncated strand 4A-mimicking peptides were taken to indicate that insertion of the loop into sheet A down to residue P14 (notation according to Schechter and Berger (14)) is necessary and sufficient for inhibition of trypsin by this serpin (11). The concept presented was that with such limited insertion the loop adopts a more rigid conformation akin to that of the loop in Kunitz type inhibitors. The consequences of this view are that inhibition is due to tight binding and that the bound inhibitor can only be cleaved slowly. The bound reactive center may approach formation of a tetrahedral intermediate, which could account for the NMR data presented by Travis et al. (15). Evidence for a fast insertion of the loop region containing residue P9 upon formation of the tPA-PAI-1 complex was recently obtained in our laboratory from studies of a PAI-1 mutant with a fluorescent probe on P9 (16), and the idea was put forward that cleavage of the reactive bond may be required to trigger such a fast, extensive insertion of the loop. This, on the other hand, is difficult to reconcile with dissociation of proteinase-serpin complexes into active enzymes and inhibitors (17).

Although the fate of the susceptible bond in the proteinase-serpin complex remains obscure, it has been well established that in order for serpins to be fully active they must possess a potential for unhindered insertion of part of the reactive center loop into β-sheet A (11, 12, 18–20). Consequently, studies of loop insertion leading to latent PAI-1 and factors that affect this process may yield significant insights into the mechanism of serpin inhibition. Several studies (21–23) have shown that such factors are pH and that the transformation of active PAI-1 to latent is much slower at weakly acidic rather than at physiological pH values. The present investigation was initiated to characterize this pH effect and the associated proton dissociation equilibrium or equilibria.
PH EFFECTS ON PAI-1 STABILITY

EXPERIMENTAL PROCEDURES

Reagents— Gel filtration columns (PD-10) were from Pharmacia (Uppsala, Sweden), and TSK-DEAE was from Merck Pharmaceutical Company (Darmstadt, Germany). Materials for SDS-PAGE and activated agarose were from Bio-Rad Laboratories (Hercules, California). N-acetylated peptides were synthesized by the University of Michigan Protein Facility (Ann Arbor, Michigan) and provided as lyophilized free acids. H-iso-leucyl-l-prolyl-l-arginine-p-nitroaniline was obtained from Chromogenix (Malmö, Sweden), and p-nitrophenyl p-guanidinobenzoate was from Sigma. Buffers were prepared from analytical grade chemicals using deionized, distilled, and degassed water.

Proteins— Human recombinant PAI-1, expressed in Escherichia coli, was a generous gift from Dr. David Ginsburg (Howard Hughes Medical Institute, Ann Arbor, Michigan) and was supplied as a frozen bacterial lysate (24). The inhibitor was purified and separated into the active and latent forms by chromatography on heparin and phenyl-substituted Sepharose by a method previously developed by two of us and described elsewhere (25). The concentration of active PAI-1 was determined spectrophotometrically at 280 nm using the extinction coefficient 0.93 ml mg⁻¹ (26) and a molecular weight of 43,000. Tryptsin from bovine pancreas (N-tosyl-phenylalanine chloromethyl ketone-treated), bovine basic pancreatic trypsin inhibitor (BPTI) (Aprotinin), and soybean trypsin inhibitor were from Sigma. The pancreatic inhibitor was dissolved in 0.15 M NaCl, and its concentration was determined spectrophotometrically at 280 nm in a pH 7.4 buffer using an extinction coefficient of 0.84 ml mg⁻¹ and a molecular weight of 6531 (27). The soybean inhibitor was immobilized to activated agarose, according to the instructions provided by the manufacturer of the gel. Tryptsin was further purified by affinity chromatography on the immobilized soybean trypsin inhibitor, essentially as described in Ref. 28. The α- and β-trypsin fractions were precipitated by dialysis in the cold against solid ammonium sulfate, corresponding to 90% saturation at equilibrium, and stored as suspensions in the cold. Only β-trypsin was used in this study, and its active site concentration was determined by titration with p-nitrophenyl p-guanidinobenzoate (29). Upon use, aliquots of trypsin were withdrawn from the suspension and collected by centrifugation. Stock solutions were prepared by dissolving the precipitate in 1 M HCl. Activase, the tPA, was a kind gift from Dr. Bruce Keyt (Genentech Inc., San Francisco, CA). It was dissolved in water to give a tPA concentration of 3.2 mg ml⁻¹ and was used without further treatment. The coordinate set for latent PAI-1 was generously provided by Dr. Elizabeth Goldsmith (University of Texas Southwestern Medical Center, Dallas, TX).

