Distortion of the Catalytic Domain of Tissue-type Plasminogen Activator by Plasminogen Activator Inhibitor-1 Coincides with the Formation of Stable Serpin-Proteinase Complexes*

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Plasminogen activator inhibitor-1 (PAI-1) is a typical member of the serpin family that kinetically traps its target proteinase as a covalent complex by distortion of the proteinase domain. Incorporation of the fluorescently silent 4-fluorotryptophan analog into PAI-1 permitted us to observe changes in the intrinsic tryptophan fluorescence of two-chain tissue-type plasminogen activator (tPA) and the proteinase domain of tPA during the inhibition reaction. We demonstrated three distinct conformational changes of the proteinase that occur during complex formation and distortion. A conformational change occurred during the initial formation of the non-covalent Michaelis complex followed by a large conformational change associated with the distortion of the proteinase catalytic domain that occurs concurrently with the formation of stable proteinase-inhibitor complexes. Following distortion, a very slow structural change occurs that may be involved in the stabilization or regulation of the trapped complex. Furthermore, by comparing the inhibition rates of two-chain tPA and the proteinase domain of tPA by PAI-1, we demonstrate that the accessory domains of tPA play a prominent role in the initial formation of the non-covalent Michaelis complex.

Plasminogen activator inhibitor-1 (PAI-1)1 is the predominant physiologic inhibitor of both tissue-type plasminogen activator (tPA) and urinary-type plasminogen activator and has been shown to play a diverse role in a variety of physiologic processes including coagulation, matrix remodeling, complement activation, tumor invasion, and angiogenesis (1). The tertiary structure of PAI-1, typical of all members of the serpin superfamily, consists of three β-sheets, nine α-helices, and an exposed flexible reactive center loop (RCL). PAI-1 is found in a metastable conformational state, which facilitates the kinetic trapping of its target proteinases in thermodynamically favorable covalent complexes (2). The serpin inhibition mechanism is initiated by the formation of a non-covalent Michaelis complex between the exposed RCL of the inhibitor and the active site of the target proteinase. Following docking, the catalytic cleavage of the RCL triggers a rapid translocation of the proteinase to the opposite pole of the inhibitor, trapping the enzyme as a stable proteinase-inhibitor complex. The ability of the serpin to maintain a stable complex with its target proteinase is partly due to distortion of the catalytic triad through compression of the proteinase against the body of the inhibitor, thereby preventing catalytic deacylation and release of the proteinase. Distortion of the proteinase has been clearly demonstrated in a crystallographic study in which 37% of the proteinase was shown to adopt a disordered structure within the trypsin-α1-antitrypsin complex (Fig. 1) (3). Furthermore, the catalytic serine (Ser-195) has been pulled more than 6 Å away from the His-57 residue, preventing proton transfer from the histidine to the serine and subsequent catalytic deacylation by the proteinase (3). Distortion of the proteinase has also been monitored by intrinsic tryptophan fluorescence within thrombin following its inhibition by an α1-antitrypsin variant in which both native tryptophan residues were mutated to phenylalanine (4). Several additional studies have also examined the distortion of the proteinase active site using [1H-15N]HSQC NMR (5), [1H]NMR (6, 7), circular dichroism (8), and proteolytic susceptibility (9) following the formation of proteinase-inhibitor complexes.

Whereas previous studies have characterized both the structure and disordered state of the proteinase within the complex, the static nature of these techniques restricts knowledge of the steps leading to the final conformational state of the complex. An understanding of the kinetic steps involved in obtaining this final complexed state would provide greater insight into the overall mechanism by which a serpin inhibits its target proteinase. To better define the kinetic steps involved in distortion of the serine proteinase and kinetic trapping, we utilized the intrinsic tryptophan fluorescence of tPA. Tryptophan fluorescence has been extensively exploited to study protein structure/function by providing a useful signal to monitor conformational changes within the protein. Although this technique is extremely useful with a single protein, fluorescence changes are difficult to interpret when observing intrinsic tryp-
Conformational Changes during Proteinase Inhibition

