Structure of a Serpin-Enzyme Complex Probed by Cysteine Substitutions and Fluorescence Spectroscopy

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ABSTRACT The x-ray crystal structure of the serpin-proteinase complex is yet to be determined. In this study we have investigated the conformational changes that take place within antitrypsin during complex formation with catalytically inactive (thrombin$_{S195A}$) and active thrombin. Three variants of antitrypsin Pittsburgh (an effective thrombin inhibitor), each containing a unique cysteine residue (Cys$_{232}$, Cys$_{P3'}$, and Cys$_{313}$) were covalently modified with the fluorescence probe *N*,*N*′-dimethyl-*N*-(iodoacetyl)-*N*′-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)ethylenediamine. The presence of the fluorescent label did not affect the structure or inhibitory activity of the serpin. We monitored the changes in the fluorescence emission spectra of each labeled serpin in the native and cleaved state, and in complex with active and inactive thrombin. These data show that the serpin undergoes conformational change upon forming a complex with either active or inactive proteinase. Steady-state fluorescence quenching measurements using potassium iodide were used to further probe the nature and extent of this conformational change. A pronounced conformational change is observed upon locking with an active proteinase; however, our data reveal that docking with the inactive proteinase thrombin$_{S195A}$ is also able to induce a conformational change in the serpin.

INTRODUCTION

Serpins are a unique family of proteinase inhibitors (Potempa et al., 1994) that in contrast to the standard-mechanism inhibitors, such as the small Kunitz and Kazal inhibitors (Bode and Huber, 1992), achieve inhibition of their target proteinase via a mobile reactive center loop (RCL). Although the conformation of the RCL during the inhibitory process is not known, its ability to assume different conformations is important in inhibition (Hopkins and Stone, 1995). In native serpins (Fig. 1 *a*) the RCL is poised at the top of the molecule ready to interact with the target proteinase. Upon proteolytic cleavage within the RCL region, the molecule opens up and the RCL is incorporated as the fourth strand of the large central A β-sheet (Fig. 1 *b*). This conformational change, termed the stressed to relaxed transition (S to R) is accompanied by a dramatic increase in heat stability and resistance to denaturants such as urea or guanidine hydrochloride (Mast et al., 1992). The x-ray crystal structures of two structural intermediates involved in this transition have been determined in which the RCL is partially inserted. In native antithrombin two residues are inserted into the top of the A β-sheet (Fig. 2 *a*) (Schreuder et al., 1994; Carrell et al., 1994; Skinner et al., 1997). The recently determined structure of antichymotrypsin Leu55Pro adopted an unusual conformation (termed δ), in which four residues of the RCL occupy the top half of the A β-sheet and the F-helix unwinds to occupy the bottom half of the sheet (Fig. 2 *b*) (Gooptu et al., 2000). The identification of these intermediates is critical, because it provides direct crystallographic evidence that it is possible for the serpin to adopt transitional states along the pathway of conformational change.

Lawrence and colleagues (1995) elegantly demonstrated that RCL cleavage at the scissile bond is critical for the formation of the final locked complex between serpin and proteinase. Furthermore, they proposed that the conformational change within the serpin is essential for trapping the proteinase at the acyl-enzyme step in the proteinase cleavage pathway. Several studies have shown that serpin conformational mobility and RCL insertion are crucial for efficient proteinase inhibition (Hopkins and Stone, 1995; Picard et al., 1999; Shore et al., 1995; Stratikos and Gettins, 1997, 1999; Wilczynska et al., 1997). For example, numerous mutations within the RCL have been shown to result in substrate-like behavior (for review see Stein and Carrell, 1995). It is proposed that such mutations disrupt efficient loop insertion and thus allow the proteinase to escape inhibition (Hopkins et al., 1993; Hopkins and Stone, 1995). Recent biophysical studies have played a major role in elucidating the structure of the serpin-proteinase complex (Wilczynska et al., 1997; Stratikos and Gettins, 1997, 1998, 1999), with a consensus emerging that the proteinase is translocated to the bottom of the serpin (Wright, 1996) (Fig. 2 *c*). Although it is clear that RCL insertion into the A β-sheet is a requirement for successful inhibition, the mechanism by which loop insertion is triggered remains unclear (Stone and Le Bonniec, 1997).