General Experimental Conditions— Incubations of PAI-1 to measure its stability or reaction with peptides were performed at 25°C in solutions containing 1 mM EDTA, 0.025 M NaOH, and one of the following buffer substances: acetic acid (pH 4.5–5.5), MES (pH 6–7), HEPES (pH 7–8), Bis-Tris (pH 8–9), CHES (pH 8.5–9.5), or CAPS (pH 10–11). For pH values below 6, 0.1 M NaCl was used. All solutions were adjusted to 0.15 M ionic strength with NaCl. Gel electrophoreses were performed under nonreducing conditions in 12 or 9% polyacrylamide gels and in the presence of 0.1% SDS (SDS-PAGE). Typically, samples for SDS-PAGE were prepared by mixing aliquots of 4–5 μM PAI-1 with a 2:3 molar excess tPA at pH 7–8, which were then incubated for about 5 min. The developed gels were stained with Coomassie Blue.

Quantitative Analysis of Gels— Transmission images of stained gels were taken with a video camera, and the gray scale intensity of each pixel was converted to absorbance. An estimate of the relative amount of protein in a band on the gel was obtained by summation of the pixel absorbances within the band after subtracting the background absorbance.

Spontaneous Inactivation of PAI-1— The rate constant for inactivation of PAI-1 as a function of pH was determined by incubating 5 μM active inhibitor in buffers ranging in pH from 4.5–13. Aliquots were withdrawn at timed intervals and assayed for active PAI-1. For this purpose, tPA was added to vortexted with 0.1 M H-iso-leucyl-l-prolyl-l-arginine-p-nitroaniline in a pH 7.4 HEPES buffer, and the amylodic activity was determined spectrophotometrically at 405 nm. The aliquot with PAI-1 was added, and its content of active inhibitor was calculated from the resulting decrease in tPA activity. The residual concentration of active inhibitor in the incubation vials was plotted against time, and the rate constant for inactivation was evaluated for each pH by fitting a first order decay function to the data. To ensure that the reduction with time in PAI-1 activity was not caused by adsorption or precipitation, selected incubation vials (–0.2 ml) were centrifuged for 5 min at 10,000 x g, and the resulting supernatants were treated with excess tPA and analyzed by SDS-PAGE.

Characterization of Inactive PAI-1— Partially or completely inactive PAI-1 was generated by incubation of the inhibitor at pH 6, 9, and 11. A fourth sample of PAI-1 was completely inactivated at pH 8.2 and then incubated at pH 11 for 4 h. Following incubation, the samples were desalted on a PD-10 column, equilibrated with 0.15 M ionic strength sodium phosphate buffer at pH 7.0, diluted to 10–20 μg of PAI-1 ml⁻¹, and analyzed. The solutions were heated at a rate of about 2°C/min in the thermostatted cell holder of a Shimadzu RF540 fluorescence spectrophotometer. Both monochromators were set to 330 nm, and the intensity of the scattered light was measured at pre-set temperatures in the range of 25–80°C. A stainless steel coated thermocouple, immersed in the sample, served as a thermometer.

Effects of pH on PAI-1 Reactivity— β-Trypsin (6 nm) was reacted with the chromogenic substrate H-iso-leucyl-l-prolyl-l-arginine-p-nitroanilide (0.2 mM) in the presence of PAI-1 (0.1–0.3 μM) or BPTI (1–3 μM) at pH 6.0, 7.0, and 8.1, respectively, and the resulting absorbance changes at 405 nm were recorded. The pseudo first order rate constant for inhibition of β-trypsin by respective inhibitor was determined by nonlinear least squares analysis of the exponential progress curves.

