Serpins are thought to inhibit proteinases by first forming a Michaelis-type complex that later converts into a stable inhibitory species. However, there is only circumstantial evidence for such a two-step reaction pathway. Here we directly observe the sequential appearance of two complexes by measuring the time-dependent change in fluorescence resonance energy transfer between fluorescein-elastase and rhodamine-α1-protease inhibitor. A moderately tight initial Michaelis-type complex $EI_1$ ($K_i = 0.38-0.52 \, \mu M$) forms and dissociates rapidly ($k_1 = 1.5 \times 10^6 \, m^{-1} s^{-1}, k_{-1} = 0.58 \, s^{-1}$). $EI_1$ then slowly converts into $EI_2$ ($k_2 = 0.13 \, s^{-1}$), the fluorescence intensity of which is stable for at least 50 s. The two species differ by their donor-acceptor energy transfer efficiency (0.41 and 0.26, respectively). $EI_2$ might be the final product of the elastase + inhibitor association because its transfer efficiency is the same as that of a complex incubated for 30 min. The time-dependent change in fluorescence resonance energy transfer between fluorescein-elastase and rhodamine-eglin c, a canonical inhibitor, again allows the fast formation of a complex to be observed. However, this complex does not undergo any fluorescently detectable transformation.

Proteolysis, a biologically important event, is regulated by protein protease inhibitors that belong to two major classes: the so-called “canonical” inhibitors and the serine protease inhibitors, called “serpins.” The former are relatively small proteins (29–190 amino acid residues) that belong to numerous structural families (1). The latter are larger proteinases (400–450 residues) that form a single family with highly conserved secondary structural elements (nine α-helices and three β-sheets) (2, 3).

Canonical inhibitors form tight reversible complexes with their cognate enzymes. X-ray crystallography shows that they have an exposed peptidic sequence, the reactive site loop, that forms a “lock and key” complex with the substrate-binding crevice of the proteinase. This Michaelian-like complex is stabilized by a large number of noncovalent bonds which account for the high enzyme-inhibitor binding energy. Its hydrolysis is probably prevented by a small distortion of the P1-P1’ linkage of the inhibitor (1).

Unlike canonical inhibitors, serpins form denaturant-stable complexes with their target proteinases and behave kinetically like irreversible inhibitors (3, 4). Their mechanism of action is not well understood mainly because the tertiary structure of their complexes with proteinases has not yet been solved. A significant number of x-ray structures of active and inactive serpins are, however, available (5–11). These structures reveal that the reactive site loop of serpins is much longer and much more flexible than that of canonical inhibitors. It may easily insert into β-sheet A to form the central strand of this unusually malleable (12) sheet. Full insertion of the P1 to P14 sequence of the reactive site loop may occur spontaneously (9) or following cleavage of the P1-P1’ bond (5). Serpins that have undergone such a β-sheet rearrangement are inactive. On the other hand, introduction into β-sheet A of a tetradecapeptide structurally related to P1-P14 inactivates serpins because it renders them unable to insert their own P1-P14 sequence (13). Thus, full loop-sheet insertion and lack of insertion both yield inactive serpins. This led to the suggestion that loop-sheet insertion modulates the inhibitory activity of serpins (14) and to the proposal of a number of models for the serpin-proteinase interaction (15–18). All these models assume that the inhibition reaction takes place in at least two steps: an initial binding followed by a structural rearrangement that stabilizes the complex. However, these steps have not been observed directly and it is not known at which rate the proteinase moves from its initial to its final position.

Just as chemical kinetics helps elucidating reaction mechanisms (19), inhibition kinetics affords insight into inhibition mechanisms. For instance, several years before publication of the first three-dimensional structure of a canonical inhibitor-protease complex, an inhibition mechanism for a canonical inhibitor has been proposed on the basis of a kinetic analysis (20). Most kinetic studies on serpins have reported measurements of $k_{\text{on}}$, the second-order inhibition rate constant, which is useful for delineating inhibitor efficiency and physiological function (21) but of poor mechanistic significance because it is a combination of rate constants for the individual reaction steps (22). On the other hand, two-step serpin-protease interactions

$$
\frac{k_1}{k_{-1}} I + EI_1 \overset{k_2}{\longrightarrow} EI_2
$$

INTERACTION 1
1The abbreviations used are: elastase, porcine pancreatic elastase (unless another type of elastase is specifically mentioned); α1PI, α1-proteinase inhibitor (α1-antitrypsin); FITC, fluorescein isothiocyanate; TMR, tetramethylrhodamine; TES, N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

The measurements were based on competition experiments in which the serpins displaced a substrate or another ligand from the active center of the proteinase (23–26). The time course of the reaction was thus followed indirectly. In addition, these experimental approaches did not provide access to $k_{-1}$ and $k_1$, but only to $K_z$, the ratio of these two constants.