In this study we use a combination of site-directed mutagenesis, fluorescence labeling and fluorescence quenching

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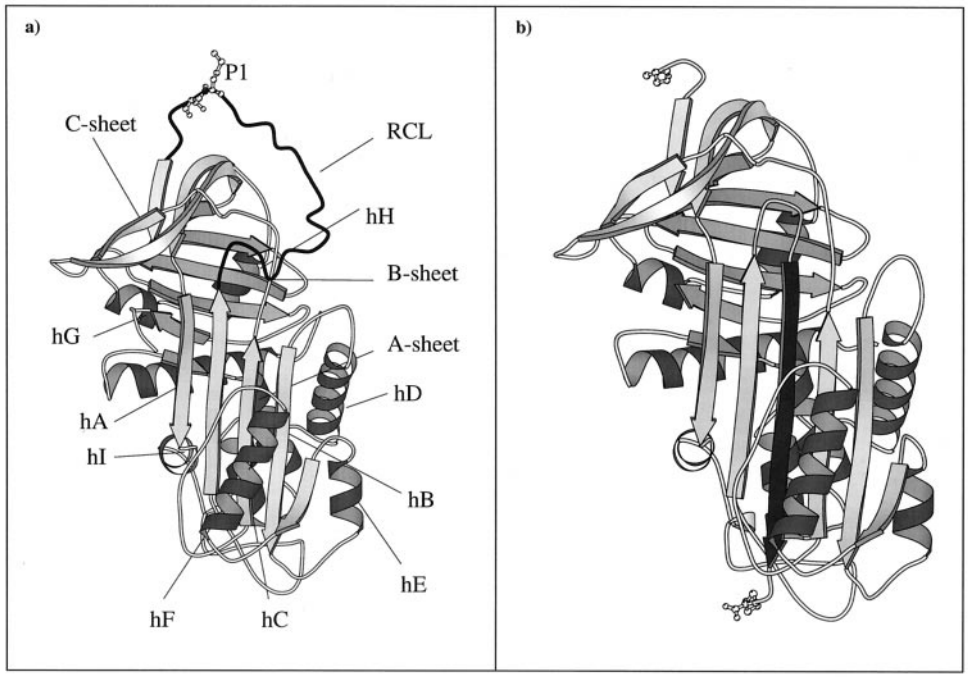

FIGURE 1 X-ray crystal structures of α_1PI. (*a*) Native α_1PI. The β-sheets and α-helices are labeled, and the RCL is shown in black at the top of the molecule.The RCL is numbered according to standard nomenclature (P_n. . . P_3-P_2-P_1-$P_{1'}$-$P_{2'}$-$P_{3'}$. . . P_n; (Schechter and Berger; 1967), P_1-$P_{1'}$ being the scissile bond cleaved by the proteinase. (*b*) Cleaved α_1PI. The RCL is buried in the center of the A β-sheet.

techniques to probe the structure of the complex further. Specifically we investigated the interaction between antitrypsin Pittsburgh (denoted α_1PI in this study) which possesses a P1 = Arg and thrombin. This serpin was used because it has high affinity for both thrombin and inactive thrombin (thrombin_{S195A}), therefore providing us with the opportunity to examine both the Michaelis-complex (E·I) and final covalent complex ($EI^{\dagger}$). Using both these thrombin forms allows us to gain insight into the structure of the initial docking complex (Cooperman et al., 1993; O'Malley et al., 1997; Stone and Le Bonniec, 1997) formed between the serpin and proteinase and then the conformational changes involved in final inhibition.

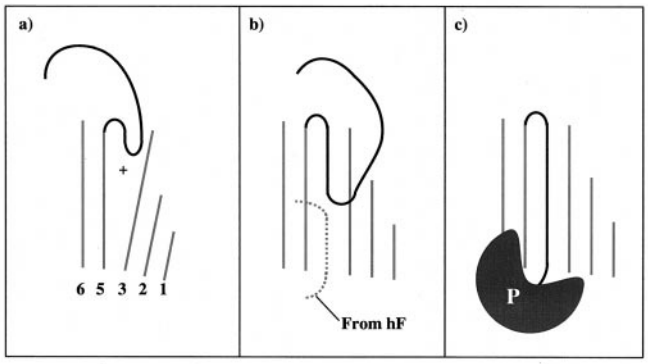

FIGURE 2 Schematic showing RCL insertion in serpins. Only the A-sheet (gray) and the RCL (black) are shown. (*a*) Native antithrombin, in which two residues (P_{15}-P_{14}) are partially inserted into the A β-sheet. A water molecule (+), located in the gap between strands s3A and s5A, forms hydrogen bonds to each of these strands. (*b*) Leu55Pro antichymotrypsin (Gooptu et al., 2000), in which four residues of the RCL (P_{15}-P_{12}) are inserted into the top half of the sheet. The bottom half of the sheet is occupied by residues that originally formed part of the F-helix (dashed line), thus fully satisfying the hydrogen bond pattern within the A β-sheet. (*c*) Full insertion with proteinase (P) attached.