Peptide-induced Inactivation of PAI-1— Stock solutions of Ac-TVA-SSTA (1 mM) and Ac-CTEASSSTA (10 mM) were prepared in 0.14 and 0.11 M NaCl solutions, respectively, and neutralized with NaOH. To compare the effect of pH on peptide binding and formation of latent PAI-1, 4–5 μM of the active inhibitor was incubated with and without Ac-TVA-SSTA or Ac-CTEASSSTA in pH 6–13. Aliquots of the mixture were treated with excess tPA and analyzed by SDS-PAGE. Because the PAI-1-peptide complexes were completely cleaved by tPA, the proportion of cleaved to latent PAI-1, determined from the bands on the gels, were taken as a measure of the ratio of peptide-bound to latent PAI-1 established prior to treatment with tPA. The effect of peptide concentration on this ratio was determined at pH 7 and 8 for Ac-TVASSSTA and at 7.4 for Ac-CTEASSSTA by the same technique. For this purpose, 4–5 μM active PAI-1 was incubated with peptide ranging in concentration from 0.02–0.2 mM Ac-TVASSSTA or 0.2–2.0 mM Ac-CTEASSSTA and analyzed by SDS-PAGE, after complete inactivation of PAI-1. The stability of the PAI-1 complex with Ac-CTEASSSTA was assessed by incubating fully active PAI-1 for 2 days at pH 7.4 at room temperature with 1 mM of the peptide. Excess peptide was removed by gel filtration, and the complex was subjected to continuous dialysis for several days. Aliquots of the dialyzed were withdrawn at timed intervals, treated with excess tPA, and analyzed by SDS-PAGE for a change of the quotient between latent and cleaved PAI-1.

RESULTS

Spontaneous Inactivation of PAI-1— Inactivation of PAI-1 was first order for the complete time course in the pH range 6–13. At pH 5 and below, the stability of the active inhibitor makes it difficult to follow the inactivation to completion, and data obtained in this pH interval were assumed to reflect a monophasic first order inactivation process. Rate constants for inactivation of pure active PAI-1, obtained between pH 4.5 and 13, are shown in Fig. 1. Two major effects of pH on the inactivation process can be seen in the investigated interval. Below pH 9 the rate constant for formation of inactive PAI-1 depends on the ionic state of a single group in the inhibitor with an apparent pKₐ of 7.6. Protonation of this group reduces the rate of inactivation of PAI-1 from 0.17 h⁻¹ at pH 9 to 0.005 h⁻¹ at pH 5 and below and is thus linked to stabilizing interactions corresponding to about 2 kcal mol⁻¹. Heat denaturation of partially and completely inactive PAI-1, generated at pH 6.0 and 9.4, respectively (Fig. 2), monitored by light scattering (24) indicated that latent PAI-1 is the major product of inactivation in this pH interval. The second effect of pH on the inactivation process becomes evident at pH 11 and above, where the rate constant for inactivation of PAI-1 increases in proportion to the OH⁻ ion. As shown in Fig. 2, PAI-1 inactivated at pH 11 for 2

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PAI-1 activity is influenced by pH. The inactivation data presented in Fig. 1 as resulting from two processes, one leading to latent PAI-1 and the other, which is induced by alkaline pH and insignificant in the neutral pH range, to denaturation.

Effect of pH on PAI-1 Reactivity—Effects of the pH on the rate constant for inactivation of PAI-1. Data were obtained kinetically (C) or by SDS-PAGE (M) as described under “Experimental Procedures.” The solid line represents the equation

\[ \log k_{\text{obs}} = \log \left( \frac{k_{\text{max}}}{1 + [H^+] K_a} + k_{\text{OH}} K_w \right) \]

with the best fit parameter values \( k_{\text{max}} = 0.167 \text{ h}^{-1}, k_{\text{OH}} = 1132 \text{ h}^{-1}, \) \( pK_a = 6.041, \) and \( pK_f = 7.58. \)

PAI-1 at the latter pH) retains the thermostability characteristic of latent PAI-1. This justifies the interpretation of the inactivation data presented in Fig. 1 as resulting from two practically irreversible processes, one leading to latent PAI-1 and the other, which is induced by alkaline pH and insignificant in the neutral pH range, to denaturation.

