Change in Environment of the P1 Side Chain upon Progression from the Michaelis Complex to the Covalent Serpin−Proteinase Complex†

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ABSTRACT: Serpins inhibit proteinases by forming a kinetically trapped intermediate during a suicide substrate inhibition reaction. To determine whether the kinetic trap involves a repositioning of the P1 side chain of the serpin following formation of the initial Michaelis complex, we used the tryptophan of a P1 M→W variant of human α_1-proteinase inhibitor as a fluorescent reporter group of the environment of the P1 side chain. The P1W variant was a valid model serpin and formed SDS-stable complexes with both trypsin and chymotrypsin with a stoichiometry of inhibition close to 1.0. Rates of inhibition of chymotrypsin for wild-type and variant α_1-proteinase inhibitor differed only ~1.8-fold. Rates of inhibition of trypsin were, however, 25-fold lower for the variant than for the wild-type inhibitor. Steady-state fluorescence spectra showed a change in environment for the P1 side chain upon forming both covalent complex with trypsin or chymotrypsin and noncovalent complex with anhydrochymotrypsin. The P1 environments in the chymotrypsin and anhydrochymotrypsin complexes were, however, different. Fluorescence quenching studies confirmed the burial of the P1 side chain upon formation of both the noncovalent and covalent complexes, but were not able to discriminate between the solvent accessibility in these complexes. Stopped-flow fluorescence measurements resolved the covalent intramolecular reaction that led to covalent complex and showed that, during the course of the covalent reaction, the environment of the P1 side chain changed consistent with a repositioning relative to residues of the proteinase active site as part of formation of the trap. This repositioning is likely to be a crucial part of the trapping mechanism.

There is now much evidence in favor of the branched pathway suicide substrate inhibition mechanism by which members of the serpin superfamily of protein proteinase inhibitors inhibit serine proteinases (*2*). In this mechanism, the proteinase recognizes the P1−P1′[1] bond of the serpin as an appropriate site for proteolytic attack, based largely on the nature of the P1 residue and the specificity of the proteinase, and forms an initial noncovalent Michaelis-like complex as the first step of a substrate-like cleavage reaction. At a later point in the reaction, the serpin undergoes a major conformational rearrangement that results in arrest of the normal substrate reaction and the kinetic trapping of an intermediate in this reaction. The kinetic well that is created by the conformational rearrangement has an energy barrier that is sufficiently high that, although the substrate reaction can still go to completion, with release of the proteinase and generation of serpin cleaved at the P1−P1′ bond, it does so on the time scale of hours to days rather than seconds (*3*). The kinetic trap thus represents a slowing of the substrate cleavage reaction by several orders of magnitude. Probably the biggest question in understanding how a generic mechanism can be used by serpins, as a class, to inhibit serine proteinases, as a class, is what is the nature of the kinetic trap. In other words, how does the conformational rearrangement of serpin and proteinase result in a dramatic slowing of the substrate reaction at a critical point, so that the reaction intermediate is a quasi-stable complex irrespective of which serpin is inhibiting which serine proteinase?

It has been shown that formation of the kinetically trapped intermediate requires participation of the active site serine of the proteinase (*4*, *5*) and that the intermediate is almost certainly an acyl ester in most (*6*−*8*), though possibly not all (*9*, *10*), cases. It has also been suggested that the nature of the major conformational change is a translocation of the proteinase from one end of the serpin to the other with the concomitant insertion of the covalently bound reactive center loop into β-sheet A as a central strand (*11*). This hingelike insertion pivots about residues P16−P15 and involves insertion of the cleaved reactive center loop with the proteinase still covalently bound to the cleaved peptide bond. Recent fluorescence resonance energy transfer measurements showing major movement of both proteinase (*12*, *13*) and

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[1] The nomenclature system of Schechter and Berger (*1*), used to describe the subsites of interaction between a proteinase and its substrate, designates the residues on either side of the substrate scissile bond as P1 and P1′. Residues N-terminal to this are designated P2, P3, ..., P*n*, and residues C-terminal are designated P2′, P3′, ..., P*n*′.

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the N-terminal part of the reactive center loop (*8*) are in keeping with this proposal. Nevertheless, this does not directly address the question of how the rate of deacylation has been slowed by orders of magnitude. There are two principal possibilities. One is that access to the active site has been restricted for the second nucleophile of the reaction, water (*14*). The second is that the insertion into β-sheet A of reactive center loop residues, together with concomitant movement of the proteinase, has disrupted the optimal contacts of the serpin reactive center loop with the active site of the proteinase in the acyl intermediate (*15*). This could be through distortion of the proteinase, including residues of the catalytic triad, movement of the acyl ester linkage, or both.

In this study, we have used tryptophan fluorescence to examine the environment of the side chain of the P1 residue of a P1 M→W variant of the serpin α_1-proteinase inhibitor (α_1PI)[2] before, during, and after formation of covalent and noncovalent complexes with proteinase or anhydroproteinase. By a combination of steady-state and stopped-flow fluorescence measurements, including quenching of steady-state fluorescence, and by comparison with wild-type α_1PI, we found that the P1 side chain undergoes an initial change in environment during formation of the noncovalent Michaelis-like complex, consistent with burial in the S1 pocket, but then undergoes a further change as part of the intramolecular changes that lead to the stable covalent complex that is consistent with movement of the P1 side chain. This change indicates that a structural rearrangement takes place in the active site, which may be a crucial part of the kinetic trap.

MATERIALS AND METHODS

Mutagenesis and Expression of Recombinant α_1-Proteinase Inhibitor. Site-directed mutagenesis to effect the change of the P1 residue from methionine to tryptophan was performed using the Quickchange method (Stratagene) using the manufacturer's protocol. The template was a double-stranded pET16b vector (Novagen) containing the α_1PI cDNA lacking the coding sequence for the first five amino acids [a deletion shown previously to enhance expression in *E. coli* without affecting activity (*16*)] and carrying a C232S mutation that has also been shown not to affect the inhibitory properties of the serpin (*12*). Briefly, two complementary primers containing the M358W mutation were annealed to the template and extended using *Pfu* DNA polymerase and thermal cycling. The sequence of the coding strand of the mismatch primer was 5′-GAG GCC ATA CCC TGG TCT ATC CCC CCC-3′ (mismatch underlined). The daughter DNA was transformed into XL1 blue supercompetent cells. Colonies were screened for the desired mutation by DNA sequencing. The mutant DNA was transformed into BL21-(DE3) cells (Novagen) for expression. Recombinant α_1PI was expressed and purified as previously described (*12, 17*), except that inclusion bodies were solubilized in 9 M urea rather than guanidine hydrochloride and accordingly dialysis after refolding and prior to application to the ion exchange column was omitted since the ionic strength was already low. Concentrations of α_1PI were determined spectrophotometrically using extinction coefficients of 27 000 M^{-1} cm^{-1} (*18*) and 32 500 M^{-1} cm^{-1} for wild-type and variant proteins, respectively. The value for the P1W variant was based on that of wild-type adjusted for the presence of an additional tryptophan (*19*). All subsequent experiments on these proteins were carried out in 20 mM sodium phosphate buffer, pH 7.4, containing 0.1 M NaCl, 0.1 mM EDTA, and 0.1% PEG8000 unless noted otherwise.