MATERIALS AND METHODS

N,*N*′-dimethyl-*N*-(iodoacetyl)-*N*′-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)ethylenediamine (IANBD) were purchased from Molecular Probes (Eugene, OR). Thrombin was purified from human plasma and characterized as previously described (Stone and Hofsteenge, 1986), and $\text{thrombin}_{(S195A)}$ was purified and characterized as previously described (Le Bonniec et al., 1993; Stone and Le Bonniec, 1997).

Production of α_1 PI variants

The construction, expression, and purification of α_1PI and the two α_1PI variants Cys_{313} and $Cys_{P3'}$ has been described (James et al., 1999; Bottomley et al., 1998). The association rate constant (k_{ass}) and stoichiometry of inhibition (SI) were measured using thrombin for both the unlabeled and labeled antitrypsin forms, as described previously (Le Bonniec et al., 1995). Fluorescent labeling of the proteins with IANBD was performed as previously described (James et al., 1999). The extent of labeling was determined using the extinction coefficient of IANBD (ϵ = 25,000 M^{-1} cm^{-1}). Cleaved antitrypsin was produced by cleavage with papain and purification by ion-exchange chromatography using Q-Sepharose.

Spectroscopic methods

Fluorescence emission spectra were recorded on a Perkin-Elmer LS50B spectrofluorimeter, using a thermostatted cuvette holder at 37°C in a 1-cm-path-length quartz cell. Excitation and emission slits were set at 2.5 nm for all spectra and a scan speed of 10 nm/min was used. The absorbance at the excitation wavelengths was monitored in all experiments and remained below 0.05 units.

Steady-state fluorescence quenching

Fluorescence quenching measurements were performed in 50 mM Tris, pH 8.0, at 37°C. Aliquots of KI (2 M stock) containing 1 mM $Na_2S_2O_3$ were added to protein solutions (200 nM) and the change in fluorescence emission intensity of the covalently bound IANBD (λ_{ex} = 480 nm) was measured. All fluorescence data were corrected for sample dilution. The quenching data were analyzed by the Stern-Volmer equation as previously described by Lehrer (1971). All data were corrected for inner filter effects where necessary.

Coordinates and model building

The coordinates of wild-type native (Elliott et al., 1996) (pdb identifier 1QLP) and cleaved (Loebermann et al., 1984) (protein data bank (PDB) identifier 7API) antitrypsin, native antithrombin (Schreuder et al., 1994; Carrell et al., 1994; Skinner et al., 1997) (PDB identifier 2ANT), and thrombin (Qiu et al., 1992) (PDB identifier 1ABJ) were obtained from the protein data bank (www.rcsb.org). Previous studies by Elliott et al. (1996) have demonstrated that the RCL of antitrypsin adopts a canonical conformation (Hubbard et al., 1991) and can be docked into the active site of chymotrypsin with relatively few steric clashes. We used similar superposition and modeling techniques to that described previously (Elliott et al., 1996; Whisstock et al., 1996) to dock antitrypsin into the active site of thrombin. Briefly, the P1 methionine residue was changed to an arginine using the mutate facility in Quanta (MSI, San Diego, CA). The P1 of the proteinase inhibitor D-Phe-Pro-Arg chloromethylketone (PPACK) in the active site of thrombin was used as a template to position the P1 arginine residue of antitrypsin into the S1 subsite of thrombin. The PPACK molecule was then removed to leave a model of antitrypsin P1 = Arg docked to thrombin. Several side-chain clashes were observed between the proteinase and the body of the serpin, and these were resolved by subjecting the model to rounds of CHARMm minimization until convergence was reached. The stereochemistry of the model was checked and all residues found to be in allowed conformations.