To test the validity of Equation 5, PAI-1 was incubated with various concentrations of peptide, and the proportion of cleaved to latent inhibitor was determined for each concentration by quantitative analysis of gels, such as the one demonstrated in Fig. 3. The results are shown in Fig. 4 (A and B). The linear dependence of \( R \) on peptide concentration, established for both peptides, is consistent with Equation 5 and confirms that peptide binding and formation of the latent inhibitor are competitive events. The small intercepts observed result from cleavage of some PAI-1 by tPA in the absence of peptides (Fig. 3, lane 0). The slopes of the regression lines in Fig. 4B, representing \( k_{\text{pep}}/k_{\text{lat}} \) for Ac-TVASSSTA at pH 7.0 and 8.1, are 16 and 20 \text{ M}^{-1} \text{ h}^{-1}, \) respectively. If peptide association \( (k_{\text{pep}}), \) in contrast to
reactive loop folding ($k_{lat}$), were pH-independent, these slopes should have differed by a factor of 4. The single line in Fig. 4A was obtained for the association of Ac-TEASSSTA at pH 7.4 and has a slope of 1.0 M$^{-2}$ s$^{-1}$. Using the best fit estimates of $k_{lat}$ (the solid line in Fig. 1), $k_{lep}$ for Ac-TVASSSTA evaluates to 0.18 and 0.74 M$^{-2}$ s$^{-1}$ at pH 7.0 and 8.1, respectively, whereas a value of 0.020 M$^{-2}$ s$^{-1}$ is obtained for Ac-TEASSSTA at pH 7.4. The effect of pH on $R$ can be seen in Figs. 5 and 6. The gel shown in Fig. 5 confirms the pH effects on the rate of formation of inactive PAI-1 reported above and indicates in a qualitative way that the proportion of peptide-bound to latent PAI-1 does not vary much with pH and, consequently, that reactive loop folding and peptide binding exhibit a similar dependence on pH. This is quantitatively confirmed by the data presented in Fig. 6 obtained by analysis of the gel in Fig. 5 and by the data obtained from similar gels developed for both peptides (not shown).

**DISCUSSION**

By a systematic study of the effects of pH on the inactivation rate of PAI-1, we have linked the observed acid stabilization of the active form of this serpin to protonation of a single functional group in the protein. To examine whether the protonated form of the group stabilizes an exposed reactive loop in a direct manner or indirectly by preventing its binding to sheet A, the reactivity of PAI-1 and BPTI toward trypsin was compared in the pH interval over which the rate of loop insertion, leading to latent PAI-1, exhibits a 15-fold increase. The comparison of the two inhibitors' rates of reaction with trypsin was performed to mask the well known effects of pH on the active site of serine proteinases and to avoid the possible complication of exosite interactions believed to be involved in formation of the tPA-PAI-1 complex (31). The absence of a significant effect of

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**Fig. 3.** Effect of peptide concentration on the proportion of peptide-bound (cleaved) to latent PAI-1 demonstrated by SDS-PAGE. PAI-1 (4 μM) was incubated for 5 days at pH 7.4 and 25 °C with increasing concentrations of Ac-TEASSSTA and analyzed by treatment with excess tPA followed by SDS-PAGE.

**Fig. 4.** Quantitative evaluation of the effect of peptide concentration on the proportion of peptide-bound (cleaved) to latent PAI-1. The ratio of cleaved to latent PAI-1, generated by incubation of the active inhibitor with Ac-TEASSSTA (A) at pH 7.4 and Ac-TVASSSTA (B) at pH 7.0 (●) and pH 8.1 (○), was determined from gels like the one shown in Fig. 3 and plotted versus peptide concentration.

**Fig. 5.** Effects of pH on the ratio of peptide bound (cleaved) to latent PAI-1 demonstrated by SDS-PAGE. PAI-1 (5 μM) was incubated at 25 °C with (+) and without (−) 1 mM Ac-TEASSSTA in buffers ranging in pH from 4.6 to 10.6.

**Fig. 6.** Quantitative evaluation of the effect of pH on the proportion of latent to peptide bound (cleaved) PAI-1. The proportion of latent to cleaved inhibitor, generated after incubation of the active inhibitor with Ac-TEASSSTA for 6 (○) and 22 h (●) were determined from gels like the one shown in Fig. 5. The dotted line is the proportion expected if peptide association leading to cleaved PAI-1 were pH-independent.