We hypothesized that labeling of one reaction partner with a fluorescence donor and of the other with a fluorescence acceptor and measuring the time dependence of the fluorescence resonance energy transfer with a fast kinetic apparatus would allow us to directly observe the individual reaction species as well as the dynamics of their interconversion. The present work checks this hypothesis by investigating the interaction of elastase with α1PI, a serpin, and comparing it with binding of elastase with eglin c, a canonical inhibitor.

**MATERIALS AND METHODS**

Porcine pancreatic elastase (elastase)$^1$ was isolated and active site titrated as described previously (27). Recombinant α1PI and eglin c expressed in *Escherichia coli* were obtained from Novartis (Basel) and were titrated with human neutrophil elastase (27, 28).

Fluorescent Labeling of α1PI, Eglin c, and Elastase—α1PI was specifically labeled on Cys-232, its single free cysteinyI residue using tetramethylrhodamine-5-maleimide (Molecular Probes). A 140 μM solution of α1PI was made up in 50 mM TES, 150 mM NaCl, pH 7.0, and reacted with a 1.5 molar excess of the labeling reagent dissolved in dimethylsulfoxide. After 1 h at room temperature, excess reagent was removed by two gel filtrations on a PD-10 column (Amersham Pharmacia Biotech) equilibrated with 50 mM Hepes, 150 mM NaCl, pH 7.4. Active site titration with human neutrophil elastase showed that α1PI was fully active after labeling. Yields varied from 0.7 to 0.9 label/molecule of α1PI as determined spectrophotometrically using ε$_{280\text{ nm}}$ = 75,000 M$^{-1}$ cm$^{-1}$ for protein-bound label. Prior reaction of α1PI with 4-N-(2-iminoethyl)aniline-2'-sulfonic acid (Protein Institute, Philadelphia, PA), a specific reagent of free thiols (29), yielded a protein that did not further bind tetramethylrhodamine-5-maleimide, indicating that the latter specifically labels Cys-232.

Eglin c (1.2 mM) was dissolved in the above TES buffer and reacted with a 3-fold molar excess of the succinimidyl derivative of tetramethylrhodamine dissolved in dimethylsulfoxide. After 1 h at room temperature, excess reagent was removed by gel filtration on a PD-10 column (Amersham Pharmacia Biotech) equilibrated with 50 mM Hepes, 150 mM NaCl, pH 7.4. Active site titration with human neutrophil elastase showed that the labeled inhibitor retained full activity. There was 0.96 molecule of label/molecule of eglin c as determined spectrophotometrically using the above ε$_{280\text{ nm}}$.

Elastase (0.8 mM) was dissolved in 0.1 M carbonate buffer, pH 9.0, and reacted with a 20-fold molar excess of FITC (Molecular Probes) dissolved in methanol. After 1 h at room temperature, excess reagent was removed by gel filtration on two PD-10 columns equilibrated and developed successively with the above carbonate buffer and with 1 mM HCl. Active site titration of FITC-elastase with nonlabeled α1PI showed that the enzyme did not lose activity during labeling. There was 1.1 label/molecule of elastase as determined spectrophotometrically using ε$_{280\text{ nm}}$ = 66,800 M$^{-1}$ cm$^{-1}$ for protein-bound FITC (30). A higher degree of labeling could not be achieved.

**Absorption and Fluorescence Measurements**—Absorption and emission spectra were recorded on a Cary 4 spectrophotometer and a SLM-8000 spectrofluorometer, respectively. Fluorescence quantum yields of free and α1PI-bound FITC elastase were measured using acryflavin as a reference ($φ =$ 0.45). Emission spectra were corrected for screening effects at both excitation and emission wavelengths (31).