To build E·I we used the x-ray crystal structure of native antithrombin as a template in the program MODELLER (Sali and Blundell, 1993) to construct a model of antitrypsin in which the RCL is partially inserted to P14. The RCL of antithrombin is three residues longer that that of antitrypsin and in a noncanonical conformation. To maintain a canonical loop in our model of partially inserted antitrypsin, the RCL was rebuilt, using the structure of native antitrypsin as a template. A similar superposition procedure to that previously described was used to generate a model between thrombin and antitrypsin Pittsburgh in which the RCL is partially inserted to P14. We observed few steric clashes between the proteinase and inhibitor, and these were resolved by rounds of CHARMm minimization. The stereochemistry of the model of E·I was checked and all residues were in allowed conformations

RESULTS

Previous kinetic studies have identified a number of intermediates involved in the serpin-proteinase inhibitory pathway (Fig. 3) (O'Malley et al., 1997; Stone et al., 1997; Stone and Le Bonniec, 1997). The aim of this study was to determine the structural changes of α_1PI as it passes through this inhibitory pathway. To achieve this goal, we have characterized four different conformers of α_1PI–native (I), noncovalent complex (E·I; formed with thrombin$_{S195A}$), covalent complex (EI†; formed with active thrombin), and cleaved (I*) (Fig. 3). Each of the four conformers was formed using three α_1PI variants, which were specifically labeled at different sites with an environmentally sensitive fluorophore (IANBD). The three Pittsburgh variants of α_1PI used in the study contained a unique single cysteine residue (see Fig. 6) positioned either at the top (α_1PI-Cys$_{P3'}$), bottom (α_1PI-Cys$_{313}$), or rear (α_1PI-Cys$_{232}$) of the molecule. Cys$_{232}$, the native cysteine residue of α_1PI, was mutated to a serine in both α_1PI-Cys$_{P3'}$ and α_1PI-Cys$_{313}$, and this was shown to have no effect upon the inhibitory properties of the proteins (James et al., 1999). Each of the three variants were expressed, purified, and covalently modified with IANBD as previously described (James et al., 1999). The labeling stoichiometry was 1:1 in all cases, and kinetic experiments demonstrated that the IANBD labeling has no effect upon the k_{ass} and SI with thrombin. In addition, SDS-PAGE analysis of the complex formed between the variants and thrombin at a 1:1 ratio demonstrated the presence of a single complex band (Fig. 4). Furthermore, far-UV circular dichroism and tryptophan emission spectra of all the labeled and unlabeled proteins were identical, suggesting that mutagenesis and IANBD labeling did not alter the structure of the serpin (data not shown). Indeed, previously we have shown that the IANBD modification does not affect the unfolding and refolding pathways of the variants (James et al., 1999). Taken together, these biochemical and biophysical data strongly suggest that the presence of the IANBD moiety had a negligible effect on the structural and inhibitory properties of the proteins.

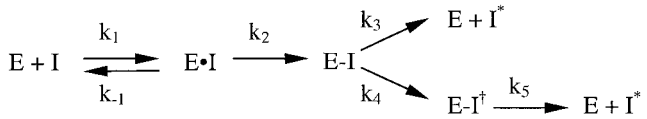

FIGURE 3 Kinetic scheme summarizing serpin-proteinase complex formation. E, proteinase; I, serpin; E·I, the noncovalent Michaelis complex; E-I, acyl enzyme intermediate before partitioning; E-I† covalent complex; I*, cleaved serpin. This scheme uses the nomenclature described by Stratikos and Gettins (1999).

The presence of the fluorescence probe IANBD provides the protein with new fluorescence properties (Fig. 5, Table 1). In the native state the emission maximum (λ_{max}) relates the position of IANBD within the protein. The labeled wild-type protein, α_1PI-Cys$_{232}$, has an emission maximum at 531 nm, whereas α_1PI-Cys$_{313}$ and α_1PI-Cys$_{P3'}$ were at 529 nm and 532 nm, respectively. These data indicate that the label on α_1PI-Cys$_{313}$ is more protected from the solvent than the corresponding label on α_1PI-Cys$_{232}$ and that the