Purification of Anhydrochymotrypsin. Anhydrochymotrypsin was prepared by alkaline β-elimination of the PMSF adduct of chymotrypsin according to published procedures (*20*). The anhydrochymotrypsin had less than 1% residual catalytic activity.

Continuous Kinetic Inhibition Assay. Rate constants for inhibition of proteinase were determined from continuously monitored progress curves of the reaction of proteinase with substrate in the presence of α_1PI. Assays were carried out at 25 °C in solutions containing 250 μM succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide for assay of inhibition of chymotrypsin or 100 μM S-2222 for assay of inhibition of trypsin. Experiments at lower inhibitor concentrations were carried out on a Shimadzu 2101PC spectrometer, whereas reactions at higher concentration were carried out using an Applied Photophysics SX-17MV stopped-flow in the absorbance mode. The lower range of P1W and wild-type α_1PI concentrations used was 2.5−22.5 nM for chymotrypsin reactions, whereas the range of serpin concentrations was 182−1100 nM for the P1W variant and 6−47 nM for wild-type α_1PI in the case of trypsin reactions. Large enough ratios of α_1PI to proteinase were used to obtain defined end points without excessive consumption of substrate. The spectrophotometric assay was started by addition of the proteinase to the assay solution containing substrate and α_1-PI. Release of *p*-nitroaniline from the substrate was monitored by the change in absorbance at 405 nm. Depending on the time required to reach an end point, the reactions were monitored for 30 min to 3 h. Stopped-flow reactions also monitored the change in absorbance at 405 nm and used concentrations of wild-type and variant α_1PI of 250 nM−2.5 μM for both trypsin and chymotrypsin reactions. Here one syringe contained proteinase, and the other contained α_1PI and substrate. Data were fitted in all cases to a pseudo-first-order exponential process taking into account the competition of substrate for proteinase (*21*). Experimentally determined K_m values of 56 μM for succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide binding to chymotrypsin and 33 μM for S-2222 binding to trypsin were used.

Stopped-Flow Experiments. Time-resolved fluorescence was measured in an Applied Photophysics SX-17MV stopped-flow fluorometer, following the change in endogenous tryptophan fluorescence. Excitation was at 280 nm, and all emission above 300 nm was collected, for experiments with both active and anhydroproteinase, by using a 300 nm cutoff filter between the reaction cell and the detector. Experiments were carried out at 25 °C under approximately pseudo-first-order conditions to ensure that a well-defined end point of reaction was reached. A ratio of α_1PI to proteinase of 5:1 was used to maximize the signal for complex formation. One syringe was filled with proteinase and the other with α_1PI. Concentrations of proteinase used were 0.065−1.0 μM for anhydrochymotrypsin and 0.065−1.5 μM for chymotrypsin. At least eight individual

[2] Abbreviations: α_1PI, α_1-proteinase inhibitor; SI, stoichiometry of inhibition, defined as the number of moles of serpin required to inhibit 1 mol of proteinase.

reactions were averaged for each concentration of α_1PI used. To confirm that chymotrypsin did not suffer significant autodigestion at this pH and concentration while in the syringe, the time dependence of cleavage was examined by gel electrophoresis under equivalent conditions of pH and concentration. From the absence of autolysis cleavage products over a time period of 120 min, determined from the appearance of the protein on SDS−PAGE, it was judged that such autolysis was not a concern in the stopped-flow experiments.

Steady-State Fluorescence Spectroscopy. Steady-state tryptophan fluorescence emission spectra of α_1PI alone and in complex with proteinase or anhydroproteinase were recorded at 25 °C on an SLM8000 fluorometer. Excitation was at 280 nm and emission recorded from 300 to 420 nm in 2 nm steps. Excitation and emission slits were both 4 nm. Samples contained 0.5 μM α_1PI and, for complexes, 0.5 μM proteinase/anhydroproteinase. Fluorescence quenching studies were carried out on 0.5 μM protein solutions (α_1-PI or α_1PI−proteinase complexes) in degassed 20 mM sodium phosphate buffer, pH 7.4, containing 0.4 M NaCl. KI was added from a stock solution containing 0.4 mM $Na_2S_2O_3$. Excitation was at 280 nm, and emission was measured at 340 nm using slits of 4 and 16 nm, respectively, for excitation and emission. A correction to the fluorescence was made for dilution.

Gel Electrophoresis. SDS−PAGE was performed on 10% gels. α_1PI samples were incubated with equimolar amounts of active proteinase for 2 min (chymotrypsin) or 3 min (other proteinases), at concentrations of ~4 μM for each component. Reactions were stopped by addition of PMSF to a final concentration of 1 mM for all proteinases. Sample buffer was added, and the mixtures were boiled for 3 min. PAGE under nondenaturing conditions was carried out on 10% gels (*22*). α_1PI (wild-type and P1W variant) was incubated with increasing amounts of anhydrochymotrypsin for 5 min at 25 °C. The ratios of anhydrochymotrypsin to α_1PI used were 0:1, 2:1, 5:1, and 10:1 for both wild-type and P1W variant.

Determination of SI. SIs were determined both by kinetic assay and by PAGE. For the kinetic assay, fixed amounts of chymotrypsin or trypsin (75 nM) were incubated with different concentrations of α_1PI for a time greater than 10 half-lives and then assayed for residual enzymatic activity by dilution into assay buffers containing either 250 μM succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide for chymotrypsin (10−20-fold dilution) or 100 μM S-2222 for trypsin (20−40-fold dilution) and determining the initial rate of reaction from the slope of the release of product with time. For determination of SI by PAGE, α_1PI (3 μg) was incubated with proteinase (0.4−1.2 μg) in 10 μL of pH7.4 buffer for 5 min and the reaction stopped by addition of PMSF. The SI was calculated by scanning densitometry of SDS−PAGE gels of these reaction mixtures. Coomassie blue stained gels were scanned, and the densities of the bands corresponding to cleaved serpin and complex were measured. The intensity of the band for complex was corrected for the contribution from the proteinase, by assuming equal staining of the serpin and trypsin per unit weight.

Determination of the Dissociation Constant for the Complex between Anhydrochymotrypsin and P1W Variant. The dissociation constant for the noncovalent complex between anhydrochymotrypsin and P1W variant α_1PI was determined by titration of 20 nM anhydrochymotrypsin with α_1PI at 25 °C and using the decrease in tryptophan fluorescence that accompanied binding, with excitation at 280 nm and emission at 340 nm. Fluorescence intensities were first corrected for the increase in intensity due to the addition of each aliquot of α_1PI, as determined from a control titration using only α_1PI P1W variant. Data were fitted to a single-site binding model by nonlinear least-squares analysis using the program Scientist (MicroMath, Salt Lake City). The fit confirmed the stoichiometry of complex formation as 1:1.