**EXPERIMENTAL PROCEDURES**

*Materials and Reagents—* Unless otherwise indicated, all of the experiments were performed in 30 mM Hepes, 0.135 M NaCl, and 1.0 mM EDTA (pH 7.4) at 25 °C. Acrylic cuvettes were coated with 0.1% polyethylene glycol to reduce protein adsorption. The chromogenic substrate Spectrozyme®TA (CHSO₄-p-HHT-Gly-Arg-pNA) was obtained from American Diagnostica Inc. All of the chromatography equipment used for protein purification was obtained from Amersham Biosciences. All of the other reagents were of analytical grade or better and were obtained from Sigma.

**PAI-1 Mutagenesis, Incorporation, and Expression of 4-F PAI-1—** Recombinant human PAI-1 was a kind gift from David Ginsburg (Howard Hughes Medical Institute, Ann Arbor, MI) and was provided in the pET3a vector. The PAI-1 gene was further cloned into the pET24d expression plasmid as described previously (13). The proteinase knock-out (TrpKO) PAI-1, a PAI-1 mutant in which all four tryptophan residues have been replaced with phenylalanine, was engineered by site-directed mutagenesis using the Muta-Gene Phagenid in vitro mutagenesis kit (Bio-Rad) using the manufacturer’s recommended protocol and the method of Kunkel (14, 15). The oligonucleotides used to generate the phenylalanine residues at positions 86, 139, 175, and 262 (PAI-1 numbering) were 5'-342dGATCTCATCCCTGTGTTAAATGGCCCCATGAGCTT111-3', 5'-303dTGTTGTGTGCTTCCAACAAATCTCTATGATGGAAT773-3', 5'-409dGGGAAAGGGATCTTAAACTGCGGCTTGTAAGT778-3', and 5'-370dGGGTGATGTTGCCTTTAAGTGATGAGCT1393-3', respectively. The mutagenesis of PAI-1 was confirmed by sequencing the entire PAI-1 gene. TrpKO and wtPAI-1 were expressed in the Escherichia coli strain BL21(DE3) and BL21(DE3)pLysS, respectively, and purified as described previously (13). 4-F PAI-1 was expressed in the E. coli strain W3110/pXa433(DE3)pLysS using the one-step analog incorporation method described previously (13). The incorporation efficiency of 4-fluorotryptophan into PAI-1 was determined at the protein structure facility of the University of Michigan by MALDI-TOF mass spectroscopy of 4-F PAI-1 in 1% acetic acid. A four-point external calibration curve was applied to the spectra to obtain better than a 0.1% mass accuracy. Protein concentrations for wtPAI-1 were determined using the absorbance at 280 nm with an extinction coefficient of 0.93 ml/mg cm⁻¹ and a M₅₀ of 43,000 (16, 17). TrpKO and 4-F PAI-1 concentrations were determined with the Bradford dye binding assay (Bio-Rad) using active wtPAI-1 of known concentration as a standard.

**Proteinases—** Recombinant tPA (Activase®) and the mutant in which serine 195 has been replaced by an alanine (Ala-tPA), rendering the protein to catalytically inactive, were provided by Genentech, Inc. The primarily single chain form of tPA was converted to two-chain tPA (tc-tPA) by passage over a 1.0-ml plasmin-Sepharose HiTrap column for approximately 1 h at 25 °C as described previously (18). The proteinase domain of tPA (P-tPA) consisting of only the catalytic domain of tPA in the pET24d expression plasmid as described previously (13). The catalytic serine at position 195 is replaced with 4-fluorotryptophan, successfully silencing all of the FTase properties of one of the proteins (10, 11). Studies characterizing structural transitions within PAI-1 to be monitored by specifically exciting the 7-azatryptophan in the presence of tPA (13). The four native tryptophans of PAI-1 were substituted with 7-azatryptophan to red-shift the absorbance, allowing the spectral transitions within PAI-1 to be monitored by specifically exciting the 7-azatryptophan in the presence of tPA (13).

