Comparative Trajectories of Active and S195A Inactive Trypsin upon Binding to Serpins*

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EI* is the encounter complex whose concentration is governed by $K_E^*$, $E_I$, a conformer in which the proteinase has been translocated, and $E_{Iac}$ is the final acylated complex, which may be further translocated or not. In this previous work, we had to use random labeling of elastase that prevented to use the FRET results for distance measurements and thus prevented the evaluation of the extent of the translocation that occurred prior to acylation. Furthermore, the kinetic parameters were such that the detailed study of the steps following the formation of $E_I^*$ was not possible.

In this paper, we further studied the reversible steps characterized by successive conformational changes. We followed the reaction of active and inactive S195A rat trypsin mutants with AT and P1R-ACT. Each protein was labeled at a single site on either a naturally present cysteine or on a cysteine added by mutation. FRET measurements at equilibrium were correlated with time-resolved fluorescence measurements for a better precision and to be able to analyze mixtures of species.

**MATERIALS AND METHODS**

**Proteins**—The single and double mutants of rat trypsinogen were expressed and purified, and activated as described previously (19). The following single mutants were prepared: Q23C, K113C, A122C, and S125C rat trypsins. Two active site modified double mutants in which Ser-195 of the catalytic triad is replaced by alanine were also obtained using the same protocol, namely K113C/S195A and A122C/S195A trypsins. Activation was done with enterokinase (Sigma) and followed by electrophoresis on SDS-acrylamide gels until completion. Active enzymes were titrated with BPTI (BioSys) using 1 mM chromogenic substrate $\Sigma$-benzoyl-arginyl-para-nitroanilide (Bachem). Concentrations of inactive mutants were estimated by measuring the optical density at 280 nm and using the calculated molar extinction coefficient of 34,000 M$^{-1}$ cm$^{-1}$. The construction of the human antichymotrypsin double variant P1Arg/S150C expression vector and the subsequent purification of the protein followed previously described methods (20, 21). The mutant was titrated with active site titrated trypsin using the chromogenic substrate $\Sigma$-benzoyl-arginyl-para-nitroanilide (Bachem). Recombinant human AT was obtained from Novartis (Basel, Switzerland) and was titrated with human neutrophil elastase (22).

**Fluorescent Labeling of Proteins**—The free cysteines of active trypsin mutants were labeled with fluorescein maleimide (Molecular Probes). Five mg of trypsin in 10 mL of 0.1 M NaCl at pH 7 were bound to 2 mL of wet benzamidine-Sepharose gel (Amersham Biosciences) to prevent autolysis of the enzymes. A 10-fold molar excess of fluorescein maleimide was added. The labeled proteins were eluted with 50 mM glycine buffer, pH 2.5, after 2 h of incubation at room temperature. The labeling ratio varied from 0.2 to 0.5 mol of fluorescein/trypsin.

**Rate of Inhibition of Trypsin by P1R-ACT in the Presence of Substrate**—The rate of inhibition was measured by adding trypsin to a mixture of P1R-ACT and substrate (\(\Delta\)-isoleucyl-prolyl-arginyl-para-nitroanilide 2.2 mM, $K_m = 2.2 \times 10^{-6}$ M) (Chromogenix) and recording the release of product with time. Rapid mixing and recording were done with a stopped-flow apparatus (Bio-Logic SFM3). The release of product was followed by monitoring the optical density at 410 nm. All measurements were performed under pseudo-first order conditions ($[I] > 10[E_I]$) with constant concentrations of rat trypsin and variable concentrations of P1R-ACT. The pseudo-first order rate constants $k_{cat}$ of trypsin inhibition were calculated by fitting the progress curves to a single exponential by non-linear regression analysis.

**Rate of Binding of Trypsin to AT**—The rate of binding of trypsin to AT was measured fluorometrically by assessing the rate of displacement of the active site probe p-aminobenzamidine from the active center of the enzyme (24). The equilibrium dissociation constant $K_d$ of the trypsin-p-aminobenzamidine complex was 2.4 $\mu$M. Trypsin (0.24 $\mu$M) was added to a mixture of AT and p-aminobenzamidine (2.4 $\mu$M) under pseudo-first order conditions. Mixing and fluorescence recording were done with the above setup of the apparatus. The excitation wavelength was 270 nm, whereas emission was monitored at $\lambda > 300$ nm using a glass filter. This experiment was done using constant concentrations of rat trypsin and variable concentrations of AT. The recorded progress curves were fitted to a single exponential function to calculate the pseudo-first order rate constants $k_{cat}$ for the binding of trypsin to AT.

**Rate of FRET Variation**—The kinetics of variation of FRET from Fl-labeled trypsins to TMR-labeled serpins was measured with the above stopped-flow using $\lambda_{em} = 450$ nm through a monochromator and $\lambda_{em} = 514$ nm through an interferential filter (Melles-Griot). For the observation of fluorescence emission of TMR-labeled P1R-ACT with time, the excitation wavelength was 550 nm through a monochromator and the emitted light was observed through a high pass filter with a cut-off at 590 nm (Melles-Griot). The least squares fittings of all traces were calculated with BioKine (Bio-Licic).

All the above experiments were done at 25 $^\circ$C in 50 mM HEPES buffer at pH = 7.4 and 0.1 M NaCl.

**Steady-state and Time-resolved Fluorescence Spectroscopy**—Fluorescence emission spectra were recorded at 20.0 ± 0.5 $^\circ$C on an SLM 48000 spectrofluorometer. The excitation and emission bandwidths were 4 and 8 nm, respectively. The spectra were corrected for inner filter effects at both excitation and emission wavelengths as described (25).

The quantum yield of the Fl-labeled enzymes was determined by using fluorescein in 0.1 M NaOH (\(\phi = 0.92\)) (26) as a reference. The quantum yield of the complex of Fl-labeled enzyme with TMR-labeled inhibitor was corrected for the fractional labeling, $f_A$, of the inhibitor by using Equation 1.

$$\phi_{Fl}^{calc} = \frac{\phi_{Fl}^{obs} \cdot (1 - f_A) \phi_{Fl}^{A}}{f_A}$$

$\phi_{Fl}^{calc}$ and $\phi_{Fl}^{obs}$ are, respectively, the corrected and measured quantum yields of the Fl dye in the Fl-enzyme/TMR-inhibitor complex. $\phi_{Fl}^{A}$ is the quantum yield of the complex of the Fl-labeled enzyme with the non-labeled inhibitor.

Time-resolved fluorescence intensity and anisotropy measurements were performed with a time-correlated, single-photon counting technique using the stable excitation pulses provided by a pulse-picked frequency tripled Ti-sapphire laser (Tsunami, Spectra Physics) pumped by a Millenia X laser (Spectra Physics). Temperature was maintained at 29 $^\circ$C. The excitation pulses were at 470 nm, with a repetition rate of 4 MHz. The emission was collected through a 4-nm band-pass monochromator (Jobin-Yvon H100) at 515 nm and a GG495 filter to reject the scattered light from the excitation beam. The single-photon events were detected with a microchannel plate Hamamatsu R3800U photomultiplier coupled to a Phillips 6854 pulse preamplifier and recorded on a multichannel analyzer (Ortec 71000) calibrated at 25.5 ps/channel. The instrumental response function was recorded with a polished aluminum reflector, and its full width at half-maximum was 40 ps. For lifetime measurements, the polarization in the emission path was set at the magic angle. For time-resolved anisotropy measurements, this polarizer was set in a vertical position. $I(t)$ and $I_0(t)$ were alternatively recorded every 5 s, by using the vertical polarization of the excitation beam without and with the interposition of a quartz crystal rotating the beam polarization factor $G$ equal to 1 in our system.