Kinetics of Interaction of FITC-elastase with TMR-α1PI or TMR-eglinc was monitored by fluorescence resonance energy transfer from FITC to TMR using a Bio-Logic SFM-3 stopped flow apparatus with a dead time of 1.7 ms (Bio-Logic, Claix, France). The reaction was done in 100 mM Hepes, 150 mM NaCl, pH 7.4, 25 °C. The excitation and emission wavelengths were 450 and 514 nm (Melles-Griot interferential filter), respectively.

**RESULTS**

**Steady-state Fluorescence of Free and Bound FITC-Elastase and TMR-α1PI—** Fig. 1 shows the fluorescence spectra of the free and bound species. It can be seen that reaction of FITC-elastase with TMR-α1PI significantly quenches the emission of FITC and enhances that of TMR, strongly suggesting fluorescence resonance energy transfer between the donor and the acceptor. To see whether part of the fluorescence intensity variation is due to a change in the environment of the label(s) following complex formation, we have recorded the spectrum of the complex formed of FITC-elastase and unlabeled α1PI as well as that formed of TMR-α1PI and unlabeled elastase (Fig. 1). In both curves, the binding of the unlabeled protein only marginally affected the fluorescence of the labeled one. This clearly indicates that all of the fluorescence intensity variation is associated with a nonradiative energy transfer process between FITC and TMR.

**Kinetics of the Interaction of FITC-Elastase with TMR-α1PI—** Fig. 2 shows a typical biphasic stopped flow trace observed upon mixing FITC-elastase with a 10-fold molar excess of TMR-α1PI. The fluorescence intensity rapidly decreases ($t_{1/2}$ ~ 60 ms), falls to a minimum and then slowly recovers ($t_{1/2}$ ~ 5 s) without reaching its initial value. We have tentatively assumed that the initial fluorescence quenching describes the formation of an initial complex $EI_1$, which slowly converts into a second complex $EI_2$ (Fig. 2). The fluorescence intensity was stable for at least 50 s (i.e. 10 $t_{1/2}$ of $EI_2$ formation) whether the reaction was run under pseudo-first-order conditions ([I]$_i$ = 10 [E]$_o$ = 7 μM) or under second-order-conditions ([E]$_o$ = 10 [I]$_i$ = 7 μM). This indicates that within this interval of time, $EI_2$ neither releases free FITC-elastase, which would enhance the fluorescence intensity, nor converts into a further complex with a different donor-acceptor transfer efficiency. The fluorescence intensity could not be recorded beyond 50 s because of photodecomposition of the label(s).

A control where FITC-elastase was mixed with unlabeled α1PI showed a fluorescence decay of about 4% over the observation time of 50 s. This small decrease was considered to be negligible and was not taken into account for further calculations. Data analysis showed that the formation of the two complexes could be described by simple exponentials: the pseudo-first-order rate constant of $EI_1$ formation ($k_{obs}$) was 11.8 ± 0.6 s$^{-1}$ for [I]$_i$ = 10 [E]$_o$ = 7 μM, whereas the first-order rate

![FIG. 1. Fluorescence resonance energy transfer between FITC-elastase and TMR-α1PI-Fig. 1 shows the fluorescence spectra of the free and bound species.](image)

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constant for the conversion of EI₁ into EI₂ (k₁₂) was 0.130 ± 0.004 s⁻¹.

To see whether the EI₁ complex is reversible or irreversible, we have recorded stopped flow traces using constant concentrations of FITC·elastase and variable concentrations of TMR·α₁PI. We have found that the minimum fluorescence intensity (Fig. 2), a measure of the concentration of EI₁, varies hyperbolically with the inhibitor concentration (Fig. 3). This rules out irreversible binding because reaction of constant concentrations of enzyme with increasing concentrations of inhibitor should lead to constant concentrations of complex if binding were irreversible. The data of Fig. 3 were thus analyzed assuming that FITC·elastase (E) and TMR·α₁PI (I) form a reversible complex EI₁ whose equilibrium dissociation constant Kᵰ may be calculated by fitting the fluorescence data to Equation 1.