**Fig. 1. Disordering within trypsin following trapping as an acyl enzyme intermediate.** The crystal structure model of the α₁-antitrypsin-trypsin complex (Protein Data Bank (PDB) structure 1EZX (3)) is shown in comparison to both trypsin (PDB structure 1J8A) and P-tPA (PDB structure 1A5H (27)). Strands and α helices are shown in purple, β sheets are represented by red. The catalytic serine at position 195 is yellow, and the tryptophan residues within the proteinases are teal. Trypsin residues within the catalytic domain are labeled. All of the structures were generated in Swiss PDB Viewer (version 3.7b2) (34) and rendered in three-dimensional using POV-Ray (version 3.1).
Sophorose column. The concentration of tc-tPA was determined from the absorbance at 280 nm using an extinction coefficient of 1.9 ml mg⁻¹ cm⁻¹ and a M₅₀ value of 63,500. The concentration of functional P-tPA was determined by active site titration with p-aminobenzamidine using a standard curve, which was determined by titrating known concentrations of tc-tPA (0–1.7 μM) into 600 μM p-aminobenzamidine (20). The data were fit to a linear equation, and the concentration of the P-tPA was extrapolated from the fluorescence following reaction with 600 μM p-aminobenzamidine.

Intrinsic Tryptophan Fluorescence of WT, TrpKO, and 4-F PAI-1—Fluorescence spectra were obtained using a Varian Cary Eclipse fluorescence spectrophotometer equipped with a Peltier controlled cell holder. Unless otherwise noted, all of the intrinsic tryptophan fluorescence spectra were obtained using 0.5 μM proteinase and 1.0–2.0 μM inhibitor at 25 °C with an excitation wavelength of 305 nm and excitation and emission slit-widths of 2.5 nm and 5.0 nm, respectively. Emission spectra were collected from 315–410 nm with a 1.0-nm step, and each data point was integrated until the signal (noise ratio) was greater than 1000 with a 10-s cutoff.

Stoichiometry of Inhibition—The stoichiometry of inhibition (S.I.) defined as the number of moles of PAI-1 required to inhibit one mole of tPA or P-tPA, was determined by measuring the residual proteinase activity following reaction with WT, 4-F, or TrpKO PAI-1 by monitoring the hydrolysis of the chromogenic substrate SpectrozymePA as described previously (13). Reactions containing 130 nM proteinase were incubated with 0–150 nM inhibitor at 25 °C for 60 min. The reaction was stopped with an equal-volume addition of 10-fold concentrated standard HEPES buffer with 0.1% polyethylene glycol 8000 and 0.1 mM SpectrozymePA, and the reaction progress was monitored by the absorbance at 405 nm. Residual proteinase activity was plotted against the molar ratio of inhibitor to proteinase, and the S.I. was extrapolated from the X-intercept of the best-fit linear regression to the data.

Apparent Second Order Rate Constant for Inhibition of Proteinases by PAI-1—The apparent second order rate constant of inhibition (kₐp) for each proteinase was determined using the competitive kinetic method (21) under pseudo-first order conditions as described elsewhere (18, 22). Reactions containing 2–30 nM proteinase were reacted with 25–175 nM of each PAI-1 variant in standard HEPES buffer with 0.5 mM SpectrozymePA at 25 °C. The reaction was monitored at 405 nm for 150–600 s, and the progress curves were fit to a single exponential equation with a steady-state linear component to derive kₐp. The kₐp value was determined by correcting kₐp for substrate competition (1 + [I]Kᵢ) and then dividing by the functional inhibitor concentration (corrected for S.I.) (23). The Kᵢ of spectrozymePA for tc-tPA has been previously reported as 77.9 μM (35). The Kᵢ for P-tPA hydrolysis of spectrozymePA was determined to be 145.0 μM using standard Michaelis-Menten kinetics.