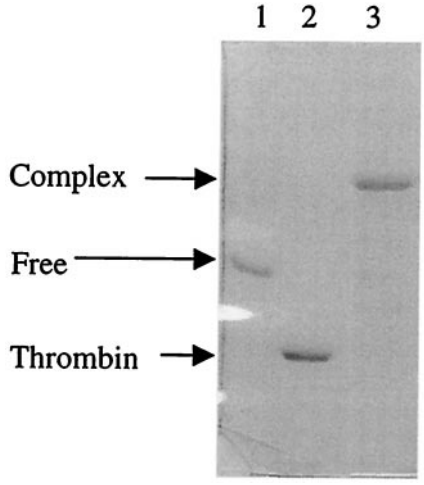

FIGURE 4 SDS-PAGE of reaction between α_1PI- Cys_{313} and thrombin. α_1PI- Cys_{313} and thrombin at 1:1 ratio were incubated at 37°C for 30 min and then analyzed by 10% SDS-PAGE. Lane 1, free α_1PI- Cys_{313}; lane 2, thrombin; lane 3, the complex between α_1PI- Cys_{313} and thrombin.

label attached to α_1PI-$Cys_{P3'}$ is highly solvent exposed on the RCL. The solvent environment of the probe is reflected in its λ_{max}, and so it is a useful indicator of structural change. Fig. 5 shows the emission spectra of all three proteins in four different conformations. The cleaved state of the protein was made by incubation of the serpin with papain. The cleaved serpin was then isolated by ion-exchange chromatography. The λ_{max} of the cleaved states shows a number of changes in comparison with the native state. The IANBD on α_1PI-$Cys_{P3'}$ and α_1PI-Cys_{232} showed no change upon cleavage, whereas a large change in λ_{max} was observed for the α_1PI-Cys_{313} in which the λ_{max} was blue shifted by 6 nm compared with the native state. All of these changes are consistent with the movement of a fragment of α_1PI caused by the insertion of the RCL into the A β-sheet as described recently (Whisstock et al., 2000). Upon formation of the noncovalent complex (E·I) with $thrombin_{S195A}$ different changes in the λ_{max} of the IANBD label were observed. As expected from the position of Cys_{232} at the back of the molecule α_1PI-Cys_{232} showed no change in λ_{max} with $thrombin_{S195A}$. However, α_1PI-Cys_{313} displayed a shift in λ_{max} of 4 nm, indicating that upon formation of E·I the region at the base of the molecule undergoes a conformational change, similar to that seen in the cleaved state. α_1PI-$Cys_{P3'}$ also displayed a change in λ_{max}, a blue shift of 2 nm, indicating that the probe became less solvent exposed upon formation of E·I. Formation of the final serpin-proteinase complex indicated further conformational change within the serpin. The λ_{max} of α_1PI-$Cys_{P3'}$ and α_1PI-Cys_{232} in complex with thrombin were identical to that of the cleaved and native states. Thrombin in complex with α_1PI-Cys_{313} exhibited a λ_{max} of 531 identical to that observed with $thrombin_{S195A}$ but not as high as that seen in the cleaved state (λ_{max} = 533 nm).

Another sensitive fluorescent approach that can reveal much about conformational change and protein-protein interactions is fluorescence quenching (Eftink and Ghiron, 1981). Both iodide and cesium were used as quenching agents to examine the structural transitions of the three variants during proteinase inhibition. Cesium, which is positively charged, was unable to quench the fluorescence of the IANBD moiety of all three variants in any form (data not shown). Iodide, however, was found to be an efficient quencher of the IANBD fluorescence. Fig. 5 shows a Stern-Volmer plot for each variant in each conformation (Lehrer, 1971), and from these plots the Stern-Volmer (K_{sv}) quenching constant was determined (Table 1). The iodide quenching yielded linear Stern-Volmer plots (Fig. 5), which implies that the fluorescence quenching takes place on a simple collisional basis. The K_{sv} for the native α_1PI-$Cys_{P3'}$ was 5.8 M^{-1}, whereas the cleaved and complex with thrombin forms were similar (7.0 M^{-1}), indicating increased accessibility of the IANBD probe in these states compared with native. In the presence of $thrombin_{S195A}$, however, the K_{sv} is significantly decreased to 4.7 M^{-1}. These data demonstrate that the IANBD label in the E·I form is less accessible either due to a conformational change or because the inactive proteinase is preventing access for the quenching agent. There are only minor changes in K_{sv} seen for the α_1PI-Cys_{232} conformations, suggesting that this region of the protein does not undergo any conformational change or that the active or inactive proteinase does not come into close proximity. α_1PI-Cys_{313} shows changes in K_{sv} upon both complex formation and RCL cleavage. The conformational states of E·I, $EI^{\dagger}$, or I* all exhibited similar K_{sv} values of approximately 8.8 M^{-1}, significantly higher than the native value of 7.4 M^{-1}. These data indicate that the region around Cys_{313} undergoes a conformational change upon formation of E·I and $EI^{\dagger}$ that is similar to that seen upon cleavage of the RCL.