Time-resolved data analysis was performed using the maximum entropy method and Pulse5 software (27, 28). For the analysis of the fluorescence decay, a distribution of 200 equally spaced lifetime values on a logarithmic scale between 0.01 and 20 ns, was used. Similarly, 200 equally spaced rotational correlation time values on a logarithmic scale were used for the analysis of the fluorescence anisotropy decay (29). We assume that each rotational correlation time is associated with all fluorescence lifetimes. The anisotropy is then defined by Equation 2.

$$r(t) = \frac{r_0}{100} \sum_{i=1}^{100} \beta_i \cdot e^{-t/\tau_i}$$

$r_0$ is the fundamental anisotropy, $\beta_i$ is the rotational correlation time and $r(t)$ is its associated relative amplitude. In all cases, the $\chi^2$ values were close to 1.0, and the weighted residuals as well as the autocorrelation of the residuals were randomly distributed around zero, indicating an optimal fit.
The average distance between the F1 dye bound to trypsin and the TMR dye bound to the inhibitor (AT or P1R-ACT) in the enzyme/inhibitor complex was calculated by fluorescence resonance energy transfer (FRET) measurements, using the quenching of F1, the donor. The efficiency of transfer was calculated by Equation 3.

\[ E = 1 - \frac{\tau_{D}}{\tau_{O}} \]  

(Eq. 3)

\( \tau_{O} \) corresponds to the 7th fluorescence lifetime of the donor (F1) in the absence of the acceptor (TMR) and \( \tau_{D} \) corresponds to the 7th fluorescence lifetime of F1 in the presence of TMR. The Förster critical distance, \( \text{R}_c \), was calculated according to Equation 4.

\[ \text{R}_c = \left( \frac{8.79 \times 10^{-24} n^2 Q_{D} \lambda_{D}^2 \lambda_{A}}{K_{\text{app}}} \right)^{1/6} \]  

(n designates the refractive index of the medium (a value of 1.333 is usually taken), \( Q_{D} \), the quantum yield of the donor, \( \lambda_{D} \), the overlap integral calculated from the overlap between the emission spectrum of the donor and the absorbance spectrum of the acceptor, and \( \lambda_{A} \), the orientational factor. Finally, using \( R_{c} \) and \( E \), the distance, \( R \), between the acceptor and the donor is calculated using Equation 5.

\[ R = R_{c} \left( \frac{1}{E} - 1 \right)^{1/6} \]  

(Eq. 5)

All measurements were done in 50 mM HEPES buffer at pH 7.4 and 150 mM NaCl. For the irreversible complexes formed of active trypsin and serpin, the concentrations were 3 \( \mu \)M for trypsin and 6 \( \mu \)M for the serpin to avoid proteolytic degradation of the complexes. For the reversible complexes formed of inactive trypsin and serpin, the concentration of inactive trypsin was 3 \( \mu \)M whereas the concentration of the inhibitors was chosen such that the association was near completion (\([I_0]\) being the largest inhibitor concentration used, the \( \text{R}_c \) complex does not accumulate to a significant extent during the inhibition reaction. Thus, for the interaction of wild-type trypsin with AT or P1R-ACT, \( K_{\text{eq}}^\ast \) is much larger than 90 or 2 \( \mu \)M, respectively. The second order association rate constants \( k_{2}^\ast / K_{\text{eq}}^\ast \) calculated from the slope of the curves in Fig. 1 (A and B) were found to be \( 1.1 \times 10^6 \text{ M}^{-1} \text{s}^{-1} \) for the inhibition of trypsin by P1R-ACT, and \( 2 \times 10^4 \text{ M}^{-1} \text{s}^{-1} \) for the inhibition of trypsin by AT.

The FI-labeled active trypsin mutants were reacted with either AT-TMR or P1R-ACT-TMR in a stopped-flow apparatus under pseudo-first order conditions (\([I_0]\) \( \gg \) \([E]_0 \)). Variations of FRET from fluorescein to tetramethylrhodamine were followed

**RESULTS**

**Kinetics of the Inhibition of Active Trypsins by AT and P1R-ACT**

Wild-type and active mutants of rat trypsin Q23CTRY, K113CTRY, A122CTRY, and S125CTRY were reacted with either AT or P1R-ACT. Both serpins were able to inhibit each trypsin variant with a 1/1 ratio. Each unlabeled or fluorescently labeled proteinase-serpin pair gave SDS-resistant complexes on SDS-polyacrylamide gels (data not shown).

The kinetic mechanism of trypsin inhibition was assessed by measuring \( k_{\text{obs}} \) as a function of serpin concentration as indicated under “Materials and Methods.” As shown in Fig. 1, \( k_{\text{obs}} \) varies linearly with the serpin concentration, indicating that the binding of the two partners conforms to a simple biomolecular reaction (\( E + I \rightarrow EI \)) (30). However, it has been shown that, for the reaction of AT with porcine pancreatic elastase (9, 13) or human cathepsin G (31), \( k_{\text{obs}} \) varies hyperbolically with the AT concentration, diagnosing a minimum two-step mechanism for the inhibition by serpins.

\[ K_{\text{eq}}^\ast = \frac{k_{2}^\ast}{k_{1}} \]

(Eq. 6)

\( EI^\ast \) is the initial encounter complex. The step \( EI^\ast \rightarrow EI \) may include several reversible or irreversible steps like translocation and acylation as shown previously (13). Scheme 2 predicts (30) the result shown in Equation 6.

\[ k_{\text{obs}} = \frac{k_{2}[I]}{[I] + K_{\text{eq}}^\ast \left( 1 + \frac{[I]}{K_{\text{eq}}^\ast} \right)} \]  

(Eq. 6)

\([I]_0\) is the concentration of the ligand that competes with the serpin for the binding of trypsin (p-aminobenzamidine or p-nitroanilide substrate) with the equilibrium dissociation constant \( K_{\text{eq}}^\ast \). Equation 6 describes a linear dependence of \( k_{\text{obs}} \) on \([I]_0\), if \([I]_0 \ll K_{\text{eq}}^\ast(1 + [I]_0/K_{\text{eq}}^\ast)\). Thus, if \( K_{\text{eq}}^\ast \gg [I]_0(1 + [I]_0/K_{\text{eq}}^\ast)\), \([I]_0\) being the largest inhibitor concentration used, the \( EI^\ast \) complex does not accumulate to a significant extent during the inhibition reaction. Thus, for the interaction of wild-type trypsin with AT or P1R-ACT, \( K_{\text{eq}}^\ast \) is much larger than 90 or 2 \( \mu \)M, respectively. The second order association rate constants \( k_{2}^\ast / K_{\text{eq}}^\ast \) calculated from the slope of the curves in Fig. 1 (A and B) were found to be \( 1.1 \times 10^6 \text{ M}^{-1} \text{s}^{-1} \) for the inhibition of trypsin by P1R-ACT, and \( 2 \times 10^4 \text{ M}^{-1} \text{s}^{-1} \) for the inhibition of trypsin by AT.
with time. The curves shown in Fig. 2 (A and B) could only be fitted with at least a double exponential model. Because the concentrations of inhibitor were not sufficient for the accumulation of the first encounter complex, the presence of a double exponential suggests that the rearrangement in our systems is at least a two-step event. With AT, the absence of a first step
during which the chromophores get closer, followed by a second step during which they get away as observed with elastase and AT in previous works (9, 13), confirms that the encounter complex did not accumulate.