Rapid Acid-quenching Technique to Determine the Limiting Rate of Serpin-Proteinase Complex Formation—The formation of SDS stable proteinase-inhibitor complexes was monitored using the rapid acid-quenching technique as described elsewhere (18). Reactions were performed under pseudo-first order conditions in which the proteinase (4.0 μM) was in 4-fold excess over inhibitor (1.0 μM) to ensure saturation of the Michaelis complex. The limiting rates (kₐlim) for generation of inhibited complex were determined by fitting the progress curves to a single exponential equation and are reported as kₐlim ± S.E. of the fit.

Stopped-flow Kinetics to Monitor Conformational Changes in the Proteinase—The conformational changes in tc-tPA and P-tPA following reaction with 4-F PAI-1 were monitored on an Applied Photophysics SX.18MV stopped-flow reaction analyzer with a 25 °C thermostated syringe chamber. To monitor changes in intrinsic tryptophan fluorescence, the reaction was excited at 300 nm and the emission fluorescence was monitored using a filter (Oriel 51255) with a cutoff below 310 nm. Unless otherwise noted, stopped-flow experiments were carried out under pseudo-first order conditions with increasing concentrations of the inhibitor (0.2–3.0 μM) in at least 4-fold excess over proteinase (0.05–0.3 μM) All of the stopped-flow traces were best fit using a nonlinear double exponential equation yielding kₐlim and kₐsec. The kₐlim data were then fit to a rectangular hyperbola, kₐlim = kₐ[I]Kᵢ + kₐ[I], where kₐlim is the limiting rate observed for the conformational change associated with the observed change in fluorescence, [II] is the inhibitor concentration, and Kᵢ is the concentration at which the kₐlim is half of the kₐlim and is defined as kₐlim + kₐ[I]Kᵢ, where kₐ and kₐ[I] are the forward and reverse rates for the formation of the non-covalent Michaelis complex, respectively.

FIG. 2. Comparison of absorption profiles for wtPAI-1, 4-F PAI-1, and TrpKO PAI-1. All of the absorbance scans were performed using 7.0 μM PAI-1. Solid line, wtPAI-1; dashed line, 4-F PAI-1; dotted line, TrpKO PAI-1.

RESULTS AND DISCUSSION

Characterization of 4-F and TrpKO PAI-1—To characterize the mechanism of proteinase distortion following complex formation, we monitored the fluorescence changes of tryptophan residues within the proteinase. To ensure that the changes in tryptophan fluorescence are solely attributed to conformational transitions within the proteinase, it is necessary to obviate tryptophan fluorescence changes within PAI-1 during the inhibition reaction. Previous work by Tew et al. (4) monitored conformational changes in thrombin during complex formation with a₁-antitrypsin by mutating the two tryptophan residues at positions 194 and 238 in the serpin to observe the tryptophan fluorescence changes in thrombin. In this study, we have included a similar mutant, TrpKO PAI-1, in which all four native tryptophan residues (86, 139, 175, and 282, PAI-1 numbering) in PAI-1 were replaced with phenylalanine. In addition, we have created a variant of PAI-1 in which all four tryptophan residues are biosynthetically replaced with the non-fluorescent 4-fluorotryptophan analog.

 Biosynthetic incorporation of 4-fluorotryptophan into PAI-1 was performed using the one-step method as described under “Experimental Procedures.” Previously, we have shown that this method limits expression of PAI-1 prior to induction, thereby maximizing analog incorporation efficiency (13). The efficiency of 4-fluorotryptophan incorporation into PAI-1 was determined by MALDI-TOF mass spectroscopy by comparing the mass of 4-F PAI-1 to that of wtPAI-1. Both the wtPAI-1 and 4-F PAI-1 spectrum consisted of only one major component with calculated masses of 42,633 and 42,700 daltons, respectively. The 67-dalton increase in the mass observed for 4-F PAI-1 is within experimental error of the 72-dalton increase expected for complete analog incorporation of wtPAI-1 (data not shown).