DISCUSSION

The minimal kinetic scheme presented in Fig. 3 illustrates the complexity of the serpin inhibitory pathway. Using the combination of α_1PI with both active and inactive proteinase, we have been able to form specific conformations along the pathway for study. The conformations of intact α_1PI (I) and RCL cleaved α_1PI (I*) have been crystallographically characterized (Fig. 1) and are easily studied. The I and I* states represent the extremes of RCL insertion, i.e., no insertion (I) and full insertion (I*). The final covalent complex ($EI^{\dagger}$) is formed between α_1PI and active thrombin, and the initial Michaelis complex (E·I) is formed with $thrombin_{S195A}$. In this study we have used a combination of protein engineering and fluorescence spectroscopic techniques to examine the structure of α_1PI in these four states.

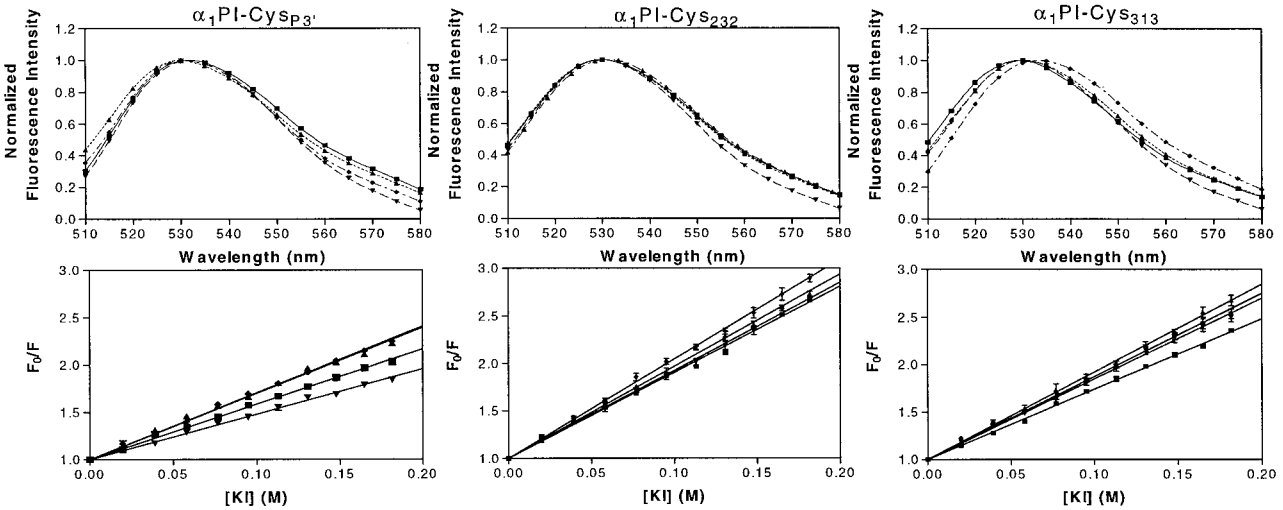

FIGURE 5 Fluorescence emission spectra and Stern-Volmer quenching of α_1PI-labeled variants. The fluorescence emission spectra (λ_{ex} = 480 nm) of the α_1PI variants are shown in the native (■), cleaved (♦), noncovalent complex with thrombin$_{(S195A)}$ (▼), and thrombin (▲). The Stern-Volmer plots for the same samples are also shown (same symbols as above). Each point is an average of five data points and from three different protein preparations. The data were analyzed by the Stern-Volmer equation (Lehrer, 1971).