To search for non covalently-bound reaction intermediates, we reacted a stoichiometric mixture of K113CTRY (1 \( \mu \)M) with AT-TMR or P1R-ACT-TMR (1 \( \mu \)M) for 15 min, a time that ensures full inhibition of trypsin. The complex was then rapidly mixed in a stopped-flow apparatus with unlabeled BPTI (4 \( \mu \)M), an efficient inhibitor of rat trypsin (32). No change in the FRET was detected during an observation time of 800 s, confirming that all the trypsin molecules were involved in an irreversible complex.

**Kinetics of the Binding of Inactive Trypsins with AT and P1R-ACT**

To study the reversible steps occurring before the acylation step, the active mutants A122CTRY and K113CTRY were further mutated on the serine residue of their catalytic triad. Both A122C/S195ATRY and K113C/S195ATRY were labeled with Fl and used for distance measurements (see below). The former was used to measure the equilibrium dissociation constant \( K_d \) of the complexes formed of inactive trypsin and AT or P1R-ACT. Increasing concentrations of AT-TMR or P1R-ACT-TMR were reacted with constant concentration of A122C/S195ATRY-Fl in a stopped-flow apparatus, and the FRET from Fl to TMR was recorded until it was stable with time. Fig. 3 shows the fluorescence intensity as a function of AT-TMR (Fig. 3A) and P1R-ACT-TMR (Fig. 3B) concentration. The calculation of the overall binding constant \( K_d \) was based on the following equation (9).

\[
\frac{\Delta F}{F_0} = \frac{[E_0] + [1]_0 + K_d}{2[E_0]} - \frac{\sqrt{[E_0] + [1]_0 + K_d^2 - 4[E_0][1]_0}}{2[E_0]} \times \frac{\Delta F_{\text{max}}}{F_0}
\]

(Eq. 7)

\( \Delta F \) is the difference between the fluorescence intensity at \( t = 0 \) (\( F_0 \)) and that at the time of completion of the reaction. \( \Delta F_{\text{max}} \) is the asymptotic value of \( \Delta F \) for infinite concentrations of inhibitor. Iterations were done on \( K_d \) and \( \frac{\Delta F_{\text{max}}}{F_0} \). For the binding of A122C/S195ATRY-Fl with AT-TMR, the least square fitting yielded \( K_d = 1.4 \) \( \mu \)M. The \( K_d^* \) value for the complex of active enzyme with AT, as estimated above was found to be larger than 90 \( \mu \)M, which is at least 64-fold higher than the overall \( K_d \) value. If we make the reasonable assumption that the binding of the initial encounter complex between active or inactive trypsin and AT are of the same order of magnitude, i.e., that \( K_d^*(\text{active trypsin}) \sim K_d^*(\text{inactive trypsin}) \), the foregoing observation provides evidence that one or several favorably equilibrated steps involving interchromophore distance changes follow the encounter of the enzyme with the serpin in the absence of any catalytic step. A122C/S195ATRY-Fl reacted with P1R-ACT-TMR with an overall \( K_d \) of 2.1 \( \mu \)M. At an inhibitor concentration of 2.1 \( \mu \)M, no accumulation of the encounter complex could be detected with the active enzyme (see above). This again indicates that least one favorably equilibrated step shifts the initial equilibrium \( E + 1 \rightarrow ET^* \) toward the final state. In correlation with the above observations, it was interesting to study the kinetics of inactive trypsin-serpin association in the same conditions. Fig. 4 shows the time dependence of the reaction of A122C/S195ATRY-Fl with AT-TMR (red trace). None of the curves obtained in pseudo-first order conditions could be fitted to a simple exponential equation. The inset to Fig. 4 illustrates the attempts to fit the traces obtained with each serpin to a single-exponential function. Only the three-exponential fits gave an acceptable description of the curves. In the example given in Fig. 4 (red trace), the least square fitting yielded 31, 2, and 0.13 s\(^{-1}\) for \( c_1 \), \( c_2 \), and \( c_3 \), respectively with 1.12, 0.55, and 0.32 for their respective amplitudes. Because the experiment was carried out with a concentration well under the estimated \( K_d^* \) and because we observed exclusively interchromophore distance changes (see under "Time-resolved Fluorescence on Trypsin-Serpin Complexes Formed with Inactive Trypsin") the observation of a triple exponential behavior reveals that at least three well separated rearrangement steps follow the initial encounter. Reaction of A122C/S195ATRY-Fl with P1R-ACT-TMR (Fig. 4, blue trace) also yielded stopped-flow traces that could only be described by a three-exponential fitting with \( c_1 = 23 \), \( c_2 = 2.1 \), and \( c_3 = 0.24 \) s\(^{-1}\) with the amplitudes of 0.74, 0.49, and 0.48, respectively. From the experiments reported above, we know that \( K_d^* \) for the active trypsin-P1R-ACT complex is much higher than 2 \( \mu \)M. If we assume again that \( K_d^*(\text{active trypsin}) \sim K_d^*(\text{inactive trypsin}) \), we may conclude that the triple-exponential behavior reflects the occurrence of at least three rearrangement steps. A122C/S195ATRY-Fl was also reacted with BPTI-TMR as a control experiment. The stopped-flow trace shown in Fig. 4 (in green) could be satisfactorily described by a single exponential decay.
ruling out any heterogeneity in the proteinase sample as being responsible for the multixponential behavior. To confirm that the reversible reaction of A122C/S195ATRY-Fl with AT-TMR or P1R-ACT-TMR is a multistep process, a mixture of 0.7 μM inactive enzyme and 4 μM serpin were incubated for 15 min and then rapidly mixed with a 4 μM solution of unlabeled BPTI used as a high affinity competitor (32). The increase of fluorescence at 415 nm caused by the decrease of FRET as the proteinase binds to the unlabeled BPTI was followed with time (Fig. 5). For both serpins a triple-exponential fitting was necessary to describe the experimental traces, indicating that at the time of mixing with BPTI, several conformations of the complex were in equilibrium. The best fits were obtained with $c_1 = 1 \text{s}^{-1}$, $c_2 = 0.056 \text{s}^{-1}$, and $c_3 = 0.0048 \text{s}^{-1}$ with the respective amplitudes of $-0.32$, $-0.36$, and $-0.23$ for AT and $c_1 = 0.77 \text{s}^{-1}$, $c_2 = 0.063 \text{s}^{-1}$, and $c_3 = 0.011 \text{s}^{-1}$ with the respective amplitudes of $-0.078$, $-0.31$, and $-0.41$ for P1R-ACT.