The absorbance spectrum of 4-F PAI-1 exhibited a 21.5% decrease in absorbance at 280 nm and is blue-shifted compared with wtPAI-1, consistent with the altered absorbance properties because of the presence of the incorporated analog (Fig. 2). The absorbance of TrpKO PAI-1 was decreased by 83.3% at 280 nm, which was expected given that all four tryptophan residues were mutated to phenylalanine. The residual absorbance within TrpKO PAI-1 (16.7%) is probably because of the absorbance of tyrosine and phenylalanine residues within the inhibitor. To characterize the fluorescence profile of 4-F PAI-1 and TrpKO PAI-1, fluorescence emission scans were performed at an excitation wavelength of 305 nm and compared with those of wtPAI-1 (Fig. 3). Similar decreases in fluorescence of 90.3 and 89.1% were observed for 4-F PAI-1 and TrpKO, respectively.
when compared with the intrinsic tryptophan fluorescence of wtPAI-1. The similar decrease in fluorescence observed for both 4-F PAI-1 and TrpKO PAI-1 suggests that the small level of 4-F PAI-1 fluorescence is probably because of excitation of tyrosine residues at 305 nm. The mass spectrometry data, absorbance profiles, and fluorescence scans all support the conclusion that 4-F PAI-1 has a 4-fluorotryptophan incorporation efficiency of >95%. Furthermore, the fluorescence data suggest that the incorporation of 4-fluorotryptophan into PAI-1 effectively silences the intrinsic tryptophan fluorescence when excited at 305 nm, similar to that of TrpKO PAI-1.

Kinetic Properties of 4-F PAI-1 Inhibition of tc-tPA and P-tPA—The mutation of an amino acid or the incorporation of a non-natural amino acid into proteins always carries the risk of altering protein function. 4-Fluorotryptophan having an additional fluorine group at the C-4 position of the tryptophan indole ring significantly changes the electronegativity of the residue and therefore may alter the packing structure within the protein (10, 24). Such an alteration may result in abnormal kinetic characteristics. To determine whether the incorporation of 4-fluorotryptophan into PAI-1 altered the kinetic inhibition mechanism, the S.I., apparent second order rate constant for inhibition (k_{\text{app}}), and the limiting rate constant for inhibition (k_{\text{lim}}) were determined using both tc-tPA and the P-tPA as target proteinases (Table I). The S.I. values for reactions of 4-F PAI-1 with tc-tPA were in close agreement with those obtained for wtPAI-1 (1.10 versus 1.14), indicating that incorporation of 4-fluorotryptophan has no significant effect on the partitioning between the substrate and inhibitor branches of the serpin mechanism with tc-tPA as the target proteinase. For reactions with P-tPA, there was a relatively small increase in S.I. (1.13 versus 1.42), suggesting a minor effect of the analog substitution on the partitioning step in the absence of the accessory domains. In contrast, the TrpKO PAI-1 mutant showed a marked increase in the S.I. to a value of 3.12, indicating that ~68% of the inhibitor becomes hydrolyzed as a substrate following loop cleavage by tc-tPA.

Deletion of the non-catalytic accessory domains of tPA, which include EGF and fibronectin-like domains in addition to the two kringle domains, resulted in a 10-fold decrease in the apparent second order rate constant (k_{\text{app}}) for the inhibition of P-tPA compared with the full-length tc-tPA (1.6 μM^{-1} s^{-1} versus 15.7 μM^{-1} s^{-1}) (Table I). The observed 10-fold decrease in k_{\text{app}} was unexpected and suggests that the inhibition rate of tc-tPA by PAI-1 is dependent upon more than just the catalytic domain of tc-tPA. This finding is consistent with several previous studies, suggesting that the kringle 2 domain of tc-tPA functions as an exosite by assisting the formation of the initial non-covalent Michaelis complex (25, 26). Upon incorporation of 4-fluorotryptophan into PAI-1, we observed no significant changes in the k_{\text{app}} values for inhibition of either tc-tPA (11.3 μM^{-1} s^{-1}) or P-tPA (2.2 μM^{-1} s^{-1}) compared with the wtPAI-1 controls (15.7 μM^{-1} s^{-1} and 1.6 μM^{-1} s^{-1}, respectively). Thus, we concluded that incorporation of 4-fluorotryptophan into PAI-1 had no effect on the overall kinetic inhibition mechanism when either tc-tPA or P-tPA was the target proteinase. On the other hand, a substantial decrease in the k_{\text{app}} value for TrpKO PAI-1 was observed with tc-tPA as the target proteinase (0.9 μM^{-1} s^{-1}), indicating that one or more of the tryptophan residues plays a significant role in the inhibition mechanism of PAI-1.