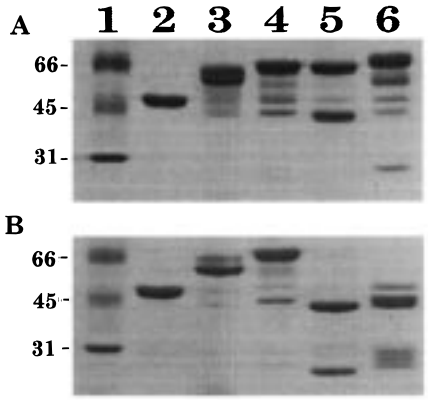

FIGURE 1: SDS−PAGE of α_1PI−proteinase incubations, showing the ability of P1W variant to form higher molecular mass stable covalent complexes with only some proteinases. Panel A, wild-type α_1PI; panel B, P1W variant. Samples are: lane 1, molecular mass standards (66, 45, 30 kDa); lane 2, unreacted; lane 3, reacted with chymotrypsin; lane 4, reacted with trypsin; lane 5, reacted with porcine elastase; lane 6, reacted with neutrophil elastase. Proteinase and serpin were incubated at ~1:1 (~4 μM each). The band with lower mobility than unreacted α_1PI is from covalent complex with proteinase; the band with slightly higher mobility than unreacted α_1PI is cleaved α_1PI. Bands visible between those of unreacted serpin and covalent complex are due to degraded complex resulting from cleavage by residual free proteinase and were not present when a lower ratio of proteinase to serpin was used.

Materials. S-2222 and succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide were from Chromogenix and Sigma, respectively. Lima bean inhibitor was from Cooper Biomedical. α-Chymotrypsin and trypsin were from Worthington and were used without further purification.

RESULTS

Ability of P1W Variant To Form SDS-Stable Covalent Complex with Trypsin and Chymotrypsin. The mutation of the P1 residue from methionine to tryptophan was made to provide an endogenous fluorescent reporter of the environment of the P1 side chain. To determine whether the mutation had altered the proteinase specificity, as judged by the ability of the mutated α_1PI to form SDS-stable complexes with proteinase, the products of reaction with pancreatic and neutrophil elastases, trypsin, and chymotrypsin were examined by SDS−PAGE (Figure 1). Both wild-type and P1W variant were able to form a covalent complex with both trypsin and chymotrypsin (Figure 1, lanes 3 and 4). In contrast, whereas wild-type α_1PI was a good inhibitor of both pancreatic and neutrophil elastases (*23*, *24*), and formed high molecular weight covalent complexes (Figure 1A, lanes 5 and 6), the P1W variant was only cleaved by these protein-

Table 1: Inhibitory Properties of Wild-Type and P1W Variant α_1PIs

	SI					
	kinetic[a]		SDS−PAGE[b]		k (M^{-1} s^{-1})[a]	
α_1PI	chymotrypsin	trypsin	chymotrypsin	trypsin	chymotrypsin	trypsin
wild-type	0.8 ± 0.02	1.2 ± 0.04	1.2 ± 0.1	1.04 ± 0.01	(1.2 ± 0.2) × 10^6	(3.1 ± 0.4) × 10^5
P1W	1.0 ± 0.04	1.1 ± 0.03	1.2 ± 0.1	1.01 ± 0.01	(2.1 ± 0.1) × 10^6	(1.3 ± 0.2) × 10^4

[a] Based on five determinations. [b] Based on four determinations.

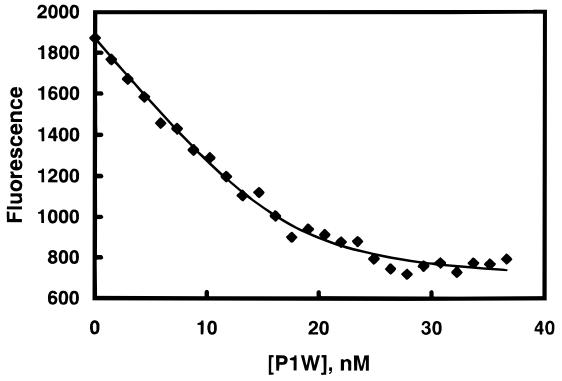

FIGURE 2: Fluorescence change upon titration of P1W α_1PI into 20 nM anhydrochymotrypsin. The fluorescence has been corrected for the contribution of the added P1W variant and thus shows only the changes associated with formation of the noncovalent complex. The solid line is the best fit of the data to a simple binding process and confirms the expected stoichiometry of 1:1 for the complex.

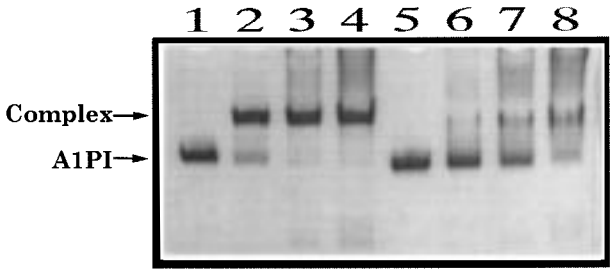

FIGURE 3: Nondenaturing PAGE showing formation of tight noncovalent complex between anhydrochymotrypsin and the P1W α_1PI variant (2 μg), but weaker association for the complex with wild-type α_1PI (2 μg). Lanes 1−4, P1W α_1PI variant; lanes 5−8, wild-type α_1PI. Lanes 1 and 5, α_1PI alone; lanes 2−4 and 6−8, incubated with increasing amounts of anhydrochymotrypsin (2, 5, and 10 equiv). The anhydrochymotrypsin does not appear on the gel because of its high p*I*. Residual chymotrypsin activity in the anhydrochymotrypsin preparation was <1%.

ases (Figure 1B, lanes 5 and 6). The stoichiometries of inhibition of the P1W variant for both trypsin and chymotrypsin were determined by two independent assays. One was by SDS−PAGE, in which the products of reaction of a fixed amount of the serpin with increasing amounts of proteinase were quantitated densitometrically following Coomassie staining of the protein bands. The other was a kinetic assay of residual proteolytic activity after incubating a fixed amount of proteinase with increasing amounts of serpin and allowing the reaction to go to completion. Both methods gave SI values similar to those of wild-type α_1PI and close to 1 (Table 1), thus showing that the M→W mutation had altered neither the basic mechanism nor the efficiency of inhibition with these two proteinases. This validates the use of chymotrypsin and trypsin for use in subsequent studies on formation of complex with P1W variant and wild-type α_1PIs.