**Variation of Tetramethylrhodamine Fluorescence in P1R-ACT-TMR upon Binding to Unlabeled K113CTRY and K113C/S195ATRY**

The TMR label of P1R-ACT-TMR is bound at position Cys-150 located at the lower end of F helix. This position is close to that of the proteinase in the final serpin-proteinase complex (3). The TMR label should therefore report the late events of the proteinase migration. To observe these events, we have recorded the kinetics of TMR fluorescence emission following stopped-flow mixing of P1R-ACT-TMR with unlabeled trypsin. The traces are shown in Fig. 6. No significant change in fluorescence takes place upon reaction with the inactive trypsin A122C/S195ATRY (trace a) or K113C/S195ATRY (trace b) under concentration conditions where binding of these variants with the inhibitor is complete. In contrast, reaction of P1R-ACT-TMR with active K113CTRY exhibits a biphasic fluorescence variation (trace c), indicating that the TMR group located at position 150 shifts from an initial to a final environment via an intermediate state. Because these experiments had to be done in second order conditions, we needed to ensure that the proteinase migration was complete during the observation time. When reacted under identical concentration conditions, P1R-ACT-TMR and K113CTRY-Fl exhibit a FRET variation indicating that during the 50-s observation time the distance between the two chromophores decreases continuously to stabilize at the end of the observation time (curve d). With the inactive enzymes, the absence of perturbation of the lower end of F helix predicts that the extent of the proteinase migration will be different from the one obtained with the active enzymes.

**Time-resolved Fluorescence on the Trypsin-Serpin Complexes Formed with Active Trypsin**

*Complexes with AT*—The fluorescence spectra (one example is given in Fig. 7), the quantum yields, and the fluorescence decays (Table I) of the four active Fl-labeled trypsin molecules are strongly similar, suggesting a similar environment for the Fl dye in these molecules. In each protein, the fluorescence decay is dominated by a major 4.02–4.09-ns component with a relative amplitude of ~90%. The minor 0.8–1.0-ns component is frequently observed in Fl-labeled proteins (33) and may correspond to a different conformational species. No changes in either the fluorescence spectra or the time-resolved parameters are observed with the addition of the unlabeled AT inhibitor. This suggests that no direct interaction between the bound inhibitor and Fl may occur in the complexes. Addition of labeled AT-TMR to each Fl-labeled enzyme variant induces a significant decrease in the steady-state fluorescence of Fl as well as in both its long-lived lifetime and relative amplitude. In addition, a 1.3–1.37-ns component appears with a relative amplitude of ~25–30%. Because the decrease in the mean fluores-
Fluorescence lifetime exactly matches with the decrease in fluorescence quantum yield in each case, no static quenching may occur. Accordingly, we could easily recalculate the relative amplitudes by taking into account the fractional labeling, $f_A = 0.76$ of the AT-TMR molecules (Table I).

In a next step, we reasonably assumed that both lifetimes of

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**FIG. 5.** Stopped-flow kinetics of the dissociation of 0.7 μM inactive trypsin A122C/S195ATRY-F1 from its complex prepared with 4 μM AT-TMR (trace 1) or 4 μM P1R-ACT-TMR (trace 2) as monitored by FRET variation. The dissociation of the complexes was provoked by rapidly adding 4 μM unlabeled BPTI as a competitor for the inactive enzyme. Each trace is the average of at least four experiments. The inset displays the first 20 s of the dissociation experiment with an expanded scale.

**FIG. 6.** Variation with time of the fluorescence emission of tetramethylrhodamine at position 150 in P1R-ACT-TMR. In traces a and b, 10 μM serpin were reacted with 10 μM unlabeled inactive trypsin derivatives A122C/S195ATRY (a) or K113C/S195ATRY (b). Trace c shows the reaction of 1.1 μM serpin with 1 μM active trypsin K113CTRY. Trace d shows the variation of FRET from K113CTRY-F1 to P1R-ACT-TMR at the same concentrations as for trace c. Each curve is the average of at least four experiments.
the trypsin-Fl/AT-TMR complexes might be correlated to the long-lived lifetime of the complex with the unlabeled inhibitor. Using this hypothesis, we calculated the energy transfer efficiencies, $E$, from Equation 3 by assuming that $\tau_{ld}$ corresponds to the long-lived lifetime of the corresponding trypsin-Fl/AT complex. The Förster distance, $R_{0}$, is calculated with Equation 4. Because both the Fl and TMR dyes are covalently bound to solvent-exposed Cys residues through flexible linkers, it is reasonable to assume that both dyes undergo a complete dynamic isotropic orientational averaging and, thus, that the orientational factor, $\kappa^2 = 2/3$. Using the quantum yields of Table I and an overlap integral, $J_{AA'} = 3.34\times10^{-13}$, a $R_0$ value of 57 Å was found for each Fl-labeled derivative. Finally, the interchromophore distances are calculated with Equation 5 and reported in Table II. Two classes of species could be clearly evidenced for each trypsin derivative. The main class corresponds to interchromophore distances of 82–90 Å and represents ~65–70% of the species. The second class corresponds to species with interchromophore distances of only 50–51 Å. According to the size of the linkers (between the dye and the Cys residue) (34), these two classes likely correspond to two distinct types of complex with a different position of trypsin on the surface of AT. The expected distances for the final covalent complex and for the encounter complex are displayed in Fig. 8 (a and b, respectively). The set of long distances of 82–90 Å considering the errors caused by the size of the chromophores clearly identify a type of complex compatible with the one crystallized by Huntington et al. (3). This species would thus be the major species, but a minor conformer with distances in the range of 50–51 Å is present. Because these distances are compatible with this com-
is the homologous position for Ser-150 in P1R-ACT. Val-222 of alaserpin is the homologous position for Cys-232 in AT. Trypsin is colored in Bank entry 1I99 (8), describing the reversible encounter complex between insect alaserpin and active site modified rat trypsin. Ser-138 of alaserpin by POV-RAY (43).

The serpins are colored in blue, and their values (in Å) are in white. Distances are materialized by straight lines in cyan, and their values (in Å) are in light brown. Helix F is colored in green. The serpins are colored in blue (β-sheets), in red and yellow (helices), and in light brown (coils). Helix F is colored in green. Distances are materialized by straight lines in cyan, and their values (in Å) are in white. Schematic structures were generated by MOLMOL (44) and rendered by POV-RAY (45).