We further demonstrated that the limiting rate (k_{\text{lim}}) for formation of stable serpin-proteinase complexes remained unaffected by the incorporation of 4-fluorotryptophan into PAI-1 by monitoring the generation of SDS stable serpin-proteinase complex over time in a rapid quench experiment. As shown in Table I, a slight decrease was found in the k_{\text{lim}} value for reactions of 4-F PAI-1 with tc-tPA (3.4 s^{-1} versus 2.3 s^{-1}); however, no such decrease was observed for reactions of 4-F PAI-1 with P-tPA as the target (1.2 s^{-1} versus 1.4 s^{-1}). These data are consistent with the incorporation of 4-fluorotryptophan not having any substantial effects on the limiting rates of the inhibition reactions with either tc-tPA or P-tPA. In contrast, the substantial reduction in the overall rate for inhibition of tc-tPA by TrpKO PAI-1 (0.9 μM^{-1} s^{-1}) was shown to be at least in part, because of a marked reduction in the rate-limiting step (0.08 s^{-1} versus 3.4 s^{-1} for wtPAI-1). Therefore, deletion of one or more tryptophan residues in PAI-1 by mutation to phenylalanine substantially decreases the rate-limiting step in the PAI-1 inhibition mechanism. Recently, a study by Blouse et al. (35) demonstrated that substitution of the highly conserved Trp-175 residue in PAI-1 with phenylalanine is capable of greatly influencing the kinetic mechanism for the inhibition of tc-tPA by altering a single rate-limiting step prior to loop insertion. Unfortunately, the critical role of the Trp-175 residue along with the cumulative effects of the other three mutations at positions 86, 139, and 262 severely limit the usefulness of TrpKO PAI-1 as a tool to study proteinase distortion during the inhibition mechanism.

Disordering of tc-tPA and P-tPA Observed by Intrinsic Tryptophan Fluorescence—The incorporation of the 4-fluorotryptophan analog into PAI-1 was shown to effectively eliminate the intrinsic tryptophan fluorescence within the inhibitor, which makes 4-F PAI-1 an excellent tool to analyze the rates of conformational changes that occur within the target proteinases during inhibition. To examine this result, the emission spectra of 4-F PAI-1 were recorded in the presence of a slight excess of tc-tPA, P-tPA, and Ala-tPA and are shown in Fig. 4. The two-chain form of tPA contains the catalytic domain of the proteinase as well as the two kringle domains and an EGF binding domain. P-tPA is a variant of tc-tPA in which the kringle domains and EGF domain have been removed, leaving only the proteinase domain. The Ala-tPA proteinase is identical to tc-tPA with the exception that the catalytic serine residue at position 195 has been replaced with an alanine, rendering the proteinase inactive. As demonstrated in Fig. 4, a 15.9% decrease in fluorescence was observed following incubation of tc-tPA with 4-F PAI-1, whereas a much larger decrease of 41.5% was observed with P-tPA. The greater fluorescence change observed for P-tPA when compared with tc-tPA is probably the result of decreased background fluorescence because of the eight tryptophan residues located in the kringle and EGF.
domains of tc-tPA (27). Furthermore, the decrease in fluorescence for P-tPA suggests that conformational changes within the proteinase can be attributed to the catalytic domain of tPA and not the accessory domains. An 8.0% increase in fluorescence was observed for Ala-tPA, which because of the inactive catalytic domain reports the formation of the initial non-covalent binding complex. To ensure that the observed fluorescence changes are not the result of changes in the background tyrosine fluorescence within 4-F PAI-1, the inhibitor was reacted with elastase to cleave PAI-1 between the P3 and P4 residues on the RCL, thus permitting loop insertion without trapping the proteinase (28). We have determined that no change in fluorescence was detected with either the elastase or 4-F PAI-1, which because of the inactive catalytic domain reports the formation of the initial non-covalent Michaelis complex (4). Conversely, an 8.0% increase in fluorescence observed during the P-tPA and 4-F PAI-1 reaction is monitoring the association rate for P-tPA and 4-F PAI-1.