Ability of P1W Variant To Form Tight Noncovalent Complex with Anhydrochymotrypsin. To compare the fluorescence of the P1 side chain in covalent and noncovalent complexes, it was first necessary to determine if the P1W variant could form a tight noncovalent complex with anhydrochymotrypsin. This was examined both by fluorescence change, using the quenching of the P1 tryptophan (see below), and by PAGE under nondenaturing conditions. A titration of the P1W α_1PI variant into 100 nM anhydrochymotrypsin gave an almost linear decrease in fluorescence up to a stoichiometry of 1:1, when corrected for the fluorescence of the added anhydrochymotrypsin, indicating very tight binding (not shown). A titration was therefore performed at a lower concentration of anhydrochymotrypsin (20 nM) (Figure 2) to determine the K_d. A value of 1.5 ± 0.6 nM and a stoichiometry of 1:1 were obtained from fitting the data, confirming very tight binding to the variant in a 1:1 complex. We have found a comparable high affinity between a serpin and an anhydroproteinase for anhydrotrypsin binding to a P1 arginine variant of α_1PI (*12*).

The binding of the P1W variant to anhydrochymotrypsin was sufficiently tight that nondenaturing PAGE showed the presence of a new band corresponding to noncovalent complex between the P1W variant and anhydrochymotrypsin for a 1:2 mixture (Figure 3, lane 2). In contrast, wild-type α_1PI gave only a weak band at the position of complex when a 1:2 mixture was run (Figure 3, lane 6). At higher ratios of anhydrochymotrypsin to wild-type α_1PI, more intensity was seen at the position of complex, but much was smeared between that position and the top of the gel (Figure 3, lanes 7 and 8). Anhydrochymotrypsin is too basic to enter the gel under the conditions of the experiment and therefore does not appear on the gel. The difference in behavior of the two complexes presumably reflects the tighter binding, and thus slower dissociation rate, of the complex of the P1W variant with anhydrochymotrypsin compared to that of wild-type α_1PI. Thus, the K_d of 1.5 nM for the complex with the P1W variant determined here compares with a published value for the wild-type complex of 55 nM (*25*).

Kinetics of Proteinase Inhibition by Wild-Type and Variant α_1PIs. The second-order rate constants for the inhibition of trypsin and chymotrypsin by both wild-type and P1W variant α_1PIs were determined by continuous assay using chromogenic substrates, as described under Materials and Methods. The mutation increased the rate of inhibition of chymotrypsin only ∼60%, from 1.2 × 10^6 M^{-1} s^{-1} to 2.1 × 10^6 M^{-1} s^{-1}, but decreased the rate of inhibition of trypsin by over 20-fold, from 3.1 × 10^5 M^{-1} s^{-1} to 1.3 × 10^4 M^{-1} s^{-1} (Table 1). The P1W variant was therefore much more selective for chymotrypsin than was wild-type α_1PI, with relative reaction rates toward these two proteinases of ∼160:1 (chymotrypsin:trypsin) for the variant but only ∼4:1 for the

wild-type. Both the increase in the rate of inhibition of chymotrypsin and the large reduction in the rate for inhibition of trypsin are in keeping with the side chain preferences of each proteinase and the effect of replacing a small hydrophobic side chain with a large hydrophobic one.

Steady-State Fluorescence Spectra of Free and Proteinase-Complexed Wild-Type and P1W Variant α_1PIs. Introduction of a tryptophan at P1 had a relatively large effect on the emission spectrum of α_1PI, which contains only two other tryptophans, at positions 194 and 238. Wild-type α_1PI gave an emission spectrum with a wavelength maximum of 336 nm. The presence of the additional tryptophan in the P1W variant resulted in an approximately 100% increase in emission intensity and a red shift of the emission maximum to 342 nm (Figure 4A), indicating a more exposed environment for the P1 tryptophan than for the two endogenous tryptophans. This is consistent with the crystal structure of α_1PI, which shows tryptophan 194 to be completely buried in a relatively hydrophobic environment and tryptophan 238 mostly buried, whereas the P1 residue is on the exposed reactive center loop and points into solution in wild-type α_1-PI (*26*, *27*).

Formation of a 1:1 noncovalent complex between anhydrochymotrypsin and wild-type α_1PI gave an emission spectrum almost identical to the summed emission spectra of the two component proteins (Figure 4B), suggesting that neither protein underwent a conformational change that was detectable by the internal tryptophans. This also suggests that the eight tryptophans in anhydrochymotrypsin are not close enough to either of the two internal tryptophans of α_1-PI to perturb the fluorescence emission of the latter by fluorescence resonance energy transfer. This is reasonable given the separations of 20 and 28 Å of the two tryptophans of α_1PI from the P1 side chain, as calculated from the crystal structure (*26*, *27*). These findings are therefore consistent with a noncovalent complex in which the anhydroproteinase has simply docked with the reactive center loop of the serpin, causing no change in structure of either the anhydroproteinase or the body of the serpin.

Formation of a 1:1 complex between anhydrochymotrypsin and the P1W α_1PI variant caused a reduction in overall fluorescence intensity (Figure 4C). Since complex formation with wild-type α_1PI caused no fluorescence change, the reduction in intensity seen with the P1W variant most probably results solely from the P1 tryptophan. This could be either a change in the emission properties of the P1 side chain alone or a coupled change that also involves tryptophans of anhydrochymotrypsin, through fluorescence resonance energy transfer with the P1 tryptophan of the α_1PI variant (*28*).

Formation of covalent complex between wild-type α_1PI and chymotrypsin resulted in very little change compared with the summed spectra of the two component proteins (Figure 4D). This was surprising, given the conformational changes that occur upon formation of covalent complex, and suggests fortuitous compensating changes in serpin and proteinase. When a similar complex was formed with the P1W variant, there was almost no reduction in overall intensity, but there was a small blue shift (2 nm) (Figure 4E). Covalent complex formation between trypsin and wild-type α_1PI resulted in about a 15% increase in intensity at the emission maximum (Figure 4F). With the P1W variant, formation of complex with trypsin resulted in a similar absolute increase in fluorescence intensity, though a smaller percentage change due to higher contribution to the starting fluorescence from the P1W variant (Figure 4G). It should be noted that an uncertainty in interpreting these changes in terms of the P1 side chain is that the replacement of methionine by tryptophan may itself cause some structural differences in the proteinase, which in turn may contribute to fluorescence changes. The stability of the fluorescence spectra with time over at least 30 min indicated the absence of a problem with proteolysis. This was confirmed by SDS−PAGE, carried out subsequently on the reaction mixtures. Bands corresponding to cleaved covalent complex were either absent or present to no more that 15% of total complex, even after 1 h of incubation.