The anisotropy decay of the free protein is characterized by three correlation times: 0.3, 1.6, and 11 ns, which may be associated to the local, segmental, and overall tumbling motions, respectively. The long correlation time is fully consistent with the 10.4-ns correlation time expected for the tumbling of a globular protein with the same molecular mass (25 kDa) as trypsin (35). The addition of either the unlabeled or the labeled enzyme does not hinder these motions. In contrast, a sharp increase of the long correlation time is observed. The measured correlation time (35 ns) is in good keeping with the 28-ns correlation time expected for the tumbling of a globular protein with the same molecular mass (25 kDa). Importantly, no correlation time corresponding to the tumbling of the isolated enzyme could be observed, suggesting that the enzyme is mainly present in its bound form. Moreover, the addition of

| Trypsin + serpin complex | $D_0^a$ | $a_0^b$ | $D_1$ | $a_1^b$ | $D_2$ | $a_2^b$ | $D_3$ | $a_3^b$ |
|--------------------------|--------|--------|-------|--------|-------|--------|-------|--------|
| A122CTRY-FI + AT-TMR     | 50     | 35     | 83    | 65     |       |        |       |        |
| Q23CTRY-FI + AT-TMR      | 51     | 30     | 90    | 70     |       |        |       |        |
| K113CTRY-FI + AT-TMR     | 50     | 35     | 82    | 65     |       |        |       |        |
| S125CTRY-FI + AT-TMR     | 51     | 33     | 86    | 67     |       |        |       |        |
| A122CS165ATRY-FI + AT-TMR| <27    | 52     | >90   | 12     | >110  | 36     |       |        |
| K113CS165ATRY-FI + AT-TMR| <27    | 58     | >110  | 9      | >110  | 33     |       |        |
| A122CTRY-FI + P-R-ACT-TMR| <27    | 35     | 12    | 7      | 13    | 103    | 40    |        |
| Q23CTRY-FI + P-R-ACT-TMR | <27    | 30     | 12    | 86     | 14    | 103    | 44    |        |
| K113CTRY-FI + P,R-ACT-TMR| <27    | 31     | 14    | 68     | 15    | 99     | 40    |        |
| S125CTRY-FI + P,R-ACT-TMR| <27    | 30     | 15    | 72     | 17    | 111    | 38    |        |
| A122CS165ATRY-FI + P,R-ACT-TMR| <27    | 22.5  | 37    | 9      | >95   | 21     | 92    | 47.5   |
| K113CS165ATRY-FI + P,R-ACT-TMR| <27    | 25     | 34    | 11     | 50    | 20     | 90    | 44     |

\(^a\) The distances were calculated from Equations 3–5, as described in the text.

\(^b\) The relative proportions of the various species are considered to be equal to the relative amplitudes calculated in Tables I, IV, and V.

\(^c\) The upper limit distances were calculated from Equations 3–5, by using $\tau_{iD} = \tau_{iDA} + SD_{iD}$ and $\tau_{iDA} = \tau_{iDA} - SD_{iDA}$, where $\tau_{iD}$ and $SD_{iD}$ are, respectively, the mean and standard deviation of the $i$th lifetime in the absence of acceptor. Similarly, $\tau_{iDA}$ and $SD_{iDA}$ are, respectively, the mean and standard deviation of the $i$th lifetime in the presence of acceptor.

**TABLE II**

Interchromophore distances and relative proportions of the species present in the various trypsin-Fl serpin-TMR complexes at equilibrium

**Fig. 8.** Expected interchromophore distances according to the two proteinase-serpin complex models available in the Protein Data Bank. Panels a and c are derived from the Protein Data Bank entry 1EZX (3) describing the covalent complex of bovine trypsin with AT. For our purposes bovine trypsin was replaced by rat trypsin (1ANE) by least square fitting on the homologous fragments with the program PREPI (see Footnote 2) in panel c. Thr-150 of AT is the homologous position of Ser-150 of P1R-ACT. Val-222 of alaserpin is the homologous position for Cys-232 in AT. Trypsin is colored in Bank entry 1I99 (8), describing the reversible encounter complex between insect alaserpin and active site modified rat trypsin. Ser-138 of alaserpin by POV-RAY (43).
BPTI (M<sub>r</sub> = 6000) to the K113CTRY-Fl/AT-TMR complex did not induce any change in the anisotropy decay (data not shown), suggesting that the complex is not reversible. This rules out the possibility that we observed remaining encounter complex. In addition, the fact that both local and segmental motions contribute to more than 60% of the anisotropy decay of complex. In addition, the fact that both local and segmental rules out the possibility that we observed remaining encounter shown), suggesting that the complex is not reversible. This ACT and TMR-labeled P1R-ACT molecules on the fluorescence P1R-ACT. In contrast, the binding of TMR-labeled P 1R-ACT to the active Fl-labeled trypsin-binding of non-labeled P1R-ACT to the active Fl-labeled trypsin depends on the distance studied in the presence of either non-labeled or TMR-labeled the fluorescence of the E1 species where Fl and TMR are close to the species associated to the long-lived lifetime in the tryptophan derivative close to the Fl-labeled trypsin-Fl/P1R-ACT complex and are thus characterized by an interchromophore distance of −35 Å. Using these assumptions, we may deduce the relative populations and the interchromophore distances for the coexisting conformations of the complexes (Table II). In each trypsin mutant-P1R-ACT complex, two classes of species could be distinguished: one with short interchromophore distances (below 36 Å) representing 42–47% of the complexes and one with long interchromophore distances (>68 Å). The set of complexes with distances below 36 Å is compatible with the type of complex revealed by Huntington et al. (3) as shown in Fig. 8c. The set of complexes with the distances above 68 Å, however, is not compatible with the former model and could even be the encounter complex as shown in Fig. 8d. As for the complexes with AT, to investigate this possibility, the anisotropy decay of K113CTRY-Fl has been studied in the presence of either non-labeled or TMR-labeled P1R-ACT (Table III).

In contrast to AT, the binding of non-labeled P1R-ACT slightly decreases the relative amplitudes of both segmental and local motions, suggesting that both motions are restricted by the bound P1R-ACT in at least some species. In contrast, addition of labeled P1R-ACT even slightly increases the amplitudes of the local and segmental motions, as compared with the free protein. Because the energy transfer almost fully quenches the fluorescence of the E1 species where Fl and TMR are close to each other, it follows that this increased motion is observed in the species with large interchromophore distances. Moreover, from the comparison with the data of the complexes with non-labeled P1R-ACT, it may be further concluded that the local and segmental motions of Fl in the latter complexes are restricted in species where the bound inhibitor is close to the Fl-labeled position. This interesting feature suggests that the fluorophores are in close contact to the side chains of the serpin in this complex. However, Fig. 8c clearly shows that this is not the case with the final complex described by Huntington et al. (3) unless the proteinase is tilted by an angle of −90°. Alternatively, internal bending resulting from the constraints described in Refs. 3, 7, and 36 could provoke these changes in the local and segmental motion of fluorescein. This feature was not present in the complex with AT, suggesting that the shape of

**Table III**

| Trypsin + serpin | 0<sup>a</sup> | β<sub>r</sub> | 2<sup>a</sup> | β<sub>r</sub> | 3<sup>a</sup> | β<sub>r</sub> | r<sub>r</sub> |
|-----------------|----------|----------|----------|----------|----------|----------|---------|
| K113CTRY-Fl     | 0.31 (± 0.05) | 35 (± 6) | 1.6 (± 0.6) | 26 (± 6) | 11 (± 1) | 39 (± 1) | 0.31 (± 0.01) |
| + AT            | 0.36 (± 0.03) | 42 (± 2) | 2.2 (± 0.4) | 23 (± 2) | 35 (± 6) | 35 (± 1) | 0.32 (± 0.01) |
| + AT-TMR        | 0.30 (± 0.02) | 40 (± 3) | 2.2 (± 0.6) | 29 (± 2) | 30 (± 3) | 31 (± 2) | 0.31 (± 0.01) |
| + P1R-ACT       | 0.34 (± 0.05) | 29 (± 3) | 2.0 (± 0.3) | 20 (± 2) | 39 (± 4) | 51 (± 2) | 0.31 (± 0.01) |
| + P1R-ACT-TMR   | 0.36 (± 0.07) | 41 (± 3) | 1.6 (± 0.2) | 25 (± 3) | 43 (± 4) | 31 (± 2) | 0.31 (± 0.01) |
| K113CS195ATRY-Fl| 0.37 (± 0.06) | 34 (± 3) | 2.9 (± 0.7) | 15 (± 4) | 13 (± 3) | 51 (± 2) | 0.31 (± 0.01) |