\[
\frac{\Delta F}{\Delta F_{\text{max}}} = \frac{[E]_o + [I]_o + K_i}{2[E]_o} - \frac{[(E)_i + (I)_o + K_i]^2 - 4(E)_i(I)_o}{2[(E)_i(4E)_o]}
\]  
\[(\text{Eq. 1})\]

where \(\Delta F\) is the difference between the minimum fluorescence intensity of EI₁ and the fluorescence intensity at \(t = 0\) while \(\Delta F_{\text{max}}\) is the asymptotic value of \(\Delta F\) for infinite concentrations of inhibitor. Nonlinear regression analysis yielded \(K_i = 0.52 ± 0.08 \mu M\). The curve calculated using this value fairly well fits the experimental points (Fig. 3).

The above equilibrium may also be described by the rate constant of complex formation and dissociation by the following interaction.

\[
E + I \xrightleftharpoons{k_1}{k_{-1}} EI_1
\]

**INTERACTION 2**

which may also be written as

\[
\frac{k_1[I]_o}{k_{-1}} E \xrightleftharpoons{k}{k_{-k}} EI_1
\]

**INTERACTION 3**

if pseudo-first-order conditions prevail. Hence, \(k_{\text{obs}}\) the pseudo-first-order rate constant for the EI₁ formation is given by

\[
k_{\text{obs}} = k_1[I]_o + k_{-1}
\]  
\[(\text{Eq. 2})\]

**Fig. 2. Kinetics of the fluorescence resonance energy transfer between FITC·elastase and TMR·α₁PI at pH 7.4 and 25 °C.** The excitation and emission wavelengths were 450 and 514 nm, respectively. The elastase and α₁PI concentrations were 0.7 and 7 μM, respectively. Curve a, FITC·elastase + unlabeled α₁PI; curve b, FITC·elastase + TMR·α₁PI. The data-recording frequency of the stopped flow apparatus was 10² s⁻¹ for observation times ≤ 1 s and 10 s⁻¹ for observation times > 1 s, which explains the apparent heterogeneity in the noise level.

To determine \(k_1\) and \(k_{-1}\), we have measured \(k_{\text{obs}}\) under pseudo-first-order conditions. Fig. 4 shows that \(k_{\text{obs}}\) is linearly related to [I], as predicted by Equation 2. The individual rate constants were calculated by linear regression analysis \(k_1 = (1.5 ± 0.02) \times 10^6 \text{ M}^{-1} \text{ s}^{-1}\) and \(k_{-1} = 0.58 ± 0.08 \text{ s}^{-1}\). The equilibrium dissociation constant \(K_i\) of the EI₁ complex, calculated from \(k_1\) and \(k_{-1}\) was 0.38 ± 0.06 μM. This value is in good agreement with that measured under equilibrium conditions and therefore provides good internal consistency to our data.

On the other hand, the fluorescence intensity corresponding to EI₂ did not change with the inhibitor concentration. In addition, \(k_{\text{obs}}\), the first-order rate constant for the conversion of EI₁ into EI₂, was also inhibitor-independent (data not shown).

These two observations are interpreted to mean that k₂ is a true first-order rate constant, i.e. that the transformation of EI₁ into EI₂ is an irreversible process. Our data therefore provide a complete kinetic description of the minimum steps of the interaction of elastase with α₁PI.
Elastase-α₁-Proteinase Inhibitor Interaction

Energy Transfer Efficiency within the FITC-Elastase-TMR-α₁PI Complexes—The transfer efficiency, $E_D$, within the complex prepared by manually mixing labeled elastase with labeled α₁PI (see legend to Fig. 1) was derived from steady-state fluorescence measurements and calculated as follows:

$$ E_D = 1 - \phi_a \phi_D $$

(Eq. 3)

where $\phi_a$ and $\phi_D$ are the quantum yields of the FITC-elastase-TMR-α₁PI complex and the FITC-elastase-α₁PI complex, respectively. $\phi_a$ was corrected to take into account the degree of labeling, $f$, of α₁PI by TMR.

$$ \phi_{D,\text{corrected}} = \frac{\phi_D \text{measured} - (1 - \phi_a) \phi_D}{f} $$

(Eq. 4)

Following corrections for screening effects, $E_D$ was found to be 0.26 ± 0.02.