To make clearer the contribution of the P1 tryptophan to the spectrum of α_1PI and to the fluorescence changes upon complex formation, fluorescence difference spectra were generated, in which the effect of P1 alone could be seen, by subtraction of a spectrum of an equivalent species in each case (see figure legend for details of the spectra used for the subtractions in each case). Because the difference spectra represent a small fraction of the total fluorescence intensity, experiments were repeated a number of times to obtain estimates of their reproducibility. Experiments were repeated 7 times for the uncomplexed α_1PI and 3 times for the complexes with anhydrochymotrypsin, chymotrypsin, and trypsin. The intensities and wavelengths given are the means $\pm$ the standard deviation. In uncomplexed P1W variant of α_1PI, the P1 tryptophan had an emission spectrum with a maximum at 349 $\pm$ 2 nm (Figure 5A), consistent with the expected very exposed environment of the P1 side chain. Upon formation of noncovalent complex with anhydrochymotrypsin, there was a large reduction in fluorescence intensity (63 $\pm$ 11%) of the P1 tryptophan (Figure 5B), consistent with burial in the relatively hydrophobic S1 specificity pocket of chymotrypsin (*29*). The wavelength maximum (352 $\pm$ 3 nm) had not changed within experimental error. The P1 tryptophan was also perturbed upon formation of covalent complexes with either chymotrypsin or trypsin, but differently from the noncovalent complex with anhydrochymotrypsin. In the chymotrypsin complex, the difference spectrum between equivalent complexes of the P1W variant or wild-type α_1PIs was significantly blue shifted (Figure 5C), with an emission maximum of 337 $\pm$ 2 nm and an overall intensity slightly reduced (8 $\pm$ 13%) relative to that of the P1 tryptophan in uncomplexed P1W variant of α_1PI (Figure 5A). In the trypsin complex, there was also a small reduction in intensity of the P1 residue (15 $\pm$ 10%), with a small blue shift to 347 $\pm$ 6 nm (Figure 5D). These difference spectra show that the P1 side chain changes its environment upon forming either noncovalent or covalent complex with proteinase. However, this environment differs most significantly between the noncovalent and covalent complexes with chymotrypsin and less between the covalent complexes with trypsin and chymotrypsin. Although the latter difference is not surprising, given the different residues that make up the S1 specificity pocket in the two proteinases, most notably the replacement of Ser189 in chymotrypsin by Asp189 in trypsin, the significant difference in wavelength maximum and intensity for the P1 tryptophan in complexes with chymotrypsin and anhydrochymotrypsin suggests a

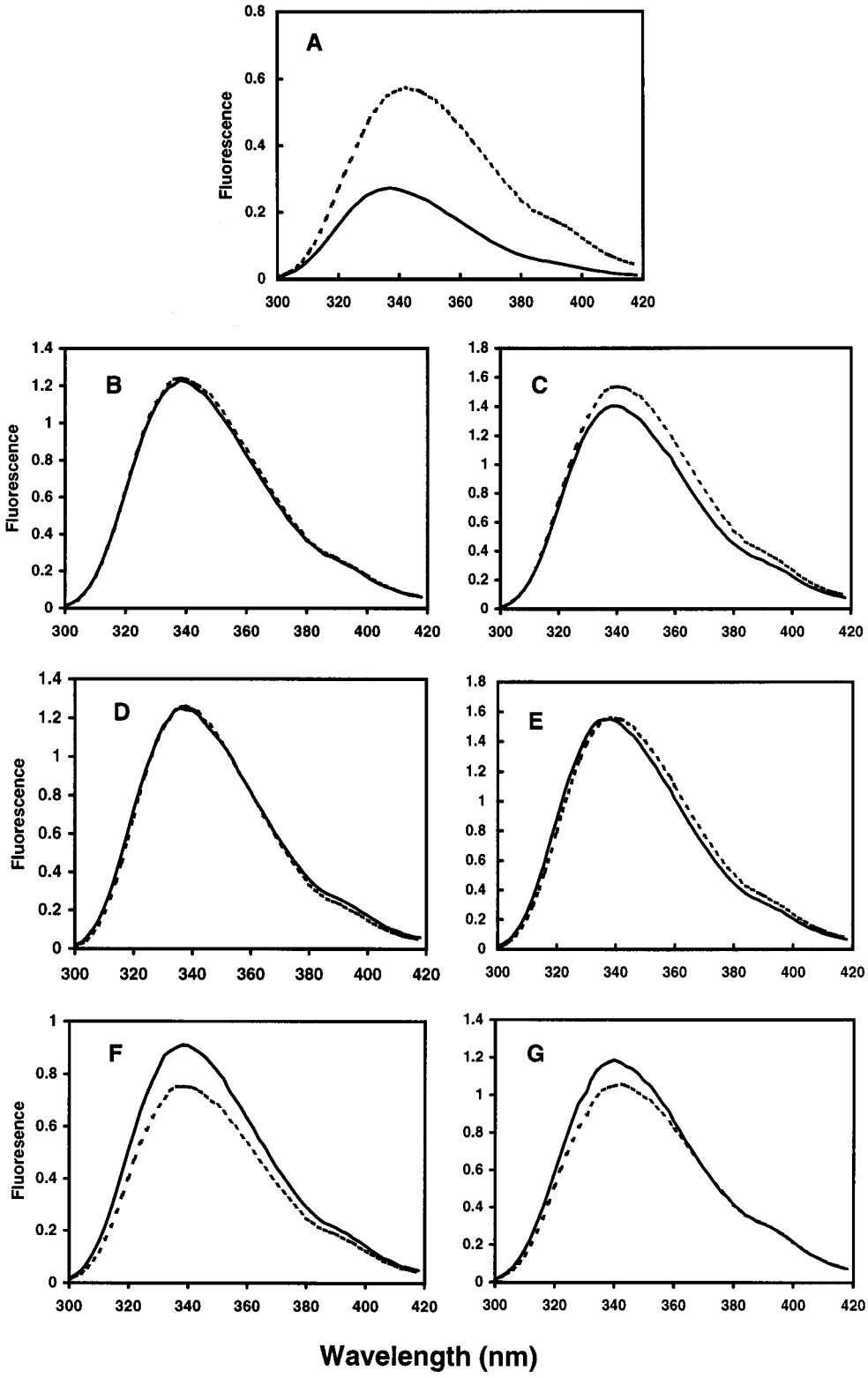

FIGURE 4: Fluorescence spectra of α_1PI wild-type and P1W (0.5 μM) either alone or in complex with anhydrochymotrypsin, chymotrypsin, or trypsin (0.5 μM). In panel A, the solid line represents wild-type and the dashed line the P1W variant. In panels B−G, the solid line is the spectrum of the complex, and the dashed line is the sum of the spectra of the two isolated components. (A) Spectra of wild-type and P1W variant α_1PI; (B) effect of formation of noncovalent complex between wild-type α_1PI and anhydrochymotrypsin; (C) effect of formation of noncovalent complex between P1W variant and anhydrochymotrypsin; (D) effect of formation of covalent complex between wild-type α_1PI and chymotrypsin; (E) effect of formation of covalent complex between P1W α_1PI and chymotrypsin; (F) effect of formation of covalent complex between wild-type α_1PI and trypsin; (G) effect of formation of covalent complex between P1W variant and trypsin.

change in positioning of the P1 side chain relative to residues in the active site of the proteinase, resulting in altered fluorescence properties.