<sup>a</sup> The rotational correlation times, 0<sub>r</sub>, the associated relative amplitude, β<sub>r</sub>, and the fundamental anisotropy, r<sub>r</sub>, were obtained from maximum entropy method analysis, as described under “Materials and Methods.”
### Table IV

**Steady-state and time-resolved fluorescence parameters of the active Fl-labeled trypsin derivatives in interaction with antichymotrypsin**

| Trypsin ± serpin | \( \alpha_0 \) | \( \tau_1^a \) | \( a_1^a \) | \( \tau_2^a \) | \( a_2^a \) | \( \tau_3^a \) | \( a_3^a \) | \( \phi \) |
|------------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
|                  | \( \% \) | \( ns \) | \( \% \) | \( ns \) | \( \% \) | \( ns \) | \( \% \) | \( ns \) |
| A122CTR-Y-FI     | 1.7 (± 0.2) | 19.3 (± 1) | 4.06 (± 0.02) | 80.7 (± 1) | 3.6 (± 0.02) | 53 (± 0.03) |
| + P, R-ACT       | 1.58 (± 0.03) | 18.2 (± 0.7) | 4.13 (± 0.01) | 81.8 (± 0.4) | 3.68 (± 0.01) | 51 (± 0.03) |
| + P, R-ACT-TMR   | 0.2 (± 0.01) | 18 (± 0.6) | 4.01 (± 0.03) | 61.4 (± 1) | 27.6 (± 0.04) | 25 (± 0.02) |
| Q23CTR-Y-FI      | 1.8 (± 0.2) | 62 (± 0.2) | 4.06 (± 0.02) | 81 (± 2) | 3.63 (± 0.02) | 53 (± 0.04) |
| + P, R-ACT       | 1.6 (± 0.1) | 19 (± 1) | 4.13 (± 0.03) | 81 (± 1) | 3.65 (± 0.02) | 51 (± 0.03) |
| + P, R-ACT-TMR   | 0.22 (± 0.01) | 17.3 (± 1) | 4.02 (± 0.01) | 62.1 (± 0.6) | 2.8 (± 0.03) | 28 (± 0.02) |
| K113CTR-Y-FI     | 1.7 (± 0.2) | 16.2 (± 1) | 4.03 (± 0.04) | 83.8 (± 1) | 3.65 (± 0.02) | 59 (± 0.03) |
| + P, R-ACT       | 1.65 (± 0.1) | 16.7 (± 0.7) | 4.15 (± 0.02) | 83.3 (± 1) | 3.73 (± 0.07) | 60 (± 0.03) |
| + P, R-ACT-TMR   | 0.22 (± 0.03) | 19.6 (± 0.9) | 4.01 (± 0.02) | 58.6 (± 0.9) | 2.66 (± 0.05) | 29 (± 0.02) |
| S125CTR-Y-FI     | 1.8 (± 0.3) | 19 (± 3) | 4.06 (± 0.06) | 81 (± 3) | 3.6 (± 0.04) | 50 (± 0.03) |
| + P, R-ACT       | 1.6 (± 0.2) | 19 (± 2) | 4.09 (± 0.04) | 81 (± 2) | 3.62 (± 0.08) | 49 (± 0.03) |
| + P, R-ACT-TMR   | 0.21 (± 0.03) | 21.9 (± 0.6) | 4.02 (± 0.02) | 53.7 (± 0.9) | 2.52 (± 0.03) | 24 (± 0.03) |

* The parameters are calculated and expressed as in Table I.

**Table V**

**Steady-state and time-resolved fluorescence parameters of the inactive Fl-labeled trypsin derivatives in interaction with AT and P, R-ACT**

The parameters are calculated and expressed as in Tables I and IV.

| Trypsin ± serpin | \( \alpha_0 \) | \( \tau_1 \) | \( a_1 \) | \( \tau_2 \) | \( a_2 \) | \( \tau_3 \) | \( a_3 \) | \( \phi \) |
|------------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
|                  | \( \% \) | \( ns \) | \( \% \) | \( ns \) | \( \% \) | \( ns \) | \( \% \) | \( ns \) |
| A122C/S195ATRY-FI | 1.1 (± 0.2) | 18 (± 3) | 4.09 (± 0.08) | 82 (± 3) | 3.55 (± 0.04) | 52 (± 0.03) |
| + AT             | 0.97 (± 0.08) | 22.2 (± 0.7) | 4.13 (± 0.03) | 77.8 (± 0.7) | 3.43 (± 0.09) | 51 (± 0.03) |
| + AT-TMR         | 0.06 (± 0.01) | 11.5 (± 2) | 4.18 (± 0.03) | 67.5 (± 4) | 3.06 (± 0.09) | 32 (± 0.02) |
| + P, R-ACT       | 1.1 (± 0.1) | 24.5 (± 1) | 4.19 (± 0.04) | 75.5 (± 1) | 3.43 (± 0.03) | 51 (± 0.03) |
| + P, R-ACT-TMR   | 0.31 (± 0.01) | 12 (± 4) | 3.96 (± 0.06) | 61 (± 2) | 2.78 (± 0.09) | 32 (± 0.02) |
| K113C/S195ATRY-FI | 1.2 (± 0.1) | 27 (± 2) | 3.8 (± 0.03) | 83 (± 2) | 3.9 (± 0.02) | 59 (± 0.03) |
| + AT             | 2.1 (± 0.2) | 17 (± 2) | 4.33 (± 0.02) | 82.1 (± 0.4) | 3.89 (± 0.06) | 58 (± 0.03) |
| + AT-TMR         | 0.06 (± 0.01) | 19.5 (± 2) | 4.43 (± 0.08) | 80.5 (± 2) | 3.96 (± 0.1) | 33 (± 0.02) |
| + P, R-ACT       | 1.9 (± 0.1) | 22 (± 2) | 4.46 (± 0.04) | 78 (± 2) | 3.9 (± 0.08) | 55 (± 0.03) |
| + P, R-ACT-TMR   | 0.19 (± 0.05) | 15.2 (± 1) | 4.20 (± 0.02) | 58.4 (± 0.9) | 2.93 (± 0.08) | 32 (± 0.03) |

* The relative amplitude of the dark species, \( \alpha_0 \), as well as the corrected relative amplitudes, \( \alpha_n \), of the complexes with AT-TMR and P, R-ACT-TMR were calculated as described in Tables I and IV, respectively. The relative amplitude of the dark species, \( \alpha_0 \), of the complexes with AT-TMR was calculated according to Equation S and divided by \( f_k \), to take into account the fractional labeling of AT.

b The quantum yields in brackets are calculated as described in Table I.
the complex is specific to each serpin-proteinase pair. In addition, the local mobility of Fl in the species with large interchromophore distances is in keeping with our assumption on the dynamic isotropic orientational averaging of the two dyes and, thus, with the use of a value of 2/3 for \( k^2 \). Finally, the absence of a correlation time corresponding to the tumbling of free protein in both complexes with P1R-ACT suggests that, as expected from the enzymatic data, most of the enzyme is in a bound form. As for AT, the anisotropy decay of the K13C/TRY-FI/P-R-ACT-TMR mixture is not affected by the addition of BPTI, implying that the long distance complexes could not be accounted for by remaining encounter complexes. Thus, irreversible complexes are present with the proteinase stuck at an intermediate position.

