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# Mapping the Serpin-Proteinase Complex Using Single Cysteine Variants of $\alpha_1$-Proteinase Inhibitor Pittsburgh*

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**To probe the covalent serpin-proteinase complex, we used wild-type and 4 new single cysteine variants (T85C, S121C, D159C, and D298C) of $\alpha_1$-proteinase inhibitor Pittsburgh. Cysteines in each variant could be labeled both in native and proteinase-complexed $\alpha_1$-proteinase inhibitors. Pre-reaction with 7-nitrobenz-2-oxa-1,3-diazole-chloride or fluorescein prevented complex formation only with the D298C variant. Label at $Cys^{121}$ greatly increased the stoichiometry of inhibition for thrombin and gave an emission spectrum that discriminated between native, cleaved, and proteinase-complexed serpin and between complexes with trypsin and thrombin, whereas fluorophore at residue 159 on helix F was almost insensitive to complex formation. Fluorescence resonance energy transfer measurements for covalent and non-covalent complexes were consistent with a location of the proteinase at the end of the serpin distal from the original location of the reactive center loop. Taken together, these findings are consistent with a serpin-proteinase complex in which the reactive center loop is fully inserted into β-sheet A, and the proteinase is at the far end of the serpin from its initial site of docking with the reactive center loop close to, but not obscuring, residue 121.**

Serpins are a family of widely distributed, structurally homologous proteins (1), many of which are inhibitors of serine proteinases (2). Whereas the many other families of protein inhibitors of serine proteinases, such as the Bowman-Birk, Kazal, and Kunitz families, inhibit target proteinases by forming tight non-covalent 1:1 complexes in which neither the proteinase nor the inhibitor undergoes significant structural change in most cases (3), serpins differ not only by apparently forming covalent 1:1 acyl enzyme complexes with their target proteinases (4), but by undergoing a major conformational change during, and as an essential part of, the inhibition process (5). Because of the requirement for conformational change as part of the inhibition mechanism, knowledge of the structure of the serpin-proteinase complex is critical for an understanding of how serpins inhibit their target proteinases through kinetic trapping of a normal covalent acyl enzyme intermediate on the proteinase substrate cleavage pathway.

A previous proposal that a major movement of the proteinase occurs following cleavage of the scissile bond (6) has been supported by two recent studies (7, 8). In one study (8) chemical cross-linking between the proteinase and the serpin in the complex, together with a measurement of the separation between P3 and P1′ residues of the serpin in the complex by fluorescence resonance energy transfer, was consistent with a location of the proteinase half-way down the flank of the serpin (Fig. 1) and in contact with helix F. The other study (7), from this laboratory, used fluorescence resonance energy transfer between fluorophores on the serpin $\alpha_1$-proteinase inhibitor ($\alpha_1$PI)[1] Pittsburgh and the proteinase to compare the inter-fluorophore separation in the normal covalent serpin-proteinase complex with that in the non-covalent complex with the non-functional anhydroproteinase. This study, although not able to precisely define the position of the proteinase in the complex, demonstrated a movement of the proteinase of at least 21 Å upon formation of the kinetically trapped covalent complex.

We describe here more extensive mapping of this serpin-proteinase complex by using wild-type $\alpha_1$PI Pittsburgh and 4 new single cysteine variants. These well separated cysteines were used as follows: (i) to probe the accessibility of the cysteine in native and proteinase-complexed serpin, (ii) to determine the effect of derivatization of the cysteine on the ability to form covalent complex, and (iii) for introduction of fluorophores, both as probes of the local environment and for fluorescence resonance energy transfer measurements. By these approaches we have been able to place further constraints on the possible structures of the serpin-proteinase complex and to show that it probably requires movement of the proteinase to the bottom of the serpin and therefore full insertion of the cleaved reactive center loop into β-sheet A. In this location the proteinase is not in contact with the outer face of helix F. Our findings are thus consistent with the model of Wright and Scarsdale (6).

## MATERIALS AND METHODS

*Site-directed Mutagenesis*—Site-directed mutagenesis was carried out on a double-stranded pET16b plasmid (Novagen) containing $\alpha_1$PI cDNA, using the Quikchange method (Stratagene). Double-stranded template DNA of two complementary primers containing the mutation was annealed and extended with *Pfu* DNA polymerase during thermal cycling. The pET16b plasmid contained an N-terminally modified $\alpha_1$PI cDNA that lacked coding sequence for the first 5 residues (10), inserted between the *Nco*I and *Bam*HI subcloning sites of the vector. The sequences for the coding strands of the mismatch primers were as follows (mismatch codons are underlined): M358R, 5′-GAG GCC ATA CCC

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[1] The abbreviations used are: $\alpha_1$PI, $\alpha_1$-proteinase inhibitor; 5-IAF, 5-iodoacetamidofluorescein; NBD, 7-nitrobenz-2-oxa-1,3-diazole; SI, stoichiometry of inhibition, defined as the number of moles of serpin required to inhibit 1 mol of proteinase by formation of SDS-stable complex; TLCK, tosyl-lysyl chloromethyl ketone; PAGE, polyacrylamide gel electrophoresis; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol.

AGA TCT ATC CCC CCC; C232S, 5′-AAC ATC CAG CAC AGC AAG AAG CTG TCC AG; T85C, G AAT TTC AAC CTC TGT GAG ATT CCG GAG G; S121C, GGC CTG TTC CTC TGT GAG GGC CTG AAG; D159C, G AAA CAG ATC AAC TGT TAC GTG GAG AAG GG; D298C, AC TGG AAC CTA TTG TCT GAA GAG CGT CCT G. All $\alpha_1$PI variants used in this study carried the Pittsburgh (M358R) mutation, and all except the wild-type Pittsburgh variant carried the C232S mutation, so that all variants contained only one free cysteine residue and all contained a P1 arginine (Pittsburgh mutation (11–13)) that conferred high affinity (5 nM) for the non-covalent complexes with anhydrotrypsin (7). All mutations were confirmed by dideoxy sequencing in the host plasmid.