The transfer efficiencies, $E_D$, within the $E_{I_1}$ and $E_{I_2}$ complexes detected by stopped flow (Fig. 2) were also calculated from the fluorescence intensities at 515 nm, a wavelength at which FITC is the only fluorescent chromophore:

$$ E_0 = 1 - I_{D,\text{obs}} $$

(Eq. 5)

where $I_{D,\text{A}}$ and $I_D$ are the fluorescence intensities of the FITC-elastase-TMR-α₁PI complex and the FITC-elastase-α₁PI complex, respectively. $I_{D,\text{A}}$ was again corrected for the degree of labeling. The screening effect was negligible in the stopped flow experiments. The $E_{I_2}$ complex, $E_2$ was 0.26 ± 0.03, a value identical to that corresponding to the elastase-α₁PI complex prepared by manual mixing.

The value of $I_{D,\text{A}}$ used to calculate $E_D$ for the $E_{I_1}$ complex was taken from the minimum fluorescence intensity (Fig. 2) of stopped flow mixtures containing 0.35 μM FITC-elastase and 11.4 μM TMR-α₁PI, which yield 97% $E_{I_1}$ complex, i.e., in which the fluorescence contribution of free FITC-elastase is negligible. After correcting for the degree of labeling, $E_D$ was found to be 0.41 ± 0.04. Possible random labeling of the lysine residues of elastase precluded interchromophore distance calculations using the $E_D$ values.

Kinetics of the Interaction of FITC-Elastase with TMR-Eglin c—Eglin c is a reversible proteinase inhibitor that belongs to the class of canonical inhibitors (1). Mixing FITC-elastase with a 10-fold molar excess of TMR-eglin c yields an exponential fluorescence quenching whose amplitude does not change during the 50-s observation time (Fig. 5). This behavior sharply contrasts with that of α₁PI (Fig. 2). The reversible binding of elastase with eglin c (22) therefore takes place in only one fluorescently detectable step. We have measured the rate constant $k_{\text{obs}}$ of fluorescence energy transfer as a function of inhibitor concentration under pseudo-first-order conditions ([I]₀ = 10 [E]₀). Fig. 6 shows that $k_{\text{obs}}$ increases linearly with the inhibitor concentration as already observed with α₁PI and described by Equation 2. The association rate constant $k_1$ calculated from this plot was found to be 1.8 × 10⁶ M⁻¹ s⁻¹, in good agreement with the value of 10⁶ M⁻¹ s⁻¹, measured by enzymatic means (22). The $k_1$ value could not be determined accurately because the eglin c concentrations used in these experiments were at least 86-fold higher than the $K_i$ of the elastase-eglin c complex ($K_i = 10^{-6}$ M; Ref. 22) so that $k_1$ [I]₀ ≫ $k_{-1}$ and $k_{\text{obs}} \approx k_1$ [I]₀ in Equation 2.

DISCUSSION

Stopped flow recording of the changes in fluorescence resonance energy transfer observed upon mixing FITC-elastase with TMR-α₁PI has enabled us to directly observe the sequential appearance of two enzyme-inhibitor complexes with significantly different donor-acceptor energy transfer efficiencies. It is commonly assumed but rarely demonstrated that the first step of the reaction of a proteinase with a serpin is the formation of a reversible Michaelis-type complex involving the substrate-binding site of the enzyme and the reactive site loop of the inhibitor. Serpin-induced displacement of ligands from the active center of proteinases (23–26) or dissociation of serpin-proteinase complexes by α₂-macroglobulin (32, 33) have provided indirect evidence for such a reversible binding step. In the present work an initial complex referred to as $E_{I_1}$ forms in less than 1 s after mixing. Rate and equilibrium measurements of the building up of this species clearly demonstrate that it is a fast equilibrating reversible complex. We believe this is the first direct evidence for a Michaelis-type complex between a proteinase and a serpin. On the other hand, the conversion of $E_{I_1}$ into $E_{I_2}$ is a true first-order process because its rate and amplitude do not depend upon the inhibitor concentration. This indicates that the formation of $E_{I_1}$ and hence the overall elastase-α₁PI interaction is an irreversible process, a view supported by enzymatic data showing that α₁PI and other serpins behave like irreversible proteinase inhibitors (4). The two-step
model presented here is probably a minimal one because fluorescence detects only those species that significantly differ in energy transfer efficiency, whereas it is not unlikely that undetectable reaction intermediates also appear during the elastase + α1PI reaction.