In summary, time-resolved fluorescence spectroscopy of mixtures of active trypsin with the two serpins reveals the occurrence of two types of irreversible complexes: one with the trypsin molecule located at a position similar to that occupied by the enzyme in the crystal of the final bovine trypsin-AT complex (3) and one with the trypsin molecule located at a position different from that occupied by the enzyme in the encounter complex and the final complex.

Time-resolved Fluorescence on Trypsin-Serpin Complexes Formed with Inactive Trypsin

Complexes with AT—The fluorescence intensity decays of both A122C/S195ATRY-FI and K113C/S195ATRY-FI are similar to those of the active derivatives and are dominated by the long-lived lifetime that represents more than 80% (Table V). Addition of non-labeled AT near saturation does not induce any significant change in the fluorescence parameters, suggesting that its binding does not modify the Fl environment. Moreover, addition of TMR-labeled AT induces only limited changes on the time-resolved fluorescence parameters of A122C/S195ATRY-FI and even no changes on the time-resolved fluorescence parameters of K113C/S195ATRY-FI. These observations are in contrast with the dramatic decrease of the quantum yields and suggest a large population of dark species. This was assessed by calculating a dark species population of 50 and 55% for the complexes with A122C/S195ATRY-FI and K113C/S195ATRY-FI, respectively (Table V). From the comparison of the relative amplitudes (corrected for the partial labeling of AT-TMR) of \( \tau_2 \) and \( \tau_1 \) in the trypsin-FI/AT-TMR complexes with the amplitudes of the corresponding lifetimes in the trypsin-FI/AT complexes, it may be reasonably assumed that the populations associated to both \( \tau_2 \) and \( \tau_1 \) lifetimes in the latter complexes are split into two populations with the binding of AT. A one population will be characterized by the same lifetime as in the complex with non-labeled AT, whereas the other one will be characterized by a very short or null lifetime. This suggests the existence of two different types of species: one complex with an interchromophore distance of less than 30 Å and one complex with an interchromophore distance larger than 90 Å (Table II). None of these distances is compatible with the proteins being arranged as in the encounter complex (see Fig. 8b). The complex displaying short interchromophore distances is compatible with the proteinase being at an intermediate position between the encounter complex and the complex formed of active trypsin and AT characterized by Huntington et al. (3). However, it is different from the intermediate complex (with distances around 50 Å) obtained with the active enzyme. This suggests that the S195A mutants remain closer to the C-terminal domain of the serpin for their most abundant complex with AT. The second type of complex with large interchromophore distances (>90 Å) is less abundant. The later set of distances either suggests the presence of free proteinase, which is unlikely because the concentration of AT was sufficient for reaching near saturation of the proteinase (see Fig. 3A) or defines a yet unknown type of complex.

Complexes with P1R-ACT—As with AT, the addition of non-labeled P1R-ACT at saturating concentration does not modify the fluorescence of either the A122C/S195ATRY-FI or K113C/S195ATRY-FI derivative. In contrast, the addition of P,R- ACT-TMR induces the appearance of a short lifetime (0.2–0.3 ns) with a relative amplitude of 12–15% and slightly decreases the long-lived lifetime as well as its relative amplitude in both trypsin mutants. These changes induce a 22–27% decrease in the mean lifetime that is not sufficient to account for the 39–46% decrease of the quantum yield. This suggests again the existence of dark species, with a calculated amplitude of 22–26%. An additional striking feature in the complexes of both proteins with P1R-ACT-TMR is that both the \( \tau_2 \) lifetime and its relative amplitude are similar to those of the corresponding complexes with non-labeled P1R-ACT. If we assume that this corresponds to the same population of molecules, the dark species as well as the species with \( \tau_1 \) and \( \tau_2 \) lifetimes in trypsin-P1R-ACT-TMR may then be related to the \( \tau_1 \) lifetime in the trypsin-FI/P1R-ACT complex. Using this hypothesis, the interchromophore distances were calculated (Table II). As with AT-TMR, two classes of trypsin-FI/P1R-ACT-TMR complexes are observed for both inactive trypsin derivatives. In the first class (representing approximately one third of the species), the distance between Fl and TMR are below 40 Å, whereas in the second class, the interchromophore distance is larger than 80 Å. The absence of a correlation time corresponding to the tumbling of the free enzyme in the anisotropy decay of the E-FI/ P1R-ACT-TMR complex suggests that the species with large interchromophore distances correspond to trypsin-serpin complexes (Table III). This conclusion is in agreement with the binding data suggesting that 90% of the enzyme molecules be bound in these conditions. As for the active enzymes, the type of complex with short interchromophore distances is compatible with the complex described by Huntington et al. (3) (see Fig. 8c). However, such a set of distances could also come from any intermediate position of the inactive proteinase close to Fl helix. Because no free enzyme is detected by fluorescence anisotropy decay, the type of complex with long interchromophore distances is either the encounter complex (see Fig. 8d) at equilibrium or a similar intermediate conformation with minor orientation changes.

In summary, time-resolved fluorescence spectroscopy of mixtures of inactive trypsin with AT or P1R-ACT also diagnoses the occurrence of two types of reversible trypsin-serpin complexes.

DISCUSSION

In this work, we compared the trajectories of active and inactive rat trypsin upon binding to AT or P1R-ACT. We reasonably assumed that the starting point of the interaction is a first encounter complex similar to that resolved crystallographically by Ye et al. (8) (see Fig. 8). The equilibrium dissociation constants \( K_d \) for the complexes formed of trypsin and AT or P1R-ACT were found to be higher than 90 and 2 \( \mu \text{M} \), respectively. This gave us the opportunity to study trypsin-serpin interactions using concentrations for which no significant accumulation of the encounter complex occurs. Under these conditions, intermediate steps in the inactive trypsin-serpin pathway could be observed. Each of the four active trypsins and each of the two inactive trypsins was labeled with fluorescein at a single cysteine residue. Likewise, the two serpins were labeled with tetramethylrhodamine at single resi-

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2 S. A. Islam and M. J. E. Sternberg, manuscript in preparation.
The Fate of Active Trypsin—The kinetics of association and rearrangement of active trypsins with AT and P1R-ACT were followed by observation of FRET with time. For all trypsin mutants in reaction with AT labeled at position 232 or P1R-ACT labeled at position 150 (see Fig. 8), the stopped-flow traces revealed a multistep process. Because the encounter complex does not accumulate, these observations are consistent with a rearrangement comprising at least two observable steps. Furthermore, the variation of fluorescence of tetramethylrhodamine at position 150 in the presence of unlabeled active trypsin also showed a two-step perturbation of the lower end of helix F. It is noteworthy that, under the same concentration conditions, the FRET between labeled active trypsin and labeled P1R-ACT shows an uninterrupted bringing nearer of the two chromophores. It should also be noticed that at the end of the experiment with unlabeled active trypsin the rhodamine fluorescence does not return to its initial value (Fig. 6c). This is in accordance with the observation of a shift of helix F visible in the crystal structure of the complex (3). This two-step perturbation of helix F is reminiscent of the observation of an intermediate structure of ACT (37) in which helix F is partially inserted into the A β-sheet. Because the binding of inactive trypsins did not perturb the tetramethylrhodamine fluorescence at position 150, we may hypothesize that an intermediate conformation similar to that found by Gooptra et al. (37) might play a role during the last events of complex formation. These last events may be essential steps in the serpin mechanism because they separate the period of complex reversibility from that of complex irreversibility. In addition, because these events were not observed with inactive trypsins, it is likely that they are related to the process of acylation.