Analysis of the native and cleaved states of α_1PI

The fluorescence approach used here shows that upon the S to R transformation a considerable conformational change takes place. There is no structural change around Cys_{232}, and this is supported by structural analysis of both I and I*. Cys_{232} is located on the B β-sheet, a region not associated with any structural reorganization during formation of the stable complex. Indeed, a structural comparison between native and cleaved antitrypsin revealed that this region forms part of the rigid scaffold upon which most serpin conformational changes occur (Stein and Chothia, 1991; James et al., 1999; Whisstock et al., 2000). The Cys residue of α_1PI-$Cys_{P3'}$, situated at the C-terminal end of the RCL, shows a small blue shift (2 nm) in λ_{max} upon RCL cleavage and an increase in K_{sv}, indicating increased accessibility to the quenching agent iodide. The most significant changes observed, however, were around Cys_{313}, which is situated at the base of the serpin molecule on a loop connecting strands 5A and 6A of the A β-sheet. Upon RCL insertion there was a large red shift in the λ_{max} (6 nm) of α_1PI-Cys_{313} and an increase in K_{sv}. These data suggest significant movement in this region that increases the exposure of the IANBD label to solvent and subsequently enhances its accessibility to the iodide quenching agent. A structural comparison between native and cleaved α_1PI revealed that the loop containing residue 313 is part of a rigid fragment that shifts significantly during the S to R transition (Whisstock et al., 2000). Thus, the fluorescent changes we observe between the native and cleaved form of the α_1PI-Cys_{313} are entirely consistent with RCL insertion and the S to R transition.

Analysis of the conformation of E·I

The E·I state, formed between α_1PI and thrombin$_{S195A}$, is equivalent to the Michaelis complex. Previously it has been shown that the affinity between the two was high enough ($K_I = 3 \times 10^{-6}$ M) (Stone and Le Bonniec, 1997) to allow formation of the complex and its study. The conformational changes involved in forming E·I have generally been inferred from kinetic studies (Stone and Le Bonniec, 1997). However, work by Gettins and co-workers using α_1PI and anhydrotrypsin suggested that no significant conformational change occurs upon formation of the noncovalent complex (Stratikos and Gettins, 1998, 1999). In this study we found

TABLE 1 λ_{max} and iodide quenching parameters for the IANBD-labeled α_1PI in four different conformations

	α_1PI-$Cys_{P3'}$		α_1PI-Cys_{232}		α_1PI-Cys_{313}	
Species	λ_{max} (nm)	K_{SV} (M^{-1})	λ_{max} (nm)	K_{SV} (M^{-1})	λ_{max} (nm)	K_{SV} (M^{-1})
Native (I)	532	5.8 ± 0.06	531	9.0 ± 0.08	527	7.4 ± 0.04
Plus thrombin$_{S195A}$ (E·I)	530	4.7 ± 0.07	531	9.2 ± 0.08	531	8.5 ± 0.09
Plus thrombin ($EI^{\dagger}$)	532	7.0 ± 0.06	531	9.7 ± 0.08	531	9.2 ± 0.08
Cleaved (I*)	532	7.0 ± 0.06	532	10.4 ± 0.09	533	8.8 ± 0.09

Each value is the average of seven separate experiments.

that the fluorescence of the IANBD label at $Cys_{P3'}$ was altered due to formation of E·I. This was demonstrated by the blue-shifted λ_{max} and the decreased accessibility of the quenching agent. The changes in K_{sv} must be due to the proximity of the inactive proteinase, as similar changes are not seen in the cleaved state. A significant difference between the previous (Stratikos and Gettins, 1999) and our present study is that we observed a conformational change around Cys_{313} upon E·I formation. This manifested itself in a 4-nm red shift in λ_{max} and an increase in K_{sv}, indicative of its increased solvent exposure. In contrast to the changes observed with α_1PI-$Cys_{P3'}$, these changes are due to a conformational change in the serpin as they are similar to those observed in the cleaved state. Therefore the docking of E to I, leading to the formation of E·I, results in insertion of the RCL into the A β-sheet, which results in movement at the base of the serpin. Additional evidence for this change comes from a recent structural comparison between intact and cleaved α_1PI, which showed that this region at the base of the molecule, which includes Cys_{313}, is required to move upon RCL insertion (Whisstock et al., 2000). These data confirm the kinetic data produced earlier that suggested that proteinase docking was enough to trigger RCL insertion (Stone and Le Bonniec, 1997).