**Kinetics of Proteinase Distortion**—To elucidate the kinetics of the two conformational changes in the proteinase, stopped-flow experiments were performed by reacting tc-tPA and P-tPA with excess 4-F PAI-1 under pseudo-first order conditions. The representative trace for the P-tPA and 4-F PAI-1 reaction shown in Fig. 5 demonstrates that a fast, low amplitude increase in fluorescence was followed by a much slower, large amplitude decrease. A comparable trace was recorded for tc-tPA, but the observed fluorescence decrease was not as pronounced as for P-tPA, which is in agreement with the fluorescence data in Fig. 4 (data not shown). The previous work by Tew et al. (4) was able to determine that the fluorescence increase observed during the initial 400 ms of the reaction was monitoring the formation of the initial non-covalent Michaelis complex between thrombin and α1-antitrypsin. Although we observed a similar rapid increase in fluorescence within the first 40 ms of the reaction, we were unable to determine whether this fluorescence change was following the association rate for P-tPA and 4-F PAI-1 because of the low signal to noise ratio and fast speed of this rate. However, several lines of evidence suggest that the initial increase in fluorescence observed during the P-tPA and 4-F PAI-1 reaction is monitoring the association rate constant. First, the data with the Ala-tPA variant demonstrated that an increase in fluorescence is detected during the formation of the non-covalent Michaelis complex (4). Conversely, P-tPA has only one tryptophan residue at position 215 that could be perturbed during the formation of a non-covalent Michaelis complex. Second, the high signal to noise ratio obtained with thrombin is likely the result of having three tryptophan residues potentially involved in the formation of the initial non-covalent Michaelis complex. The large decrease of tryptophan fluorescence is probably characteristic of a more solvent-exposed environment for one or more tryptophan residues in the proteinase.

**Table I**

| Kinetic data for wild type PAI-1, 4-fluorotryptophan PAI-1, and TrpKO PAI-1 with tPA and P-tPA |

All of the kinetic experiments were performed in 0.03 M HEPES, 0.135 M NaCl, 1.0 mM EDTA at pH 7.4 and 25 °C and are reported as the average ± S.E.

|                      | wtPAI-1 | 4-Fluorotryptophan PAI-1 | TrpKOPAI-1 |
|----------------------|---------|--------------------------|------------|
|                      | tPA     | P-tPA                    | tPA        |
|                      | P-tPA   |                          | P-tPA      |
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|                      |         |                          | tPA        |
4-F PAI-1 (10^7 M⁻¹ s⁻¹) compared with thrombin and α₁-antitrypsin (−10³ M⁻¹ s⁻¹) (29).