*Expression and Refolding of Recombinant $\alpha_1$PI Variants*—The plasmids containing the mutated $\alpha_1$PI genes were transformed into BL21 (DE3) cells (Novagen). 5 ml of an overnight culture was used to inoculate 0.5 liters of LB broth containing 125 mg/ml ampicillin. Cells were grown at 37 °C in a shaking water bath to an $A_{600\ nm}$ of 0.5. IPTG (0.4 mM) was added to induce expression of $\alpha_1$PI, and the cells were allowed to grow for a further 2.5–3 h at 37 °C. Cells were harvested by centrifugation at 4000 × *g* for 10 min, and the cell pellet was collected and washed twice with cold PBS. The cell pellet was resuspended in 10 ml of buffer A (50 mM Tris, pH 8.0, 50 mM NaCl, and 1 mM EDTA) containing 1 mM phenylmethylsulfonyl fluoride, 1 mM β-mercaptoethanol, and 0.2 mg/ml lysozyme. The suspension was sonicated using four 30-s pulses. Inclusion bodies were harvested by spinning the lysed cells at 10,000 × *g* for 45 min. The pellet containing the inclusion bodies was washed twice with buffer A containing 0.5% Triton X-100 (Sigma). The inclusion bodies were finally dissolved in 10 ml of buffer A containing 8 M guanidinium hydrochloride by vortexing and sonication. Any non-solubilized inclusion bodies were spun down at 10,000 × *g* for 15 min. The typical yield of solubilized protein at this stage was ~20 mg, determined by BCA assay, from 0.5-liter cell culture. Solubilized denatured $\alpha_1$PI was refolded by dropwise dilution over a period of 20 min of the 8 M guanidinium hydrochloride solution into 250 ml of a 4 °C solution of 10 mM sodium phosphate buffer, pH 6.5, containing 1 mM EDTA and 1 mM dithiothreitol. The diluted solution was extensively dialyzed against 10 mM sodium phosphate buffer, pH 6.5, containing 1 mM EDTA to remove the guanidinium hydrochloride. After dialysis the solution was centrifuged for 30 min at 4 °C and 8000 × *g* and filtered through a 0.2-μm filter to remove any precipitated protein.

*Purification of Monomeric $\alpha_1$PI*—The refolded $\alpha_1$PI contained dimers and higher order oligomers in addition to monomers. Monomeric $\alpha_1$PI was purified in two steps. The first step was ion exchange chromatography on DE52 (Whatman), using a linear 0–0.25 M NaCl gradient. This gave a sharp early eluting peak for the monomer and a broader later eluting peak for higher order aggregates. The pooled monomeric fractions were dialyzed against 20 mM Bis-Tris-propane buffer, pH 6.5, containing 1 mM EDTA and rechromatographed, where necessary, on a MonoQ column equilibrated in the same buffer and eluted by a 0–0.3 M NaCl gradient. Monomeric $\alpha_1$PI eluted as a very sharp single peak. The purity of all preparations was confirmed by SDS-PAGE and by PAGE under non-denaturing conditions to confirm that all material was monomeric. Preparations were dialyzed against 20 mM sodium phosphate, pH 7.4, 100 mM NaCl, 0.1 mM EDTA, 0.1% PEG8000, quickly frozen in aliquots, and stored at −70 °C until needed.

*Preparation of Anhydrotrypsin and β-Trypsin*—Anhydrotrypsin was prepared from commercial crystallized trypsin (Sigma) by alkaline β-elimination of the phenylmethylsulfonyl fluoride adduct according to published procedures (14). Following the reaction, the solution was treated with Phe-Phe-Arg-chloromethyl ketone (20 μM) to inhibit any remaining or regenerated active trypsin and acidified to pH 3.0. β-Anhydrotrypsin was purified from the reaction mixture by chromatography on a soybean trypsin inhibitor affinity matrix. The absence of proteolytic activity in the product was confirmed by activity assay using the chromogenic trypsin substrate S-2222. β-Trypsin was prepared from TPCK-treated commercial trypsin by affinity chromatography using the same soybean trypsin inhibitor affinity matrix.

*Preparative Labeling of $\alpha_1$PI with Fluorescein or NBD*—All $\alpha_1$PI variants were labeled with fluorescein by reaction of the single free cysteine with 5 iodoacetamido-fluorescein (Molecular Probes, Eugene, OR). The protein (10–40 μM) was reacted with a 2-fold molar excess of dithiothreitol for 15 min at room temperature and then with a 10–15-fold molar excess of 5-IAF. The reaction was allowed to proceed overnight at 4 °C. Excess reagent was removed by dialysis for 24 h against 10,000 volumes of 20 mM sodium phosphate, pH 7.4, 100 mM NaCl, 0.1 mM EDTA, 0.1% PEG8000. The extent of labeling was determined spectrophotometrically using the absorbance at 495 nm for determination of the fluorescein concentration and the absorbance at 280 nm, corrected for the contribution from fluorescein at this wavelength, which was determined to be 25% of the absorbance at 495 nm based on the spectrum of the adduct of IAF with β-mercaptoethanol, to determine the protein concentration. For all preparations the extent of labeling was close to 1 eq per mol or less, with a range from 0.59 to 1.06. This range represents the determined stoichiometries for preparations made at different times under somewhat different reactant concentrations and does not necessarily reflect intrinsic differences in reactivity of the various cysteines. Where comparisons of labeling efficiency are made elsewhere, reactions were carried out under identical conditions for each $\alpha_1$PI species. Extinction coefficients of 27,000 (15) and 82,000 $M^{-1}$ $cm^{-1}$ were used for $\alpha_1$PI and fluorescein, respectively.

$\alpha_1$PI variants were labeled with NBD by reaction with NBD-chloride. $\alpha_1$PI, 10–20 μM, in 20 mM sodium phosphate buffer, pH 7.4, containing 100 mM NaCl, 0.1 mM EDTA, and 0.1% PEG8000, was first reduced by addition of dithiothreitol in slight molar excess and incubation at room temperature for 15 min. A 10-fold excess of NBD-chloride was added and the reaction allowed to proceed overnight at 4 °C in the dark. The sample was then dialyzed against the same buffer for 24 h at 4 °C. The extent of labeling was calculated spectrophotometrically using the extinction coefficient of NBD at 420 nm of 13,000 $M^{-1}$ $cm^{-1}$ and an extinction coefficient for the protein at 280 nm of 27,000 $M^{-1}$ $cm^{-1}$. The contribution of NBD at 280 nm is small. Labeling stoichiometries of 0.8–1.0 were obtained.

*Preparative Labeling of β-Trypsin and β-Anhydrotrypsin with Tetramethylrhodamine Isothiocyanate*—β-Trypsin and β-anhydrotrypsin were labeled with tetramethylrhodamine isothiocyanate while immobilized on soybean trypsin inhibitor-agarose beads (i) to permit equivalent reaction conditions for anhydrotrypsin as for trypsin without concern for autodigestion by free trypsin, and (ii) to provide a ready means of selecting only those labeled proteins that were still active in binding to protein inhibitors. About 300 μl of wet soybean trypsin inhibitor-agarose beads were equilibrated with 0.1 M sodium citrate buffer, pH 4.0, and then mixed with 700 μl of either β-trypsin or β-anhydrotrypsin, followed by gentle rotation for 30 min at 4 °C. The beads were washed twice with 700 μl of 0.1 M citrate buffer, pH 4.0, to remove any unbound protein and then four times with 700 μl of 0.1 M sodium carbonate, pH 9.0, to raise the pH. The beads were resuspended in 700 μl of 0.1 M sodium carbonate, pH 9.0, and 10 μl of a 10 mM solution of tetramethylrhodamine isothiocyanate in *N*,*N*-dimethylformamide were added. The reaction was allowed to proceed, with gentle rocking, at room temperature for 2–4 h, depending on the degree of labeling wanted. The beads were then washed four times with 700 μl of 10 mM sodium carbonate, pH 9.0, to remove excess reagent and any free protein. Labeled protein was eluted with 500 μl of 0.2 M sodium citrate, pH 2.4. The eluate was dialyzed overnight against 1000 volume of 1 mM HCl containing 10 mM $CaCl_2$ and centrifuged at 14,000 × *g* for 10 min to remove any precipitated material. The extent of labeling was determined spectrophotometrically using extinction coefficients of 62,000 $M^{-1}$ $cm^{-1}$ at 550 nm and 35,800 $M^{-1}$ $cm^{-1}$ at 280 nm for tetramethylrhodamine and trypsin, respectively. The absorbance at 280 nm was first corrected for the contribution at that wavelength from tetramethylrhodamine, which was found empirically to be 28% of the absorbance at 550 nm. The extent of label incorporation was 0.47 mol/mol for trypsin and, for two separate preparations of anhydrotrypsin, 0.30 and 0.71 mol/mol.