Stopped-Flow Measurements of Complex Formation. Whereas the steady-state fluorescence spectra of the covalent complex of the P1W variant with chymotrypsin showed that

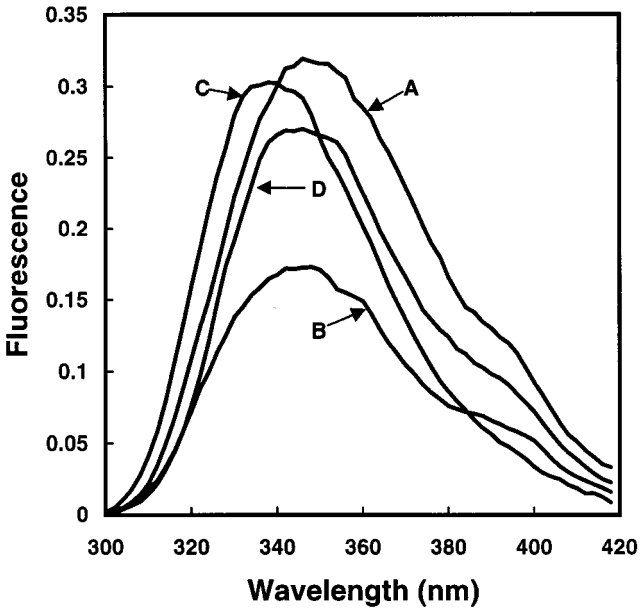

FIGURE 5: Fluorescence difference spectra showing the environment of the P1 tryptophan of P1W variant α_1PI. (A) Difference between spectra of P1W and wild-type α_1PI; (B) difference between spectra of noncovalent complexes of anhydrochymotrypsin with P1W variant and with wild-type α_1PI; (C) difference between spectra of covalent complexes of chymotrypsin with P1W variant and with wild-type α_1PI; (D) difference between spectra of covalent complexes of trypsin with P1W variant and with wild-type α_1PI. Spectra used to generate these differences were obtained using 0.5 μM α_1-PI or α_1PI−proteinase complexes.

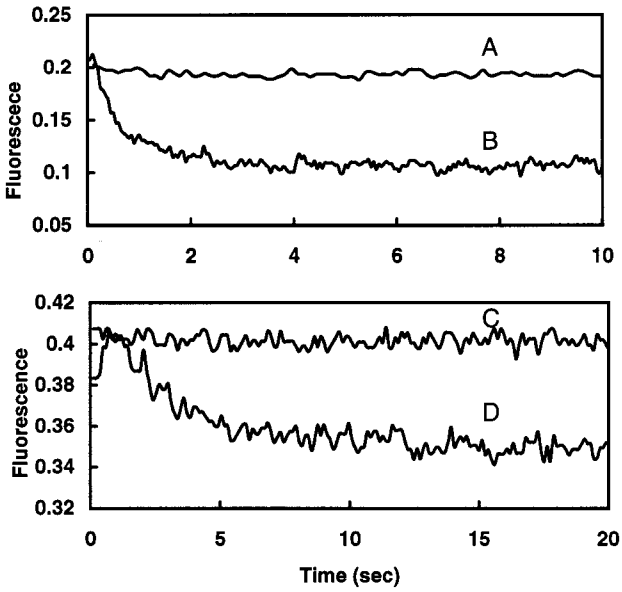

FIGURE 6: Changes in P1 tryptophan fluorescence during complex formation from stopped-flow measurements. (A) Anhydrochymotrypsin−wild-type α_1PI; (B) anhydrochymotrypsin−P1W α_1PI; (C) chymotrypsin−wild-type α_1PI; (D) chymotrypsin−P1W α_1PI. The *y*-axis scale has an arbitrary zero not related to the total fluorescence intensity of the mixture.

the P1 side chain changed its environment upon complex formation and that this environment was not the same as for the noncovalent complex with anhydrochymotrypsin, these spectra alone could not show whether the change occurred as a result of the initial encounter of α_1PI with chymotrypsin or during a subsequent step of the substrate reaction. To try and resolve the temporal sequence of changes, we used stopped-flow fluorescence measurements of the interaction of chymotrypsin and anhydrochymotrypsin with both wild-type and P1W variant α_1PIs.

The simpler experiment to interpret was complex formation between anhydrochymotrypsin and α_1PI, since only a single-step reaction was expected, involving formation of a Michaelis-like complex. The control experiment of wild-type α_1PI with anhydrochymotrypsin gave a negligible change in fluorescence intensity upon complex formation (Figure 6A), showing that the two internal tryptophans were not sensitive to formation of the noncovalent complex. In contrast, the P1W variant gave a reproducible decrease in fluorescence intensity that could be well fitted to a monoexponential process (Figure 6B), indicating that the environment of the P1 tryptophan changed upon formation of the complex. These kinetic traces were also consistent both with the steady-state fluorescence spectra (Figure 4B,C), which showed no change for wild-type α_1PI, but a reduction for the P1W variant, and with the fluorescence difference spectra (Figure 5), which show that the P1 tryptophan is ~63% quenched upon complex formation with anhydrochymotrypsin.

The control experiment of wild-type α_1PI with chymotrypsin, to give the covalent complex, gave almost no change in the kinetic trace (Figure 6C), again consistent with the negligible alteration in fluorescence intensity seen in the steady-state spectra (Figure 4D). In contrast, complex formation with the P1W variant showed a small slow decrease that was complete after about 15 s, and that could be well-fitted to a monoexponential decay (Figure 6D). Michaelis complex formation alone would be expected to result in a large decrease, as seen above for the anhydrochymotrypsin complex. Careful comparison of the summed fluorescence intensities of the two component solutions prior to mixing with that of the initial intensity of the mixture showed very similar values so we can be sure that we are not missing a large but very rapid decrease at very early time points. The failure to see early large changes is likely due to the absence of a buildup of this intermediate species.

To determine whether the slow time-dependent changes seen resulted from initial association or from subsequent intramolecular changes, the concentration dependence of the changes was investigated for both the covalent and noncovalent complexes. The pseudo-first-order rate constant for the covalent reaction between the P1W variant and chymotrypsin was determined over a concentration range of 0.05−1.5 μM α_1PI, with a fixed inhibitor-to-proteinase ratio, and showed a saturable concentration dependence (Figure 7). This behavior was consistent with a two or more step reaction involving initial formation of a reversible Michaelis complex followed by irreversible conversion to a stable complex. Fitting the data by the expected hyperbolic equation gave a K_m value of 0.12 ± 0.03 μM and a limiting rate constant of 0.46 ± 0.03 s^{-1}. This indicated that the fluorescence change was reporting on the environment of the P1 tryptophan as the Michaelis complex was transformed into the stable complex. This is also consistent with the smaller quench observed in forming the covalent complex. Combining the K_m value with the limiting rate for the intramolecular process gave a value for k_{cat}/K_m of 2.6×10^6 M^{-1} s^{-1}. This compares well with the directly determined second-order rate constant for the reaction of 2.1×10^6 M^{-1} s^{-1} (Table 1).

