Mapping the Serpin-Proteinase Complex Using Single Cysteine Variants of α₁-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 α₁-proteinase inhibitor Pittsburgh. Cysteines in each variant could be labeled both in native and proteinase-complexed α₁-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 Cys121 greatly decreased affinity and molecular mass of complexes with trypsin and thrombin. Whereas fluoresphore at residue 159 on helix F was almost insensitive to complex formation, fluorescence resonance energy transfer decreased significantly between native, cleaved, and proteinase-complexed serpin and between complexes with trypsin and thrombin, whereas fluorescence at residue 159 on helix F was almost insensitive to complex formation. Fluorescence resonance energy transfer measurements for covalent complexes and non-covalent complexes were consistent with a location of the proteinase at the center of the serpin proteinase complex 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.

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 α₁-proteinase inhibitor (α₁PI)1 Pittsburgh and the proteinase to compare the interfluorophore 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 α₁PI 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 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 α₁PI cDNA, using the Quikchange method (Stratagene). Double-stranded template DNA of two complementary primers containing the mutation was annealed and extended with Phi DNA polymerase during thermal cycling. The pET16b plasmid contained an N-terminally modified α₁PI cDNA that lacked coding sequence for the first 5 residues (10), inserted between the NcoI and BamHI subcloning sites of the vector. The sequences for the coding strands of the mismatch primers were as follows (mismatch codons are underlined): M338R, 5'-GAG GCC ATA CCC

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1 The abbreviations used are: α₁PI, α₁-proteinase inhibitor; 5-IAF, 5-idoacetamidofluorescein; 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-[hydroxyethyl]-propane-1,3-diol.
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 containing β-trypsin and NBD-β-trypsin with different mutations 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 α1PI species. Extinction coefficients of 27,000 (15) and 82,000 M⁻² cm⁻¹ were used for α1PI and fluorescein, respectively.

Preparative Labeling of α1PI Variants with NBD-β-Trypsin—α1PI Variants were labeled with NBD-β-trypsin with tetramethylrhodamine isothiocyanate while immobilized on soybean trypsin inhibitor 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 anhydrotrypsin, followed by gentle rotation for 30 min 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 α1PI Variants to Form SDS-Stable Monomers—α1PI Variants were labeled with tetramethylrhodamine isothiocyanate immobilized on soybean trypsin inhibitor-agarose beads 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% guanidinium hydrochloride, 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. Iodoacetate was added to 30 mM and allowed to react for 30 min at room temperature. The denatured labeled trypsin was diluted into 800 μl of 50 mM Tris, pH 8.0, 50 mM NaCl, 1 mM EDTA, 10 mM CaCl₂, 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, pH 2.4) over 30 min. Fractions were collected over a period of 20 min.

The tryptic digest was chromato-
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 μg of α1PI was reacted for 10 s with trypsin at different molar ratios, ranging from 0.3:1 to 2:1 trypsin:α1PI, with α1PI fixed at 5–10 μM. 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 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 α1PI 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 intensifying films bands corresponding to cleaved or uncleaved 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 μg of either the Pittsburgh variant of α1PI or the cysteine mutants (T85C, D159C, S121C and D298C) in a total volume of 20 μl were reacted with 1 μg of β-trypsin (excess of α1PI to ensure that complex was not degraded by excess proteinase) to form the stable trypsin-α1PI complex. TLCK was added after a few seconds to a final concentration of 25 μM. In all reaction mixtures 1.5 μl of 1.8 mM IAP was added (final concentration was about 100 μM), 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 IAP 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 β-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—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 fluorescence intensity 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 α1PI and unlabeled β-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 labeled at 1 μM labeled trypsin. 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.