*Characterization of the Sites of Labeling on β-Trypsin*—The sites of labeling in β-trypsin were identified by N-terminal sequencing of tetramethylrhodamine-labeled peptides isolated from a tryptic digest of the labeled protein. 140 μg of labeled β-trypsin was freeze-dried and dissolved in 50 μl of 8 M guanidinium hydrochoride, 50 mM Tris, pH 8.0, 50 mM NaCl, 1 mM EDTA. Dithiothreitol was added to 10 mM and the mixture incubated at room temperature for 30 min. Iodoacetic acid was added to 30 mM and allowed to react for 30 min at room temperature. The denatured labeled trypsin was diluted into 600 μl of 50 mM Tris, pH 8.0, 50 mM NaCl, 1 mM EDTA, 10 mM $CaCl_2$ containing 5 μg of active β-trypsin and digested in the dark at 37 °C for 2 h, after which an additional 5 μg of β-trypsin was added. A second addition was made after a further 2 h, and the reaction was allowed to continue overnight. The tryptic digest was chromatographed on a C-18 reverse phase column, using a linear gradient from 80% buffer A (0.1% trifluoroacetic acid in water), 20% buffer B (0.1% trifluoroacetic acid, 90% acetonitrile, 9.9% water) to 35% buffer A, 65% buffer B. Peaks were monitored both for absorption at 280 nm and fluorescence at 550 nm. Two major peaks of approximately equal fluorescent intensity were obtained, as well as several much smaller ones, and were submitted for N-terminal sequence determination.

*Assay for Ability of Labeled or Unlabeled $\alpha_1$PI Variants to Form Covalent Complex*—The ability of the $\alpha_1$PI variants to form SDS-stable

covalent complex with trypsin was assayed by 10% SDS-PAGE of the reaction products and visualization of the complex either by Coomassie staining for unlabeled complex or by fluorescence intensity for labeled complex. Typically 2–3 $\mu$g of $\alpha_1$PI was reacted for 10 s with trypsin at different molar ratios, ranging from 0.3:1 to 2:1 trypsin:$\alpha_1$PI, with $\alpha_1$PI fixed at 5–10 $\mu$M. This was sufficient time for the reaction to have gone to >99% completion based on the published rate constant for this reaction (7) and confirmed empirically by the absence of unreacted serpin in lanes where the proteinase was in excess. This also confirmed that the serpin was >95% active.

*Calculation of Stoichiometry of Inhibition*—The stoichiometry of inhibition (SI) was calculated by scanning densitometry of SDS-PAGE gels. Coomassie Blue-stained gels were scanned, and the density 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. This was justified by a standard curve for trypsin and $\alpha_1$PI which showed comparable staining for both proteins on a weight basis and a linear dependence between amount of protein and band intensity in the range used for the experiments. This method was considered accurate for SI values in the range 1.1 to 5, corresponding to 91 to 20% complex, but incapable of determining SI where complex bands were so faint as to be not visible. SI values for fluorescein-labeled serpins were determined in an analogous way, except intensity of the fluorescent bands corresponding to cleaved and complexed bands were used, and no correction was needed for contribution from (unlabeled) trypsin. No error was thereby introduced for complex formed by unlabeled serpin. In cases where the degree of fluorescein labeling was close to 100%, so that all covalent complex was also fluorescent, or where labeling did not affect complex formation, independent quantitation of SI by both fluorescence and Coomassie Blue staining gave good agreement.

*Ability to Label Cysteines in Covalent Complex*—5–6 $\mu$g of either the Pittsburgh variant of $\alpha_1$PI or the cysteine mutants (T85C, D159C, S121C and D298C) in a total volume of 20 $\mu$l were reacted with 1 $\mu$g of $\beta$-trypsin (excess of $\alpha_1$PI to ensure that complex was not degraded by excess proteinase) to form the stable trypsin-$\alpha_1$PI complex. TLCK was added after a few seconds to a final concentration of 25 $\mu$M. In all reaction mixtures 1.5 $\mu$l of 1.8 mM IAF was added (final concentration was about 100 $\mu$M), and the reaction was allowed to proceed for 2 h at 4 °C. Dithiothreitol was added to a final concentration of 1 mM, and the mixture was incubated at room temperature for 10 min (to inactivate any unreacted probe). The samples were then subjected to SDS-PAGE analysis (12% acrylamide). A control reaction of IAF with TLCK-treated trypsin alone was carried out and showed no labeling of trypsin under the conditions used.

Similar reactions were also carried out with thrombin as the proteinase, using comparable conditions as for the reactions with $\beta$-trypsin except that reaction was carried out for 8 min at room temperature (sufficient for complete reaction of the thrombin), and Phe-Pro-Arg-chloromethyl ketone was added to inactivate any free thrombin.

*Fluorescence Measurements*—All fluorescence measurements were made on a SPEX fluorolog scanning fluorimeter. NBD spectra were acquired by exciting at 420 nm and scanning from 440 to 580 nm. Fluorescein and rhodamine spectra were recorded by exciting at 340 nm and scanning from 460 to 640 nm. All slit widths were 4 nm. Measurements were made at 25 °C. For time courses the emission signal was monitored at 515 nm, where the contribution of rhodamine fluorescence is negligible. For energy transfer measurements the labeled serpin was between 50 and 150 nM, and the proteinase was at 2–3 times the serpin concentration. 1 mM benzamidine was included in the cuvette as a competitive inhibitor of trypsin to slow down the reaction. NBD spectra were acquired at concentrations of 156 nM for the S121C variant and 400 nM for the D159C variant. The buffer used for all measurements was 20 mM sodium phosphate, pH 7.4, containing 100 mM NaCl, 0.1 mM EDTA, and 0.1% PEG8000.