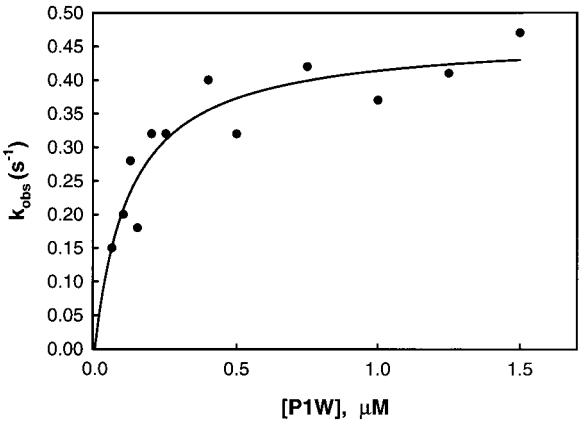

FIGURE 7: Concentration dependence of observed pseudo-first-order rate constant for reaction of chymotrypsin with P1W α_1PI variant. Reactions were carried out under pseudo-first-order conditions with a 5:1 ratio of P1W α_1PI to chymotrypsin. The solid line is the fit to a rectangular hyperbolic function.

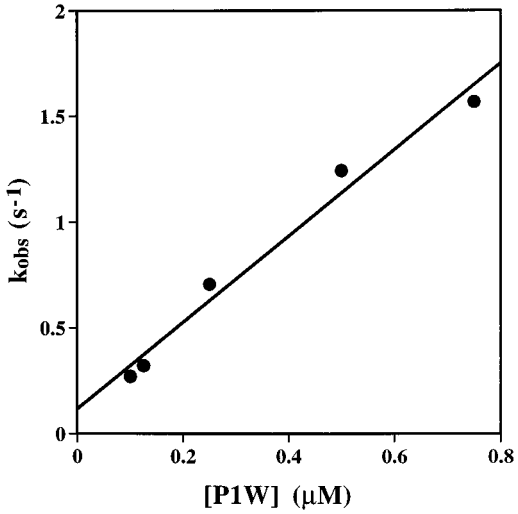

FIGURE 8: Concentration dependence of pseudo-first-order rate constant for association of anhydrochymotrypsin with P1W variant. Reactions were carried out under pseudo-first-order conditions with a 5:1 ratio of P1W α_1PI to anhydrochymotrypsin. The solid line is the linear least-squares fit of the data.

In contrast to the saturability of the rate of fluorescence change for the covalent reaction, the observed rate constant for the noncovalent reaction between P1W α_1PI and anhydrochymotrypsin showed a linear dependence on inhibitor concentration, at fixed inhibitor-to-proteinase ratio (Figure 8), as expected for a fluorescence change that was monitoring the association of the two proteins. The rate constant for the noncovalent association obtained from the slope was 2.04×10^6 M^{-1} s^{-1}. The dissociation rate constant obtained from the intercept on the *y*-axis could not, with confidence, be distinguished from zero, consistent with the high affinity measured for the interaction by equilibrium binding measurements ($K_d = 1.6 \pm 0.3$ nM) and the expected small value for this rate constant obtained as $K_d \times k_{ass}$($=3 \times 10^{-3}$ s^{-1}). Stopped-flow studies were not possible for the trypsin/anhydrotrypsin pair with each of the α_1PI species since the affinity of the anhydrotrypsin for both wild-type and P1W variant α_1PIs was too weak to allow complete saturation of the serpin at accessible anhydrotrypsin concentrations.

Accessibility of P1 Side Chain from Fluorescence Quenching Measurements. Given the different environments of the P1 side chain in the noncovalent and covalent complexes as well as the time-dependent changes in environment during formation of the final covalent complex for the reaction with chymotrypsin, we tested whether there was a concomitant change in solvent exposure of the tryptophan side chain between noncovalent and covalent complexes, using iodide quenching of tryptophan fluorescence.

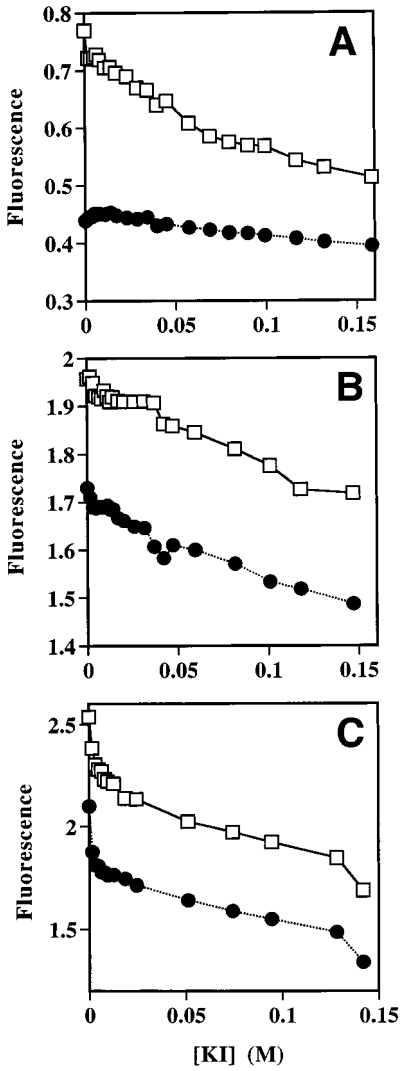

FIGURE 9: Fluorescence quenching of tryptophans in α_1PI, wild-type, and P1W variant, and in complexes with anhydrochymotrypsin and chymotrypsin. (A) Free α_1PI; (B) noncovalent complex with anhydrochymotrypsin; (C) covalent complex with chymotrypsin. In all panels, filled circles represent wild-type α_1PI and open squares represent P1W variant. Protein concentrations were 0.5 μM in all cases.

Wild-type α_1PI showed relatively poor accessibility of the two tryptophans to iodide, from the shallow slope of the quenching as a function of [I^-] (Figure 9A). This is consistent with the mostly buried locations of these tryptophans. Quenching of the P1W variant showed two phases, one similar to that of wild-type and a second that represented much higher quenchability, that must correspond to the P1 tryptophan residue (Figure 9A). Upon formation of noncovalent complex with anhydrochymotrypsin, the P1 tryptophan became very much less accessible, as judged by approximately constant difference between the quenching curves of complexes of wild-type and P1W α_1PIs (Figure 9B). This is consistent with the expected burial of the P1

tryptophan in the S1 pocket of the proteinase and the observed 63% reduction in intensity of the fluorescence signal (Figure 4B). Within the sensitivity of the method, the covalent complexes of chymotrypsin with both wild-type and P1W variant α_1PIs also showed parallel quenching curves, indicating similar low accessibility of the P1 tryptophan as in the noncovalent complex (Figure 9C).

