Evidence That Translocation of the Proteinase Precedes Its Acylation in the Serpin Inhibition Pathway*

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The inhibition of proteinases by serpins involves cleavage of the serpin, acylation, and translocation of the proteinase. To see whether acylation precedes or follows translocation, we have investigated the pH dependence of the interaction of fluorescein isothiocyanate-elastase with rhodamine α1-proteinase inhibitor (α1PI) using two independent methods: (i) kinetics of fluorescence energy transfer which yields $k_{2,r}$ the rate constant for the fluorescently detected decay of the Michaelis-type complex (Mellet, P., Boudier, C., Mely, Y., and Bieth, J. G. (1998) J. Biol. Chem. 273, 9119–9123); (ii) kinetics of elastase-catalyzed hydrolysis of a substrate in the presence of α1PI, which yields $k_{2,a}$, the rate constant for the conversion of the Michaelis-type complex into irreversibly inhibited elastase. Both rate constants were found to be pH-independent and close to each other, indicating that acylation, a pH-dependent phenomenon, does not govern the decay of the Michaelis-type complex and, therefore, follows translocation. On the other hand, anhydro-elastase reacts with α1PI to form a Michaelis-type complex that translocates into a second complex with a rate constant close to that measured with active elastase, confirming that acylation is not a prerequisite for translocation. Moreover, the anhydro-elastase-α1PI complex was found to be thermodynamically reversible, suggesting that translocation of active elastase might also be reversible. We propose that serpins form a Michaelis-type complex $E_{1M}$, which reversibly translocates into $E_{1r}$, whose acylation yields the irreversible complex $E_{1c}$.

$$K_j E + I \rightleftharpoons E_{1M} \rightleftharpoons E_{1r} \rightleftharpoons E_{1c}$$

**REACTION 1**

Serpins are a superfamily of proteins that arouse increasing interest. There are now more than 130 serpin entries in the Swiss-Prot data bank originating mainly from eukaryotic organisms but also from some viruses. In higher organisms they are present extracellularly but also in various intracellular compartments. A large number of these proteins are active serine proteinase inhibitors (1), but inhibition of some cysteine proteinases has also been reported (2). The most striking feature of the inhibition of proteinases by serpins is the formation of an irreversible inhibitory complex. The structure of native serpins includes a long flexible and exposed reactive site loop anchored to a highly conserved scaffold made of nine α-helices and three β-sheets (3). Some non-inhibited proteinases cleave the exposed loop, which inserts into β-sheet A as a new β-strand (4). Inhibited proteinases form a SDS-stable complex with serpins, which is probably an acylenzyme (5–7) between the carbonyl group of the P1 site of the loop and the catalytic serine of the proteinase. During the time separating the formation of the first binding complex and the formation of the final complex, a conformational change leading to the translocation of the proteinase was observed (8). The location of the proteinase within the stable complex is still a matter of debate (9–11). Only the x-ray crystallography of the complex, not available at present, would reveal precisely how the irreversible complex is held together and at which site the proteinase finally binds.

Proteolysis may be regulated by another type of inhibitors, the canonical inhibitors. By contrast with serpins, these smaller proteins have a rigid and short reactive site loop that forms a reversible lock-and-key complex with proteinases. The adaptive advantages by which evolution has selected serpins rather than canonical inhibitors in many regulatory processes are presently not easy to understand. Canonical inhibitors may have loose or strict specificities and low or high affinities for proteinases. Many of them have such low $K_i$ values that the inhibition may be pseudo Irreversible. Thus, irreversible inhibition is not a unique feature of serpins. Similarly, the modulation of the activity by an effector like heparin extensively studied with the serpin antithrombin III (12) has also been observed with the canonical mucus proteinase inhibitor (13). Moreover, serpins are large inhibitors that are more sensitive than canonical inhibitors to denaturation and to point mutations even at amino acids distant from the reactive loop (reviewed in Ref. 14). However, serpins have been selected by evolution to regulate delicate proteolytic events like the blood coagulation cascade, the complement cascade, or fibrinolysis. It is perhaps the complicated multistep process leading to the irreversible complex that is the basis of the specificity of serpin inhibitors.