To estimate the efficiency of energy transfer between fluorescein and rhodamine in the covalent complex, the fluorescence spectrum of the fluorescein-labeled serpin was recorded, and trypsin, either unlabeled or rhodamine-labeled, was then added to the cuvette in the presence of 1 mM benzamidine and the reaction followed by monitoring the change of fluorescein fluorescence at 515 nm. When a plateau was reached the fluorescence emission spectrum of the mixture was recorded. The amount of energy transfer for each variant was determined from the observed reduction in fluorescein fluorescence corrected for any contribution that arose solely from complex formation, which was determined from a control reaction using fluorescein-labeled $\alpha_1$PI and unlabeled $\beta$-trypsin. A correction was also made for the SI in every case, calculated as described above, although this was mostly a small correction. Percentages of energy transfer reported in Table III also take into account the stoichiometry of rhodamine labeling and are scaled to the efficiency expected at 1 mol/mol of label. The justification for such a scaling is that, for different degrees of labeling, a linear dependence of efficiency of transfer was found. Scaling to 1:1 treats each trypsin as having either zero or one labels and that label at any of the positions is equivalent. Although this is not likely to be strictly accurate, it should give a minimum value for the efficiency of energy transfer. Confirmation that the end point represented complete reaction of the serpin was from SDS-PAGE analysis of the trichloroacetic acid-precipitated reaction products, which also showed no evidence of cleavage of the proteinase in the complex by excess active proteinase.

TABLE I

*Properties of $\alpha_1$PI cysteine variants and their fluorescein-labeled conjugates with respect to inhibition of trypsin and thrombin and formation of covalent complex*

| Cys position | Ability to form complex[a] | SI[b] | Ability to form complex when labeled[a] | SI[b] when labeled | Ability to label Cys in complex[a] |
|---|---|---|---|---|---|
| 85 | Normal | 1.06 | Normal | 1.13 | Yes |
| 121 | Normal | 1.07 | Reduced[c] | 1.06[d] | Yes |
| 159 | Normal | 1.06 | Normal | 1.06 | Yes |
| 232 | Normal | 1.10 | Normal | 1.10 | Yes |
| 298 | Normal | 1.05 | Abolished | ≫10 | Yes |

[a] Applies to both $\beta$-trypsin and thrombin, unless noted otherwise.
[b] SI for reaction with trypsin. Uncertainty in SI is no more than 0.02 for SI values close to 1.
[c] Reduced ability to form complex with thrombin, no significant perturbation of complex formation with trypsin.
[d] SI for 121C variant with thrombin was >5.

For fluorescence resonance energy transfer measurements on noncovalent complexes between anhydrotrypsin and serpin, fluorescein-labeled $\alpha_1$PI (30–50 nM) and rhodamine-labeled or unlabeled anhydrotrypsin (80–100 nM) were mixed and the spectra recorded. For each $\alpha_1$PI variant four series of spectra were recorded; labeled $\alpha_1$PI alone, labeled $\alpha_1$PI with unlabeled anhydrotrypsin, labeled $\alpha_1$PI with labeled anhydrotrypsin (0.3 label/mol), and labeled $\alpha_1$PI with labeled anhydrotrypsin (0.71 label/mol). The efficiency of energy transfer was estimated from the reduction in fluorescein emission intensity in the doubly labeled complex compared with complex with unlabeled anhydrotrypsin. The values were scaled to 1.0 label/mol and the results for the two different preparations of labeled anhydrotrypsin averaged. Spectra are the average of three consecutive scans.

*Preparation and Fluorescence Spectra of Different NBD-labeled Species*—NBD-labeled S121C variant was reacted with trypsin, papain, or thrombin as described in the figure legend for Fig. 4. One aliquot of the reaction mixture was used for SDS-PAGE analysis, and another aliquot was diluted to a final concentration of 156 nM $\alpha_1$PI and the emission fluorescence spectrum recorded as described above. The Coomassie Blue-stained gel was scanned and the density of the bands used to estimate both the completeness of each reaction and the SI. Every reaction was found to be more than 95% complete.

## RESULTS

*Accessibility of Engineered Cysteines in Native and Proteinase-complexed Variants*—Previous studies on a recombinant Pittsburgh variant of $\alpha_1$PI (P1 Met → Arg) have shown that the cysteine at position 232 is quite accessible to nucleophiles (7), consistent with its exposed location in the crystal structure of $\alpha_1$PI. We found here that cysteine 232 is also accessible in the complex, since it could be comparably labeled with 5-IAF while in complex with both $\beta$-trypsin and thrombin, as judged by the intensity of fluorescence associated with the band of complex on SDS-PAGE (Table I, gel not shown). To carry out similar accessibility studies at different sites on the serpin, we created four new variants, each containing a single free cysteine at strategic locations on the serpin surface (Fig. 1). The choice of sites was guided by proposed models for the serpin-proteinase complex (6, 8, 9), with the aim of creating one or more variants that had a cysteine that might be accessible when the serpin was uncomplexed but inaccessible in complex.

The ability of $\alpha_1$PI to be labeled with fluorescein either alone

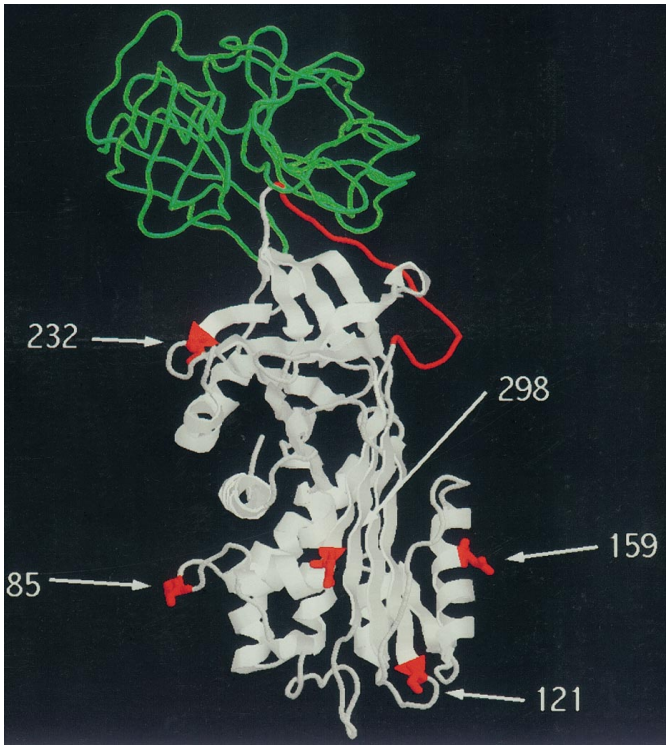


FIG. 1. **Modeled structure of $\alpha_1$PI docked with trypsin, showing the location of the sites of mutation of single residues to cysteine used in this study.** The coordinates are from pdb1smf (trypsin) and pdb7api ($\alpha_1$PI). The reactive center loop is shown here as *red*. Trypsin (*green*) is docked on the P1 residue in a theoretical structure that should resemble the non-covalent, Michaelis-like complex for the initial interaction. This structure is also expected to describe rather well the interaction of the anhydroproteinase with the serpin. Residue 159 is on helix F.