Molecular modeling studies (15, 16, 18) and biochemical investigations (17, 34) indicate that in the course of its reaction with a serpin, the protease moves more or less far away from its initial position, whereas the serpin’s reactive site loop inserts more or less deeply into β-sheet A. It has also been demonstrated that protease cleaves the P1–P’₁ bond of serpins and form acyl-enzyme intermediates between the serine residue of their catalytic site and the P₁ residue of the inhibitors. This cleavage is believed to trigger the above conformational changes and, as a consequence, to stabilize the final complex by preventing the hydrolysis of the acyl-enzyme (35, 36). These findings may help discuss the nature of the EI₂ complex and the significance of k₂, the rate constant of its formation. The stopped flow detected complex is probably structurally identical to the stable complex prepared by manual mixing and incubated for 30 min because the two species have identical donor-acceptor transfer efficiencies. As a consequence, EI₂ is likely to be identical with the elastase-α₁PI complex, which Lawrence et al. (35) and Wilczynska et al. (36) have shown to be a translocated acyl-enzyme. Although k₂ describes the first-order fluorescence increase that accompanies the conversion of EI₁ into EI₂, it is not necessarily the rate constant for the translocation of elastase. The enzyme’s acylation and translocation are separate events characterized by two individual rate constants, k_{acylation} and k_{translocation}. Because the EI₁ to EI₂ conversion is a simple exponential process, k₂ might have either one of the following two meanings: (i) k₂ ∝ k_{acylation}, i.e., k_{acylation} < k_{translocation} or (ii) k₂ ∝ k_{translocation}, i.e., k_{acylation} ≫ k_{translocation}. The second assumption implies that the acyl-enzyme would be exposed to water during the long lasting translocation process (t₁/₂ = 5.3 s). Because enzyme translocation is required to prevent hydrolysis of the acyl-enzyme, active enzyme would be released during the translocation process. This is not the case because the 1:1 elastase-α₁PI complex is enzymatically inactive (4). We may, therefore, tentatively conclude that k₂ is the rate constant for the acylation of elastase. We are not unaware that the above reasoning is somewhat speculative. For instance, despite its having an energy transfer efficiency identical to that of the stable complex prepared by manual mixing, EI₂ might not be the final inhibitory complex because it might still undergo fluorescently silent structural changes. On the other hand, the acylation and the translocation rates might not be as different from each other as we have hypothesized. Clearly, the only way to solve these uncertainties would be to directly measure the rate of acylation.

Like the serpin α₁PI, the canonical inhibitor eglin c (1) rapidly forms a Michaelis-type complex with elastase, but this complex does not undergo the characteristic conversion seen with the elastase-α₁PI complex. This is in accord with crystallographic data showing that the P₁–P’₁ bond of eglin c is not cleaved within the enzyme-inhibitor complex (37) and that there are no gross structural differences between free and protease-bound eglin c (38). Thus, kinetics of fluorescence resonance energy transfer unambiguously discriminates the serpin α₁PI from the canonical inhibitor eglin c.

Most extracellular serpins play a proteolysis-preventing function. The data collected using our new approach may help to better delineate this function. In vivo, a protease is usually released into a milieu containing both serpin and substrate. Knowledge of $K_i$ and $[I]_90$, the in vivo inhibitor concentration, will allow prediction of whether the substrate may effectively compete with the serpin for the binding of the protease; competition is possible if $[I]/K_i \sim [S]/K_m$. Besides, the magnitude of $k_−$ will predict the rate at which a preformed EI₁ complex may be dissociated by substrate. Last, the magnitude of $k_2$ will predict the apparent reversible or irreversible nature of the inhibition in vivo. If $k_2$ is fast, for example say $k_2 > 10^{-2} s^{-1}$ ($t_1/₂ < 1 min$), the inhibition will be irreversible within a few minutes. However, if $k_2 < 10^{-2} s^{-1}$, the inhibition may be reversible for a prolonged time, a view that is generally overlooked.

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