For fluorescence resonance energy transfer measurements on non-covalent complexes between anhydrotrypsin and serpin, fluorescein-labeled α1PI (30–50 nM) and rhodamine-labeled or unlabeled anhydrotrypsin (80–100 nM) were mixed and the spectra recorded. For each α1PI variant four series of spectra were recorded, labeled α1PI labeled with fluorescein alone, labeled α1PI with unlabeled anhydrotrypsin, labeled α1PI with labeled anhydrotrypsin (0.3 label/mol), and labeled α1PI with labeled anhydrotrypsin (0.71 label/mol). The efficiency of energy transfer was estimated from the reduction in fluorescence 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 reacted for a final concentration of 156 nM for α1PI 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 α1PI (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 α1PI. We found here that cysteine 232 is also accessible in the complex, since it could be comparably labeled with 5-IAP while in complex with both β-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 α1PI to be labeled with fluorescein either alone

| Cys position | Ability to form complex | SIa | Ability to form complex when labeledb | SIb when labeled | Ability to label Cy in complexc |
|--------------|------------------------|-----|-------------------------------------|-----------------|------------------------------|
| 85           | Normal                 | 1.06| Normal                              | 1.13            | Yes                          |
| 121          | Normal                 | 1.07| Reduced                            | 1.06            | 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 β-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.
or in complex with either β-trypsin or thrombin was assessed by SDS-PAGE of the reaction mixtures examined by fluorescence (not shown). The time used for reaction of α₁PI 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 β-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 fluorescence (not shown). The time used for reaction 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 β-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 β-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 β-trypsin (Fig. 3 and Table II), the species was cleaved loop-inserted α₁PI rather than covalent complex with β-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 β-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 complex labeled α₁PI. 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

![Fig. 1. Modeled structure of α₁PI 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 (α₁PI). 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 P.](image-url)
b in the presence of 1 mM benzamidine. The spectrum was recorded as S121C and D298C variants.

... was cleaved the reaction. For this labeled variant the end product of the reaction soon as the fluorescence had reached a plateau, indicating completion of the reaction of 2.8 mM variant with 0.05 mM 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 incubating 2.8 mM with 5 s, followed by addition of 60 μM TLCK and dilution into the cuvette. Papain cleavage used 50 nM papain, 5 mM 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.

... of complex formation on NBD and fluorescein fluorescence

| Cysteine position | Proteinase          | Fluorophore NBD | Fluorophore Fluorescein |
|------------------|---------------------|-----------------|-------------------------|
| 85               | Trypsin             | Not measured    | No effect               |
| 121              | Anhydrotrypsin      | +215%, 6-nm blueshift | -20%, 2-nm blueshift |
| 159              | Thrombin            | No effect       | No effect               |
| 232              | Trypsin             | Not measured    | No effect               |
| 298             | Trypsin             | Small decrease (-15%) | +100% increase, 4-nm blueshift |

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**Table II**

Effect of complex formation on NBD and fluorescein fluorescence

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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.

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**Fig. 4. NBD fluorescence spectra for the 121 and 159 positions.**

Spectra 1 and 2 are of the α1PI 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.

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mixture 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.
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 αPI labeled at Cys232 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 αPI (7), we obtained 4 sets of energy transfer efficiencies for Cys232-labeled Pittsburgh αPI (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 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 αPI 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 αPI 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 Cys232-labeled Pittsburgh αPI (7), we obtained 4 sets of energy transfer efficiencies for covalent and non-covalent complexes with αPI (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.

**DISCUSSION**

We have described here the use of the Pittsburgh variant of αPI 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 αPI is that its affinity for anhydrotrypsin is high enough to
Mapping the Serpin-Proteinase Complex

![Diagram of proposed model of the serpin-proteinase complex](http://www.ncbi.nlm.nih.gov/pubmed/15588)

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 α₁PI (1pdb7api) and thus assumes full loop insertion for this model. β-Sheet A is colored cyan with the exception of the residues P1-P5 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 α₁PI 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. Whistock 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 β-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 β-sheet A, it becomes immediately understandable why cysteine at 298 can be modified both in native and proteinase-complexed α₁PI 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 α₁PI. 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 α₁PI. 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 β-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 XIa, 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 of the acyl ester linkage, no disruption of the catalytic site of the proteinase, and hence no kinetic trap.

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