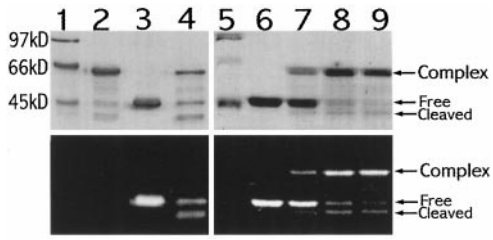


FIG. 2. **SDS-PAGE of reactions of fluorescein-labeled S121C and D298C variants with trypsin.** Derivatization with fluorescein of the cysteine at position 298 blocked the formation of SDS-stable complex, as judged by the absence of a fluorescent band for the complex (the variant is only about 50% labeled and thus the unlabeled portion still forms complex that can be detected by Coomassie Blue staining but is invisible by fluorescence detection.) *Gray background* indicates Coomassie Blue staining. *Black background* indicates fluorescence detection. *Lanes 1* and *5* are molecular massweight markers. *Lanes 2–4* are for the D298C variant (1.8 $\mu$g) and *lanes 6–9* for the S121C variant (1.6 $\mu$g). Amounts of trypsin were 0.33 $\mu$g (*lanes 4* and *7*), 0.66 $\mu$g (*lane 8*), and 0.99 $\mu$g (*lanes 2* and *9*). In the presence of excess proteinase (*lane 9*) there is an additional fluorescent band between that of complex and intact serpin, which is evidence for cleavage of the complex by free proteinase. It should be noted that this was not the case for energy transfer experiments, where different conditions were used.

or in complex with either $\beta$-trypsin or thrombin was assessed by SDS-PAGE of the reaction mixtures examined by fluorescence (not shown). The time used for reaction of $\alpha_1$PI with proteinase was sufficient for the reaction to go to completion in all cases. Surprisingly, all four new sites could be labeled with the large fluorescein moiety both before and after reaction with $\beta$-trypsin or thrombin (Table I). The stoichiometries of incorporation of label were judged to be qualitatively similar for all variants, based on visual inspection of photographs of fluorescein fluorescence, and showed little difference between native and proteinase-complexed forms, indicating that none of the sites was significantly obscured by $\beta$-trypsin or thrombin in the complex.

*Effect of Cysteine Modification on Complex Formation*—We also determined whether covalent modification of any of the cysteines with fluorescein, *before* reaction with proteinase, affected the subsequent ability of the serpin to inhibit $\beta$-trypsin, to establish if the presence of a bulky group impeded movement of the proteinase during its migration to its final position in the complex. All variants except D298C could still form covalent complex with $\beta$-trypsin, as judged by the formation of covalent complex visible on SDS-PAGE (gel not shown). The SI values, estimated from quantitation of the bands of complex and cleaved $\alpha_1$PI on the gels (not shown), were close to 1 for variants 85, 121, 159, and 232 labeled with fluorescein (Table I), showing that the relative fluxes along the inhibitory and substrate branches of the serpin pathway had not been significantly affected by the labeling. For the D298C variant, the attachment of fluorescein resulted in a sufficiently large reduction in the flux along the inhibitory branch of the serpin pathway (16) that no covalent complex was detectable by SDS-PAGE examined by fluorescence. Instead, the only fluorescent product was substrate-cleaved $\alpha_1$PI. A comparison of the different outcomes of the reactions of the 121 and 298 variants is shown in Fig. 2. Attachment of the smaller NBD fluorophore also resulted in a greatly increased SI for the 298 variant. However, since NBD fluorescence could not be seen on the SDS gel and some unlabeled variant was still present and competent to form complex, we cannot be sure that the inhibition pathway was completely blocked. The fluorescein-labeled S121C variant, while showing a normal SI with $\beta$-trypsin of close to 1, gave an SI of >5 with thrombin (Table I). Thus, depending on the proteinase used to form complex, labeling of either of the positions 121 and 298 resulted in perturbations of the SI.

*Sensitivity of Fluorescent Label to Complex Formation*—The sensitivity of fluorophores at each of the cysteine sites to proteinase-induced changes was examined. Fluorescein at positions 85, 159, and 232 was little perturbed by formation of complex with $\beta$-trypsin (Table II) (spectra not shown). Fluorescein at 121, however, gave a 20% reduction in intensity and a red shift of ~3 nm upon complex formation. Since this fluorophore gave almost no change upon substrate-like cleavage with papain (Fig. 3), it seems that it is the presence of the proteinase, rather than simply reactive center loop insertion and accompanying conformational changes, that results in the 20% enhancement of fluorescein fluorescence in the covalent complex. Although fluorescein at position 298 showed a 100% fluorescence enhancement upon reaction with $\beta$-trypsin (Fig. 3 and Table II), the species was cleaved loop-inserted $\alpha_1$PI rather than covalent complex with $\beta$-trypsin, since the label blocked the inhibitory pathway (see above).

Because fluorescein is not a very sensitive reporter group of changes in local environment, we further examined the environment of the cysteines at 121 and 159 in complex, using the much more responsive NBD label. Formation of complex with $\beta$-trypsin resulted in a 215% enhancement and 6-nm blue shift for NBD at position 121 but almost no change for NBD at position 159 (Fig. 4). The effect of complex formation with thrombin was complicated by the increased SI for complex formation with S121C-NBD, so that the observed NBD emission spectrum was the sum of spectra for both cleaved and complexed labeled $\alpha_1$PI. However, we observed that the SI was temperature-dependent, so that reactions carried out at 4 °C (SI = 2.1) and 37 °C (SI = 1.75) gave different percentages of

TABLE II
*Effect of complex formation on NBD and fluorescein fluorescence*

| Cysteine position | Proteinase | Fluorophore | |
|---|---|---|---|
| | | NBD | Fluorescein |
| 85 | Trypsin | Not measured | No effect |
| | Trypsin | +215%, 6-nm blueshift | −20%, 2-nm blueshift |
| 121 | Anhydrotrypsin | No effect | −5%, 2-nm blueshift |
| | Thrombin | > +262%[a] | Not measured |
| 159 | Trypsin | No effect | No effect |
| | Thrombin | No effect | Not measured |
| 232 | Trypsin | Not measured | No effect |
| | Anhydrotrypsin | Not measured | 10% decrease |
| 298[b] | Trypsin | Small decrease (~15%)[b] | +100% increase, 4-nm blueshift[a,b] |

[a] The enhancement must be greater than that seen in the spectrum of a mixture of thrombin-serpin complex and cleaved serpin, since complex has greater NBD emission intensity than cleaved serpin.
[b] Value is for cleaved variant, since no complex is formed.