There are at least three steps in the inhibition process by serpins. Each of these steps will decide on the choice of the proteinase and on its fate. The first reversible binding step is governed by the equilibrium dissociation constant for the given proteinase-serpin pair. Several examples covering a wide range of equilibrium dissociation constants have been described in the literature, indicating that this step is effectively used to discriminate the target proteinases. It is important to note that from this step on, the proteinase activity is inhibited (15). The

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Mechanism of Proteinase Inhibition by Serpins

The kinetic constants for the formation and disappearance of $E_1M$ were as follows: $k_1 = 1.5 \times 10^6 \text{M}^{-1} \text{s}^{-1}$, $k_{-1} = 0.58 \text{ s}^{-1}$, and $k_{2f} = 0.13 \text{ s}^{-1}$, the latter being the rate constant for the translocation of elastase. However, several questions remained unanswered about the translocation step. 1) Is the translocation of the proteinase triggered by the cleavage of the serpin reactive site loop? 2) Is translocation rate-limiting or is it preceded by another step that controls it (i.e. is acylation a prerequisite for translocation)? 3) After which step is the enzyme-inhibitor binding irreversible? This paper attempts to answer these questions.

EXPERIMENTAL PROCEDURES

Elastase was isolated according to the procedure of Shotton (16) with an additional purification step on S-Sepharose (Amersham Pharmacia Biotech) and active site titrated as described previously (17). Recombinant α1PI and eglin c expressed in Escherichia coli (Novartis, Basel) were obtained with human neutrophil elastase (17, 18). Buffers solutions at pH 5.5, 6, 6.5, and 7.1 were made with 50 mM NaOAc and 0.15 mM NaCl. Buffer solutions at pH 7, 7.5, 8, and 8.5 were made with 50 mM HEPES and 0.15 mM NaCl.

Preparation of Anhydro-elastase—Anhydro-elastase was prepared by alcali treatment of phenylmethanesulfonyl fluoride-treated elastase (20 mg) as described by Ako et al. (19). It was isolated by affinity chromatography as described by Williams et al. (20), except that the column was made with gellan gum instead of turkey ovomucoid inhibitor. The affinity gel was prepared by coupling 100 mg of gellan gum with 0.5 g of epoxy-activated Sepharose (Amersham Pharmacia Biotech) according to the manufacturer’s instructions. The fractions of elastase that retained affinity for gellan gum but had negligible catalytic activity were pooled. The pooled sample retained less than 0.09% of the activity expected from a sample of elastase with the same absorbance at 280 nm.

Fluorescent Labeling—Labeling of elastase with FITC (Molecular Probes), of α1PI with tetramethylrhodamine-5-maleimide (Molecular Probes), and of eglin c with the succinimidyl derivative of tetramethylrhodamine (Molecular Probes) were done as described previously (8). Anhydro-elastase was labeled with FITC in the same conditions as elastase. The tentative concentration of the stock solution of FITC-anhydro-elastase was measured by comparing its absorbance at 280 and 495 nm with that of a titrated solution of active FITC-elastase. The absorbances at 280 and 495 nm gave concentrations of 3.1 $\times 10^5$ and 2.6 $\times 10^5$ M, respectively. Since a more precise value was not needed, we arbitrarily used a concentration value of 3 $\times 10^{-5}$ M for all experiments.

Preparation of a Binary α1PI-Peptide Complex—5.9 mg of the synthetic P1-P14 peptide Ac-Thr-Glu-Ala-Ala-Gly-Ala-Met-Phe-Leu-Glu-Ala-Ala-Ala-Ala-Ala-NH2 (Nesystems, France) was added to 1.5 ml (0.35 mM) of a 105 mM solution of TMR-α1PI in 50 mM HEPES, 150 mM NaCl, pH 7.5. The mixture was incubated for 60 h at 37 °C in the presence of sodium azide and dialyzed against the above buffer. Peptide insertion was considered complete since no residual inhibitory activity on elastase could be detected. Absorbance at 555 nm was used to determine the peptide-inserted TMR-α1PI concentration.

Kinetics of Hydrolysis of Suc-Ala3-pNA by FITC-elastase as a Function of pH—The initial rates of Suc-Ala3-pNA (Bachem) hydrolysis by FITC-elastase were measured spectrophotometrically at 410 nm in the buffered solutions described above. The concentration of Suc-Ala3-pNA was varied between 0.216 and 2.16 mM in the presence of a final concentration of 1% N-methylpyrrolidone. The FITC-elastase concentration was 3 $\times 10^{-6}$ M throughout. At each pH $K_m$ and $V_m$ were obtained through non-linear least-square fits of the data to the Michaelis-Menten equation.