To determine the limiting rates for the conformational changes within the proteinases, increasing concentrations of 4-F PAI-1 (0.2–3.0 μM) were reacted with both tc-tPA (Fig. 6, panel A) and P-tPA (Fig. 6, panel B) under pseudo-first order conditions. The data for the large decrease in fluorescence were best fit to a double exponential function with the majority of the fluorescence change (−90%) being attributed to the large global distortion of the proteinase domain that accompanies complex formation. The remainder of the fluorescence change (−10%) is much slower and can be attributed to a secondary rearrangement or reorientation of one or more tryptophan residues during the final trapping of the proteinase in the proteinase-inhibitor complex. The saturating dependences of \( k_{\text{obs}} \) for proteinase distortion on 4-F PAI-1 concentration were fit to a rectangular hyperbola yielding \( k_{\text{lim-1}} \), values of 2.7 s⁻¹ and 1.0 s⁻¹ for tc-tPA and P-tPA, respectively. These values are in close agreement with the rates of final complex formation for tc-tPA (2.3 s⁻¹) and P-tPA (1.4 s⁻¹) reported in Table I. The \( k_{\text{lim-2}} \) values for the second observed rate, \( k_{\text{obs-2}} \), are considerably slower for both tc-tPA (0.6 s⁻¹) and P-tPA (0.14 s⁻¹) compared with the fast, large amplitude rates (2.7 and 1.0 s⁻¹). Although we are unable to definitively attribute this slower rate to a specific event during the inhibition mechanism, the slow nature of this rate suggests that a conformational change must occur after the initial global distortion of the proteinase catalytic domain. Furthermore, \( k_{\text{obs-2}} \) is not the conformational step that is required to trap the proteinase because these rates are much slower than those observed for formation of the SDS-stable proteinase-inhibitor complexes as measured by the rapid-quenching experiments in Table I. A hypothesis published by Gettins (30) suggests that the final form of the inhibited complex exists not as a static structure but rather in a dynamic equilibrium between the active proteinase and disordered proteinase conformations. In support of this hypothesis, trypsin has been shown to undergo a Ca²⁺-dependent conformational change from the trapped, disordered proteinase form to a catalytically active form, facilitating the catalytic deacylation of the acyl enzyme intermediate (31). It is possible that \( k_{\text{obs-2}} \) is monitoring the development of the equilibrium that exists between the disordered and active conformations of the proteinase following the initial rapid distortion of the proteinase.

Although previous studies have established that the structure of a serine proteinase undergoes a conformational change following its trapping by a serpin, an understanding of the kinetic mechanism of this conformational change and how it is involved in trapping the proteinase is limited. Previous work by Tew et al. (4) demonstrated that replacing the two native tryptophan residues of α₁-antitrypsin with phenylalanine silenced the intrinsic tryptophan fluorescence permitting the conformational changes within thrombin to be monitored. However, it was not clear whether distortion of thrombin could be directly attributed to trapping of the proteinase as a stable enzyme inhibitor complex, particularly because thrombin has been shown to exist in several conformational states (32). The development of a novel 4-F PAI-1 variant has allowed us to examine the tryptophan fluorescence changes during proteinase distortion. Most importantly, in this study, we demonstrate a direct correlation between the distortion rate of TPA and the formation of the stable proteinase-inhibitor complex. Furthermore, the detection of a slow secondary conformational rearrangement within the proteinase structure, which occurs after the initial global conformational change within the proteinase, suggests that another event could be involved in the stabilization of the trapped complex. We have also shown that the formation of the initial Michaelis complex perturbs the intrinsic tryptophan fluorescence in the tPA catalytic domain, suggesting that a conformational change may occur within the proteinase during docking with PAI-1. This observation is in agreement with the work by Dekker et al. (33) in which they proposed that formation of the initial Michaelis complex perturbs the intrinsic tryptophan fluorescence in the tPA catalytic domain, suggesting that a conformational change may occur within the proteinase.

Fig. 6. The limiting rates of proteinase distortion. tc-tPA (panel A) or P-tPA (panel B) (0.05–0.3 μM) was reacted with increasing concentrations of 4-F PAI-1 (0.2–3.0 μM) in a stopped-flow experiment as described under “Experimental Procedures.” The average \( k_{\text{lim-1}} \) (filled circle) and \( k_{\text{lim-2}} \) (open circle) values for at least six experiments are plotted against the concentration of 4-F PAI-1 and fit by a rectangular hyperbola to yield the \( k_{\text{lim-1}} \) and \( k_{\text{lim-2}} \) respectively. Data are reported as the averaged value ± S.E.
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