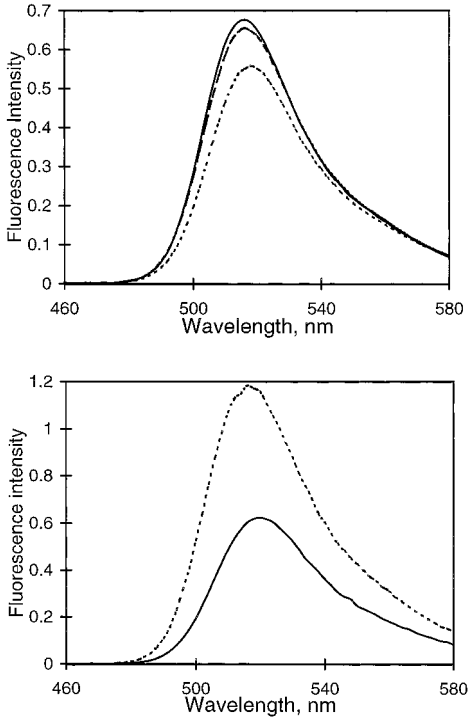


FIG. 3. **Fluorescence emission spectra of fluorescein-labeled S121C and D298C variants.** *Upper panel*, S121C-fluorescein (63 nM). *Solid line*, native; *dashed line*, papain-cleaved; *dot-dashed line*, covalent β-trypsin complex (1:1). Papain-cleaved variant was made by incubating 2.8 μM variant with 0.05 μM papain for 20 min at 37 °C, followed by addition of iodoacetamide to 3 mM. The spectrum was recorded of a dilution of this mixture. Trypsin complex was made by reaction of 2.8 μM β-trypsin with 2.8 μM variant in the presence of 1.8 mM benzamidine for 5 s, followed by addition of 60 μM TLCK and dilution into the cuvette. *Lower panel*, D298C-fluorescein (66 nM). *Solid line*, native variant; *dashed line*, variant reacted with 116 nM β-trypsin in the presence of 1 mM benzamidine. The spectrum was recorded as soon as the fluorescence had reached a plateau, indicating completion of the reaction. For this labeled variant the end product of the reaction was cleaved $\alpha_1$PI rather than stable complex, as indicated by SDS-PAGE analysis. This is because introduction of the fluorescein group adversely affects complex formation (see text).

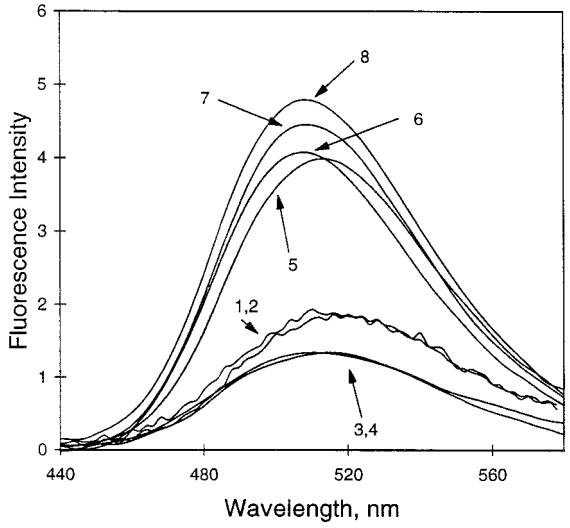


FIG. 4. **NBD fluorescence spectra for the 121 and 159 positions.** *Spectra 1* and *2* are of the $\alpha_1$PI D159C variant (400 nM) that has been labeled with NBD-Cl, either alone or in complex with thrombin, respectively. These spectra are indistinguishable. *Spectra 3-8* are for the S121C variant (156 nM) labeled with NBD-Cl and scaled to the same NBD concentration as spectra 1 and 2. *Spectrum 3* is in the absence of proteinase; *spectrum 4* is the complex with anhydrotrypsin (300 nM); *spectrum 5* is papain-cleaved; *spectrum 6* is trypsin covalent complex; *spectrum 7* is thrombin reaction mixture from 4 °C reaction (SI = 2.1); and *spectrum 8* is thrombin reaction mixture from 37 °C reaction (SI = 1.75). Reactions with papain, trypsin, and thrombin were carried out at higher protein concentrations and then diluted into the cuvette. Papain cleavage used 50 nM papain, 5 μM variant reacted for 10 min at 37 °C. Thrombin reactions were at 6.6 μM thrombin and 5 μM variant for 60 s at 37 °C and 50 min at 4 °C. Trypsin reaction was at 4.3 μM trypsin, 3.8 μM variant for 5 s at 25 °C. Proteinase reactions were stopped by addition of iodoacetamide for papain, Phe-Phe-Arg-chloromethyl ketone for trypsin, and Phe-Pro-Arg-chloromethyl ketone for thrombin.

complex (48 and 57%, respectively) and thus different NBD emission spectra. We found that both samples gave emission spectra with intensities higher than for trypsin-S121C-NBD complex (Fig. 4), with the sample that contained more thrombin complex giving the highest intensity. Therefore, although we cannot give an accurate value for the enhancement for formation of the thrombin-S121C-NBD complex, since the difference in the percentage of complex was only about 9%, we can be sure that it is higher than both the enhancement for the comparable trypsin complex and that seen in the spectra of mixtures of cleaved and thrombin-complexed species (262% enhancement). No change in NBD emission spectrum was found for formation of thrombin complex with D159C-NBD (Fig. 4). Since position 159 has been implicated elsewhere as being close to the proteinase in the complex (8), such insensitivity of NBD at this site to complex formation with both trypsin and thrombin was not expected if that model were correct.

Given the sensitivity of the NBD at position 121 to different proteinases, we also examined the effect on this fluorophore of cleavage of the reactive center and of formation of a non-covalent complex with anhydrotrypsin. Formation of a tight non-covalent complex with anhydrotrypsin gave no change in NBD fluorescence (Fig. 4). Papain cleavage, which occurs at P7-P6 (17), gave a 200% enhancement of NBD fluorescence at this position, although with no shift in wavelength maximum