Distance measurements made on the final irreversible complexes yielded two sets of results for each serpin. The first set of distances confirms the previous observation (3) that the proteinase is dragged from the upper part to the lower part of the serpin molecule. In addition, time-resolved fluorescence anisotropy measurements made with one trypsin mutant revealed some steric constraints in the complex formed with P1R-ACT but not with AT. This strongly suggests that the fine structure of the complex may be different for each proteinase-serpin pair. Such a heterogeneity has also been recently proposed by Plotnick et al. (38) on the basis of different complex breakdown kinetics. The second set of distances observed on the final complexes clearly differs from the first one by an average of 30–40 Å. All four trypsin variants displayed this feature, thus eliminating the possibility of a fortuitously unfavorable side chain orientation. The two sets of distances correspond to irreversible complexes because their presence was insensitive to the dissociation action of BPTI. In addition, they were not an artifact caused by the presence of free trypsin as diagnosed by fluorescence anisotropy. The large difference between these two sets of distances clearly demonstrates that two different complexes are simultaneously present in the solution. This observation agrees with that of Calagaru et al. (39), who showed that the final complex might be in equilibrium between two forms, one in which the enzyme is in an active conformation and the other in which the conformation is inactive. We may therefore suggest that our two conformers are also in equilibrium. This hypothesis could provide an alternative explanation to the observations of Plotnick et al. (38), who showed that different serpin-proteinase pairs have different breakdown mechanisms. If one assumes on the one hand that there is an equilibrium between two forms of the final complex, one in which some catalytic activity of the proteinase is left and one in which the catalytic activity is fully abolished and on the other hand that the proportion of these two forms varies with each serpin-proteinase pair, we may easily explain the data of Plotnick et al. (38). Our data may also provide an explanation to the apparently contradictory results obtained by another team in two consecutive papers relating cross-linking experiments on the same serpin-proteinase pair (40, 41). In the first paper using one type of cross-linking agent, the authors came to the conclusion that the enzyme was trapped in an intermediate position, whereas, in the second paper with a different cross-linking agent, the proteinase seemed to be in a position compatible with a full loop insertion. If two complexes in equilibrium were present, it is possible that one of the cross-linking molecules favored one type of complex and thus shifted the equilibrium.

The Fate of Inactive Trypsin—The overall equilibrium dissociation constants for the interaction of inactive trypsin with AT or P1R-ACT are 1 and 2 μM, respectively. We have shown that both these constants are well below $K_i^*$, the equilibrium dissociation constant for the encounter complexes. Consequently inactive trypsin and both serpins associate at concentrations well below $K_i^*$. It may thus be concluded that one or several subsequent complexes accumulate after the formation of the first complex. The association traces for both serpins evidence at least three steps until equilibrium is reached (see Fig. 4). Because our fluorescence variations are purely generated by FRET variations, these steps are characterized by a rearrangement of the complexes. The multistep behavior of this rearrangement was confirmed by the competition experiment with BPTI, which showed slow multistep dissociation of the complexes (see Fig. 5). Thus, if we include the anhydroelastase–AT system described previously (13), we have three enzyme–serpin systems that undergo conformational changes following binding without requiring the catalytic machinery. Single residue labeling enabled us to evaluate the extent of translocation of the inactive proteinase. The kinetic data predicted several species at equilibrium. In accord with these data, time-resolved fluorescence data revealed two sets of distances for each inactive proteinase-serpin pair. For binding with AT one set of distances was lower than 30 Å, whereas the other was larger than 90 Å. None of these distances is compatible with the enzyme remaining at the encounter complex position (see Fig. 8c). For binding with P1R-ACT one set of distances was found to be compatible with a complex in which inactive trypsin occupies a position identical to or close to that it occupies in the encounter complex. The second set of distances might be compatible with the inactive trypsin occupying a position close to that occupied by active trypsin in the final complex (see Fig. 8c) or an intermediate position very close to helix F. Because the fluorescence of tetramethylrhodamine at position 150 showed no perturbation upon binding to inactive trypsin, it is likely that this second set of distances defines a complex in which the trypsin does not migrate beyond helix F. In any case, the latter set of distances is far from compatible with inactive trypsin occupying a position identical to that it occupies in the encounter complex (see Fig. 8d). These results are in apparent contradiction with those of Olson et al. (17), who suggest that binding of serpins with inactive proteinases is a single-step process that does not involve any conformational change. However, their results were obtained with other serpin–proteinase pairs, namely plasminogen activator inhibitor I with trypsin or tissue plasminogen activator. This discrepancy may also suggest that the steps following the initial binding do not necessarily give...
rise to an accumulation of rearranged complexes resulting from unfavorable equilibrium dissociation constants. Consequently, these species would remain undetected. It is likely that, in the near future, when more examples of detailed kinetic data will be available from various serpin-proteinase pairs, all possible situations will be observed. Another work failed to observe any rearrangement upon binding to an inactive trypsin (16). The serpin used contained seven stabilizing mutations providing the molecule with the thermal stability of ovalbumin (42). Such a perturbation in the metastability is expected to impair the fine mechanism of serpin functioning and is unlikely to reflect the mechanism of inhibition by the wild-type serpin.

**Consequences for the Inhibition Mechanism**—The structure of the encounter complex between S195A rat trypsin and alaserpin (8) has interesting new features compared with the structure of canonical proteinase-inhibitor complexes. On the one hand, unusual interactions with the catalytic triad of the enzyme might impair catalysis, and, on the other hand, a loosening of the internal interactions around P14 of the serpin may get narrower in physiological conditions when competitors such as other inhibitors or substrates are present. As seen by following the rhodamine fluorescence at position 150 in P-R-ACT, the proteinase has to be catalytically active to be able to trigger its complete translocation, although important movements may occur without acylation. Thus, with active proteinases, acylation followed by a further rearrangement perturbing the helix F must take place following the first set of rearrangements. No reversibility of these steps could be observed. Finally, the presence of several irreversible complexes at reaction completion suggests that there may be an equilibrium between several irreversible complexes, one of them being similar to the one described by Huntington et al. (3). Scheme 3 is probably valid for any proteinase-serpin pair that forms exclusively inhibitory complexes. However, one proteinase-serpin pair may differ from another by the ability to accumulate or not the encounter complex or any other intermediate species. In addition, the lifetime of each intermediate species may also vary so that their observation may be difficult. These features may be sufficient to explain the diversity of the reactivities within the serpin superfamily.

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Comparative Trajectories of Active and S195A Inactive Trypsin upon Binding to Serpins

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