What is the extent of RCL insertion in E·I? The structures of native antithrombin and the δ conformation of Leu55Pro antichymotrypsin reveal that the serpin structure is able to adopt at least two different partially inserted conformations (Fig. 2, *a* and *b*) (Schreuder et al., 1994; Carrell et al., 1994; Skinner et al., 1997; Gooptu et al., 2000). Previous modeling studies have shown that RCL insertion in antitrypsin beyond P14 without cleavage is not sterically feasible unless the first strand of the C-sheet (s1C) is released (Whisstock et al., 1996). This prediction is supported by the structure of δ antichymotrypsin in which s1C is distorted to allow RCL insertion to P_{12} (Gooptu et al., 2000). The antichymotrypsin RCL is four residues longer than that of α_1PI, suggesting that extensive distortion of s1C would be required for RCL insertion to P_{12} in α_1PI. A superposition of native α_1PI and native antithrombin (in which the RCL is inserted to P_{14}) reveals that a substantial conformational change accompanies the insertion of just two residues (Whisstock et al., 1996). Furthermore, these data reveal a shift in the rigid fragment containing residue 313. We therefore predict that the initial encounter complex formation between α_1PI and $thrombin_{S195A}$ (Fig. 6 *a*) triggers RCL insertion to P_{14} to form a stable noncovalent complex (Fig. 5 *b*). We cannot, however, exclude the possibility of further RCL insertion in the noncovalent complex with the concurrent release of s1C.

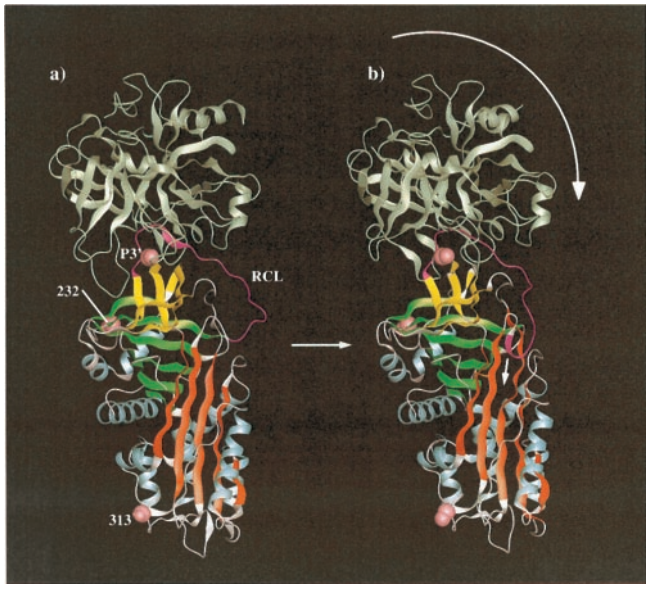

FIGURE 6 Molecular model of the initial docking complex (*a*) and noncovalent Michaelis complex E·I (*b*). The A β-sheet is in red, the B β-sheet in green, and the C β-sheet in yellow. The RCL is labeled and in magenta. Residues 232, 313, and P3′ are represented by van der Waal spheres. The proteinase is at the top of the figure in light green. We predict that initial docking (*a*) is followed by RCL insertion to P14 (*b*).

Analysis of EI†

Our data describe a covalent complex in which the proteinase has moved from the top of the serpin to some position on the A β-sheet of the molecule. Our data are not sufficient to place the proteinase in a specific position, although we are able to narrow down its location with respect to the serpin. The λ_{max} of α_1PI-$Cys_{P3'}$ in complex with thrombin is similar to the native state, suggesting a similar solvent environment for the probe in both states (i.e., not covered by the proteinase in the complex). The K_{sv} value of the EI† form is the same as the cleaved state, suggesting that the C-terminal residues of the scissile bond are in a similar environment. Therefore, the proteinase has moved significantly from its initial docking position. Our data with the α_1PI-Cys_{313} indicate that thrombin is not situated directly over this residue at the base of the serpin. This is due to the increased solvent exposure of the label on α_1PI-Cys_{313} in the presence of thrombin. This is in contrast to recent results from experiments that placed a probe at Cys_{314} and found it covered by trypsin. Trypsin is a much smaller proteinase than thrombin and this may go some way toward explaining the difference (Stratikos and Gettins, 1999).

In conclusion, the data presented here using α_1PI and $thrombin_{S195A}$ clearly demonstrate that proteinase docking is enough to trigger RCL insertion into the A β-sheet, which has allowed us to present a model of the initial docking complex. However, although we are not able to precisely map the position of the proteinase in the final covalent complex it is clear that it has significantly moved from its initial position at the top of the serpin.

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