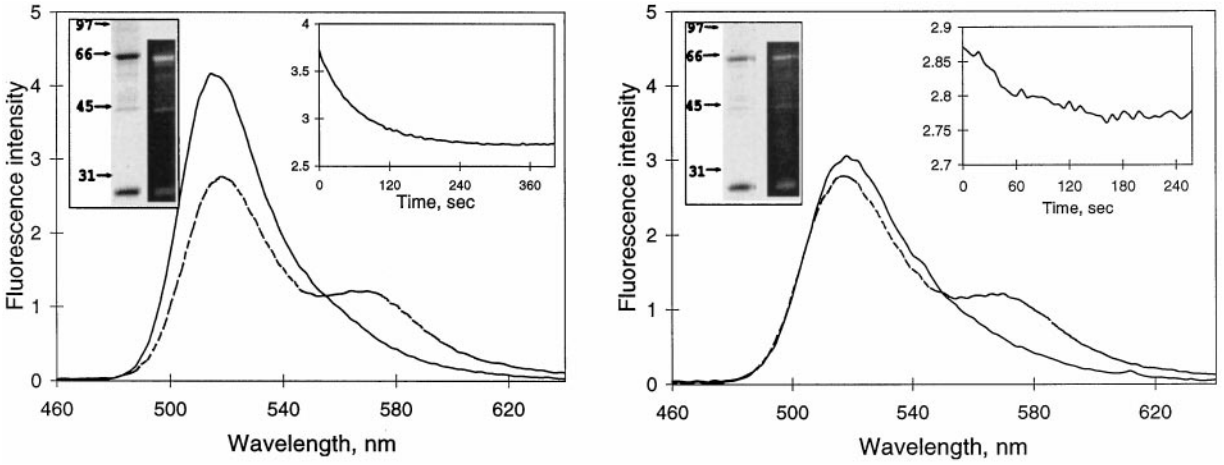


FIG. 5. **Changes in fluorescein fluorescence of $\alpha_1$PI-labeled variants upon complex formation with rhodamine-labeled trypsin.** *Left panel*, S121C variant. Fluorescein fluorescence spectra are shown for the free $\alpha_1$PI (*solid line*) and the complex (*dashed line*). The time course of this change appears on the *right inset*. The final product of the reaction was analyzed by SDS-PAGE (*left inset*) and detected by Coomassie stain (*left lane*) and fluorescence (*right lane*), revealing more than 95% complete reaction toward complex formation (slow mobility band). *Right panel*, D159C variant, same presentation. For both reactions the concentration of $\alpha_1$PI was 50 nM and the concentration of rhodamine-trypsin was 100 nM.

compared with the native protein (Fig. 4). The blue shift of 6 nm upon complex formation with β-trypsin but absence of such shift upon insertion of the cleaved reactive center loop again indicates a strong effect of the proteinase, which is in addition to any effects of serpin conformational change and is therefore likely to be due directly to the presence of the proteinase in the complex.

*Fluorescence Resonance Energy Transfer between Serpin and Proteinase*—We have previously shown that there is a very large difference in fluorescence resonance energy transfer between covalent and non-covalent complexes of rhodamine-labeled trypsin (or anhydrotrypsin) and $\alpha_1$PI labeled at $Cys^{232}$ with fluorescein, consistent with a large change in position of the proteinase as a result of covalent complex formation (7). In the present study we used the new cysteine-containing variants to extend such measurements to define better the location of the proteinase in the covalent complex. Before doing this we determined the sites of attachment of the rhodamine label by peptide mapping of a tryptic digest of labeled β-trypsin. This showed that, although less than an average of one label was incorporated per trypsin, the label was distributed over several sites. Two major peptides, one accounting for 16 and one 20% of the rhodamine fluorescence, were sequenced and shown to correspond to peptides starting with sequences *X*LX*X*AP and VCNYV, respectively. This identified the labeled lysines as 159 and 239.

Given this heterogeneity of the rhodamine label on trypsin, it was not possible to use fluorescence resonance energy transfer quantitatively to triangulate the location of the proteinase in the complex. We therefore restricted use of fluorescence resonance energy transfer measurements to qualitative pairwise comparisons of the relative energy transfer between the covalent and non-covalent complexes and between covalent complexes with label at different sites. Determination of fluorescence resonance energy transfer for a given serpin-proteinase pair was made from time-dependent changes in donor (fluorescein) fluorescence after mixing of the fluorescein-labeled $\alpha_1$PI variant with rhodamine-labeled β-trypsin, as described under "Materials and Methods."

Such time-dependent changes are shown for formation of covalent complexes between either fluorescein-labeled D159C or S121C $\alpha_1$PI and rhodamine-labeled β-trypsin (Fig. 5). For all complexes examined, the time courses were well fitted to a single step bimolecular reaction. Including previously published energy transfer efficiencies for $Cys^{232}$-labeled Pittsburgh $\alpha_1$PI (7), we obtained 4 sets of energy transfer efficiencies for covalent and non-covalent complexes with $\alpha_1$PI (Table III), corrected as described for any fluorescence changes due solely to complex formation. Whereas label at 232 gave higher efficiency of transfer to proteinase in the non-covalent complex, label at 85 and 121 gave higher efficiency of transfer in the covalent complex. Label at 159 gave low efficiency of transfer for both types of complex. These results are consistent with the proteinase being close to the bottom of the serpin, whereas the anhydroproteinase is expected to be at the top of the serpin, where it can interact with the intact reactive center loop in a manner analogous to complexes between canonical serine proteinase inhibitors and their target proteinases (3). Because of the qualitative treatment of these energy transfer efficiencies, it is not possible to be more precise about the location of the proteinase, especially as a result of the uncertainties in the orientation factors for each of the donor-acceptor pairs. This means that the model of Wilczynska *et al.* (8) cannot be absolutely ruled out on the basis of these fluorescence resonance energy transfer measurements, since it is possible that the proteinase could be close to residue 159 but have an orientation such that the observed efficiency of transfer is small, despite a small interfluorophore separation.

TABLE III
*Fluorescence resonance energy transfer in complexes with β-trypsin or anhydrotrypsin*

| Position of fluorescein | Fluorescence energy transfer | |
|---|---|---|
| | Trypsin | Anhydrotrypsin |
| | % | |
| 85 | 34.5 | 19 |
| 121 | 22.5 | 10 |
| 159 | 7.7 | 10 |
| 232 | 18[a] | 77[a] |
| 298 | Not measurable[b] | <2 |

[a] From Ref. 7.
[b] No detectable complex formed (very high SI).

## DISCUSSION

We have described here the use of the Pittsburgh variant of $\alpha_1$PI and four new single cysteine-containing derivatives to map the covalent complex that this serpin forms with proteinases. The advantage of the Pittsburgh variant over wild-type $\alpha_1$PI is that its affinity for anhydrotrypsin is high enough to