**Fig. 7.** Effects of pH on PAI-1 fluorescence. The emission spectra were recorded for active PAI-1 at pH 6.0 (solid line) and 9.4 (dotted line) as described under "Experimental Procedures." The inset shows how the average emission intensity in the 380–400 nm region increases with pH; the line represents a protonic dissociation function with a $pK_a$ of 7.6. Fluorescence units are arbitrary and the Y axis of the inset corresponds to 0.025 such units.
pH Effects on PAI-1 Stability

\[
\frac{[I]_{\text{max}}}{[I]_{\text{min}}} = \frac{K_2}{1 + K_3} C_i \quad \text{(Eq. 6a)}
\]

\[
\frac{[I]_{\text{min}}}{[I]_{\text{max}}} = \frac{K_1}{1 + K_3} C_i \quad \text{(Eq. 6b)}
\]

where \( C_i \) is the total concentration of active inhibitor. Let \( k_{\text{ins}} \) be the intrinsic rate constant for loop insertion in the open inhibitor, and the observed rate constant will be given by

\[
k_{\text{obs}} = k_{\text{ins}} \left( \frac{[I]_{\text{max}}}{[I]_{\text{min}}} \right) \quad \text{(Eq. 7)}
\]

From the data presented in Fig. 1, we have \( [I]_{\text{max}}/[I]_{\text{min}} = 35 \). In view of Equations 6a and 6b, this means that \( K_1 < 1/34 \) and \( K_1 \ll K_3 \). The actual position of these equilibria will determine the relationship between \( K_2 \) and \( K_4 \) in Scheme 1 and the experimentally found constants, \( K_6 \) and \( K_7 \), presented in Fig. 1. Clearly, \( pK_6 \) corresponds to the pH at which \( [I]_{\text{max}} \sim 2 \times [I]_{\text{min}} \). Hence

\[
\frac{1 + K_2 K_4}{K_2 + K_2 K_4 + K_1 (1 + K_2 K_4)} = \frac{2}{1 + K_1}
\]

(Eq. 8a)

which, with \( K_1 \ll 1 \), simplifies to

\[
K_6 = K_3 \left( 1 - \frac{K_4}{K_2} \right)
\]

(Eq. 8b)

Because

\[
\frac{K_4}{K_2} \ll 1
\]

(Eq. 9)

the value reported for \( pK_6 \) in Fig. 1 (6.0) must be a close estimate of \( pK_6 \), the \( pK_6 \) of the noninteracting ionizing group.

A \( pK_6 \) of 6 is consistent with that of a histidine residue slightly perturbed by a hydrophobic environment or a neighboring positive charge. In the search for a candidate among the 13 histidine residues of human PAI-1, we studied the structure of the latent inhibitor and focused on histidine residues that could have a direct effect on binding of peptides to sheet A. This approach is justified by the fact that apart from the reactive center loop, the differences between serpins with 5 or 6 strands in sheet A are predominantly observed in this sheet (33–35) and its immediate vicinity. More profound changes may accompany formation of latent PAI-1 (6) but are not likely to be required for insertion of the octamer peptides. One possible candidate then is His-143 in helix F. Helix F is part of a loop that connects strand 1A and strand 3A and covers the lower half of sheet A in all inhibitory serpins. It appears that the loop containing helix F must move out of the way in order for the reactive loop to insert completely as strand 4A and that factors that stabilize the helix should slow down the insertion. In the latent inhibitor the presumably neutral His-143 (the crystals were grown at pH 8.2 (36)) is seen at hydrogen bond distance to the \( \gamma \)-oxygen of Thr-142 and the carbonyl oxygen of Trp-139, both in helix F. In the active inhibitor, a positive charge on the histidine in addition to structural differences may allow the imidazolium ring to rotate into a different position, where it may stabilize helix F by neutralizing its dipole field or by forming a hydrogen bond to sheet A. Although there is no suitable acceptor within distance for the latter bond in latent PAI-1, it can be seen from the differences between latent PAI-1 and nonrelaxed serpins (35) that sheet A in the formers has expanded in the region beneath helix F. In the active inhibitor the side chain oxygen of Thr-94 in strand 2A and one of the nitrogens of His-143 may very well be within hydrogen bond distance. The spatial relationships pertinent to this discussion are illustrated by the stereodigram of the \( \alpha \)-carbon backbone.
active PAI-1 are maintained at 37 °C. This means that active PAI-1 basically should be about 2.5 times more stable in cells (pH = 6.8) than in plasma (pH = 7.4). Obstructions to blood flow through active tissues could lead to a local acidification through accumulation of CO₂ followed by a shift of intermediary metabolism toward fermentation with a concomitant prolongation of the lifetime of active PAI-1.

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