DISCUSSION

In this study, we introduced a tryptophan at the P1 position of α_1PI to examine the change in environment of the P1 side chain of a serpin upon formation of both noncovalent and covalent complexes with proteinase, to determine whether a change in environment of the P1 side chain occurs after formation of the initial Michaelis-like complex, since this would help to discriminate between different mechanisms for formation of the kinetic trap whereby serpins inhibit proteinases. The introduction of tryptophan at P1 changed the target proteinase specificity and rate of reaction with proteinase but did not alter the serpin inhibition mechanism or the SI for inhibition of trypsin or chymotrypsin. The variant was therefore a valid model serpin for such basic mechanistic studies. Steady-state fluorescence measurements showed that the P1 side chain changed environment both in covalent complexes with chymotrypsin and trypsin as well as in a noncovalent complex with anhydrochymotrypsin. This was shown by the perturbation of the P1 fluorescence for all three complexes. Since the noncovalent complex with anhydrochymotrypsin should be a good model for the first-formed Michaelis-like complex and therefore an intermediate in the covalent reaction with active chymotrypsin, the finding that the fluorescence properties of the P1 side chain in covalent and noncovalent complexes were very different in intensity (differing by ~50%) and also different in wavelength maxima (337 vs 352 nm, respectively) suggested that one or more additional steps occurred during the conversion of the Michaelis complex into the stable covalent complex that was detectable by changes in fluorescence of the P1 tryptophan. This was confirmed by stopped-flow measurements on the two types of complex, which showed that, during covalent complex formation, there was an intramolecular change that followed association of the two proteins and that resulted in a change in fluorescence of the P1 residue. The intramolecular fluorescence change occurred with a rate constant of 0.46 s^{-1}. This fluorescence change thus reports either the rate-limiting step directly or a subsequent step that occurs at a faster rate. Although it is not possible from the present results to say which of these possibilities is correct, it can be said that the changes that occur at, or after, the rate-determining step of the inhibition reaction involve a change in the environment of the P1 side chain, since the end-point fluorescence spectrum is that of the final covalent complex. Rate constants for this step have been reported for a number of other serpin−proteinase pairs, and cover a range from 3 s^{-1} for tissue plasminogen activator with PAI1 (*30*) to 140 s^{-1} for antithrombin−factor Xa−heparin ternary complex (*31*), with intermediate values for thrombin−antithrombin (5 s^{-1}) (*32*) and chymotrypsin−α_1-antichymotrypsin (7 s^{-1}) (*33*). This rate constant thus shows great variation as a function of the specific serpin−proteinase pair. The ~15-fold lower rate observed here for the P1W variant of α_1PI with chymotrypsin compared with α_1-antichymotrypsin with chymotrypsin may be due to the proline at P2 in the former compared with leucine in the latter. The failure to detect any large fluorescence change prior to progression to the trapped acyl enzyme intermediate, despite the observation of a large fluorescence change for the P1 tryptophan upon formation of the Michaelis complex itself, requires that there must be an additional intermediate formed relatively rapidly between the Michaelis complex and the acyl intermediate, so that there is no significant buildup of the Michaelis complex. There is precedent for such an additional fast step in the reaction between chymotrypsin and α_1-antichymotrypsin (*34*). From fluorescence quenching studies, we further showed that the P1 side chain is much more exposed prior to complex formation with either anhydrochymotrypsin or chymotrypsin, though differences in the accessibility of the P1 tryptophan in the two types of complex could not be detected by this method.

Beyond Michaelis complex formation, the rate-limiting step for most serine proteinase cleavages of peptide substrates is formation of the acyl enzyme intermediate (*35*). The same is likely to be true for serpins acting as suicide substrate inhibitors of serine proteinases. Conversion of the tetrahedral intermediate into the acyl enzyme intermediate involves cleavage of the peptide bond, and in serpins it is thought that this permits the reactive center loop of the serpin to insert into β-sheet A. The expected structural changes that occur during and after the rate-determining step of the serpin−proteinase reaction are thus (i) formation of the acyl enzyme intermediate and (ii) insertion of the reactive center loop into β-sheet A, with concomitant translocation of the covalently bound proteinase. There is now evidence that the proteinase in the final complex is located at the end of the serpin distal from the initial location (*12*, *13*). Since it is also thought that the structural change that results in formation of the kinetic trap only occurs once the final structure has been attained (*36*), it is likely that the fluorescence change for the P1W variant seen by stopped flow occurs as the loop completes its insertion into β-sheet A and thereby brings about alteration in the structure of the proteinase as a result of the proteinase impinging upon the distal end of the serpin. This structural change furthermore appears to involve a readjustment of the position of the P1 side chain within the active site of the proteinase.

It is also of note that, upon formation of the tight noncovalent complex between anhydrochymotrypsin and *wild-type* α_1PI, there is very little change in tryptophan fluorescence of either protein, in either steady-state or stopped-flow fluorescence measurements. The absence of change for the noncovalent complex provides further evidence for the fundamental difference in structure between the initial noncovalent complex that is formed upon docking of the two proteins and the final covalent serpin−proteinase complex. Whereas the initial complex most probably involves no conformational change within the body of the serpin or the proteinase, formation of the covalent complex involves changes in the active site of the proteinase, as evidenced by changes in the environment of the P1 side chain as the complex progresses from the initial noncovalent structure to the final covalent complex. The major fluorescence changes of the P1 residue upon formation of the covalent complex may arise from changes in the serpin, in the proteinase, or in both. It is known from earlier studies

that formation of cleaved, loop-inserted α_1PI results in an increase in endogenous tryptophan fluorescence (*37*). Since formation of covalent complex with trypsin and chymotrypsin gave different effects on fluorescence intensity for wild-type α_1PI (large increase vs little effect, respectively), yet would be expected to have similar contributions from changes within the serpin, this suggests that the proteinase also undergoes a structural change that, in addition to causing changes in the active site, alters the structure of the proteinase as a whole and hence of its fluorescence, in agreement with previous work on the serpin−proteinase pair of antithrombin−thrombin (*38*).

There is independent evidence in support both of a change in the catalytic residues of the proteinase in the covalent complex and for more general structural changes in the proteinase. ^{1}H NMR has shown that the proton that is shared between the histidine and aspartate of the catalytic triad has different spectroscopic properties in noncovalent complexes and in covalent serpin complexes with proteinase (*39*, *40*). The nature of this difference is still speculative (though we provide evidence here for direct changes in location of the P1 side chain), but may involve a change that makes the active site more like that of the zymogen form of the proteinase (*40*), thus explaining the reduced catalytic efficiency for breakdown of the acyl ester intermediate. Calorimetry has shown that trypsin in complex with α_1PI is several degrees more stable than when it is free (*40*). The same study, as well as others, has also shown increased susceptibility of certain loops within the proteinase to cleavage by uncomplexed proteinase (*40*−*42*). This accounts for the tendency of serpin−proteinase complexes to be degraded to lower molecular weight species, when examined by SDS−PAGE under reducing conditions.

In conclusion, the present findings support a mechanism of formation of the kinetic trap that involves an alteration in the structure of the proteinase in the covalent complex, but not in the noncovalent complex. This structural alteration affects the environment and position of the P1 side chain in the active site and is most probably a manifestation or cause of the reduced catalytic efficiency of the proteinase that represents the kinetic trap.

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