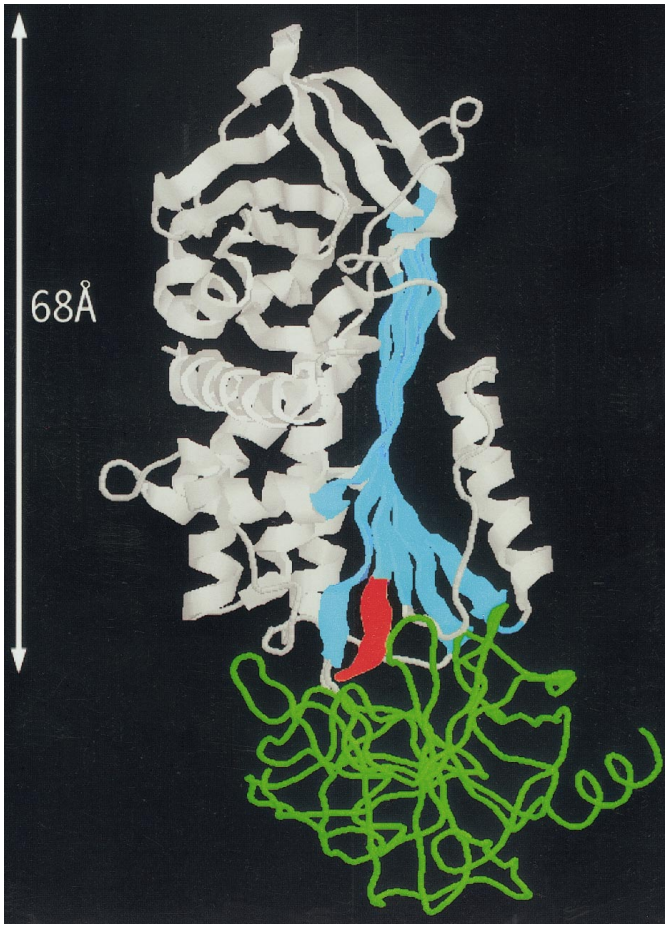


FIG. 6. **Proposed model of the serpin-proteinase complex that is consistent with results presented in this study, showing the proteinase at the distal end of the serpin.** The location of the proteinase is relatively close to both residues 85 and 121 to account for the resonance energy transfer findings, but not so close that either is obscured, to be consistent with the ability to label each site while in complex with proteinase. The structure of the serpin is that of the cleaved $\alpha_1$PI (1pdb7api) and thus assumes full loop insertion for this model. $\beta$-Sheet A is colored *cyan* with the exception of the residues P1-P3 of the reactive center loop which are shown in *red*.

allow examination of both non-covalent and covalent complexes. Previous studies using fluorescence resonance energy transfer between fluorophores on trypsin and $\alpha_1$PI Pittsburgh had shown that the proteinase undergoes a major change in location from its initial site of docking with the reactive center loop (7). However, because only a single distance constraint was used it was not possible to distinguish between three different models for the complex, each of which involves a very different extent of proteinase translocation. Whisstock *et al.* (9) have proposed a modest movement of the proteinase from the initial site of docking, with insertion of the reactive center loop into $\beta$-sheet A up to P12. Wilczynska *et al.* (8) have proposed a greater movement, involving alignment of the proteinase with the flank of helix F and the cleaved reactive center loop inserted up to P5. The third proposal, from Wright and Scarsdale (6), involves the proteinase moving completely to the end of the serpin distal from the initial docking site, which requires complete insertion of the cleaved reactive center loop in a manner similar to that of substrate-cleaved serpin. Our present findings, taken as a whole, are consistent with the third model of full loop insertion and full movement of the proteinase (Fig. 6). The basis for this conclusion is as follows. (i) If complex formation involves complete insertion of the cleaved, but still covalently bound, reactive center loop into $\beta$-sheet A, it becomes immediately understandable why cysteine at 298 can be modified both in native and proteinase-complexed $\alpha_1$PI yet not permit complex formation when labeled beforehand, since the reactive center loop, with the large proteinase covalently bound, would need to insert past this residue. A large group, such as fluorescein, might impede such insertion, thereby allowing only substrate cleavage to occur, as observed. (ii) With proteinase at the distal end of the serpin in covalent complex (*bottom* in Fig. 6) and at the proximal end in non-covalent complex (*top* in Fig. 1), the relative efficiency of fluorescence resonance energy transfer should be greater in the anhydro complex than in the covalent complex for fluorophore at 232, but the reverse for fluorophore at 121, as was found. The expectation for position 85 is also that it would be closer in the covalent than the non-covalent complex and thus give higher efficiencies of fluorescence resonance energy transfer in the covalent complex, again as found. (iii) The proteinase would be expected to be close to residue 121 but not obscure it. Introduction of label at this position might therefore influence the rate of achievement of the final complex, through steric effects and thus affect the SI. This is indeed the case for position 121, where the effect on SI is dependent both on the size of the label and the size of the proteinase. Thrombin, which is ~50% larger than trypsin, gives a much higher SI than does trypsin for both the fluorescein and NBD derivatives, although the effect is less for the smaller NBD, consistent with steric clashes during complex formation being most pronounced for the larger proteinase with the larger label. (iv) With proteinase at the bottom of the serpin and in the vicinity of residue 121, the emission spectrum of fluorophore at this position should be responsive to complex formation but in a different way than from simple loop insertion. Indeed NBD at position 121 can discriminate between non-covalent complex, covalent complexes with different proteinases, and cleaved $\alpha_1$PI. The large changes in NBD fluorescence for cleavage or covalent complex formation contrast with the absence of perturbation for formation of the anhydro complex and thus strongly indicate that the covalent complex is very different from that of the non-covalent complex with respect to perturbation of the serpin structure. In this regard, the covalent complex is much more similar to cleaved, loop-inserted serpin, as is expected for the model of Wright and Scarsdale (6). It is important to note that NBD label at 121 shows a 6-nm blue shift when complex is formed with trypsin but no such wavelength shift in papain-cleaved $\alpha_1$PI. Similarly, the intensity of the NBD emission spectrum of the thrombin complex is much higher than for that of the thrombin-cleaved serpin. Both of these results indicate that the presence of the proteinase, either trypsin or thrombin, causes significant spectral changes that are distinct from changes caused solely by loop insertion and associated conformational change. Although our results are most consistent with full insertion of the reactive center loop and placement of the proteinase at the distal end of the serpin, they do not allow a precise positioning of the proteinase, except that it must be close enough to position 121 to perturb fluorophore at this position but not so close as to prevent labeling of cysteine in the S121C variant when in complex with either $\beta$-trypsin or thrombin.

The model of Wright and Scarsdale (6) is also the one that is philosophically most consonant with the requirements and restrictions for stable serpin-proteinase complex formation. Thus, a given serpin can often form SDS-stable complexes with many different proteinases that differ greatly in size and shape. The Pittsburgh variant used here is a good example, in that it can inhibit trypsin, elastase, thrombin, C1s, factor XIIa, plasmin, and urokinase (18, 19). Similarly a given proteinase may be able to form complexes with different serpins. Throm-

bin is inhibited by antithrombin, heparin cofactor II, protease nexin 1, plasminogen activator inhibitor 1, and protein C inhibitor. It is hard to conceive a model for the complex that involves a specific interaction between serpin and proteinase in the final trapped complex and can yet accommodate such a wide array of different proteinases. The model favored here, however, has as the only requirement a conformationally strained acyl ester linkage between the P1 residue at the very bottom of β-sheet A and the active site serine of the proteinase, for which there is some experimental evidence (20–22). This is common to all pairs of serpin-proteinase complexes.

Another satisfying aspect of such a model is that it has been found experimentally that the P1-P1′ bond must be exactly 14 residues from the hinge point for insertion into β-sheet A, which makes it just long enough upon complete insertion to have residues P2 and P1 protrude from the end of the sheet and provide enough of a linker to reach into the proteinase active site and thereby to impose a particular non-optimal conformation for the acyl group in the proteinase-active site. If the scissile bond were closer to this hinge point the acyl intermediate could not be trapped by such a full insertion mechanism. If the scissile bond were further away the resulting peptide that extends beyond β-sheet A would be so long that there could be no constraint imposed on the conformation of the acyl ester linkage, no disruption of the catalytic site of the proteinase, and hence no kinetic trap.

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