Kinetics of Fluorescence Resonance Energy Transfer—Kinetics of interaction of FITC-elastase or FITC-anhydro-elastase with TMR-α1PI or TMR-eglin c were monitored by fluorescence resonance energy transfer from fluorescein to tetramethylrhodamine using a Bio-Logic SF3 stopped flow apparatus with a dead time of 1.7 ms (Bio-Logic, Ciba, France). The concentrations were 5 $\times 10^{-7}$ M for FITC-elastase and FITC-anhydro-elastase and 8 $\times 10^{-5}$ M for TMR-α1PI and TMR-eglin c. The excitation and emission wavelengths were 450 nm (monochromator) and 515 nm (Melles-Griot interferential filter), respectively.

pH Dependence of the Rate of Inhibition of Elastase by α1PI in the Presence of Suc-Ala3-pNA—The rate of inhibition was measured by adding elastase to a mixture of α1PI and substrate and recording the release of product as a function of time. Mixing and recording were done with the above stopped-flow apparatus. For comparison purposes FITC-labeled elastase was used in these experiments, although its fluorescence properties were not needed. Its concentration was 1/10 or less the concentration of the inhibitor to maintain pseudo-first order conditions. The substrate was 2 mM Suc-Ala3-pNA in the presence of a final concentration of 1% N-methylpyrrolidone. At each pH the rate of inhibition was measured with initial concentrations of α1PI varying between 1 $\times 10^{-6}$ M and 5 $\times 10^{-6}$ M.

Differential Scanning Microcalorimetry—Spectra were performed on a DASM-4 microcalorimeter. For analysis and integration, the software ORIGIN (Microcal Inc.) was used. The thermograms were recorded in 20 mM phosphate buffer at pH 7.4 with a protein concentration of 1.5 mg/ml. The heating rate was 1 °C/min between 20 and 120 °C.

RESULTS

Kinetics of Hydrolysis of Suc-Ala3-pNA by FITC-elastase as a Function of pH—In proteolysis by serine proteinases, the acylation and deacylation steps are controlled by the ionization state of the histidyl residue of the catalytic triad. Thus, both steps are pH-dependent. The extent of the variation of the catalytic constant by pH is specific to the proteinase and to the steps are pH-dependent. The extent of the variation of the catalytic constant by pH is specific to the proteinase and to the function of pH is a characteristic of the proteinase. The extent of the variation of the catalytic constant by pH is specific to the proteinase and to the function of pH is a characteristic of the proteinase. The extent of the variation of the catalytic constant by pH is specific to the proteinase and to the function of pH is a characteristic of the proteinase. The extent of the variation of the catalytic constant by pH is specific to the proteinase and to the function of pH is a characteristic of the proteinase.
[E]₀ = 8 × 10⁻⁶ M, which yields nearly saturation of the enzyme as an EIₜ complex and hence the highest amplitude at pH 7.4 (8). Under these concentration conditions, we were able to follow the reaction down to pH 5.5 despite an important loss in the quantum yield of fluorescein. At all pH values we observed the same biphasic trace as described previously. Fig. 2 illustrates this observation with two examples, one at pH 8.5, the other one at pH 5.5. The difference in the amplitudes of the signal is mainly due to changes in the quantum yield of fluorescein with pH but also to the variations of the settings of the detector. kₐ₀ₑ did not significantly vary with pH (data not shown), suggesting that formation of EIₜ does not involve ionic interactions. Interestingly, k₂f was also pH-independent from pH 5.5 to 8.5 (Fig. 3).

Interaction of FITC-elastase with the Binary TMR-α₁PI-Peptide Complex—Insertion of a 14-mer peptide corresponding to the P₁ to P₁₄ sequence of the reactive site loop into β-sheet A of serpins is known to change them from inhibitors to substrates by preventing loop insertion (21). As a control experiment and to investigate the nature of the rearrangement monitored by our fluorescence transfer method, we observed the reaction of FITC-elastase with 14-mer peptide-inserted TMR-α₁PI at pH 7.5 and at concentrations identical to those used with free TMR-α₁PI. In Fig. 4, three steps are visible: (i) a fast exponential decrease in fluorescence corresponding to the formation of the initial Michaelis-type complex, (ii) a steady-state phase corresponding to the expected turnover of peptide-bound TMR-α₁PI whose concentration is 10-fold higher than that of elastase, and (iii) a very slow (~100 s) non-exponential return of the fluorescence intensity to its initial value. Such a return to the initial fluorescence intensity indicates that elastase and α₁PI are again separated from each other following substrate-like cleavage of the reactive site loop. Thus, the exponential translocation (t₁/₂ ≈ 5 s) observed with free TMR-α₁PI does not take place with peptide-inserted TMR-α₁PI. It may therefore be concluded that the interchromophore distance change observed with active α₁PI is related to the insertion of the reactive site loop into β-sheet A.

Effect of pH on the Rate of Inhibition of Elastase by α₁PI in the Presence of Suc-Ala₃-pNA—The rate of inhibition, i.e. the

![Graph of k_cat vs pH](image)

**Fig. 1.** Plot of the catalytic rate constant k_cat of FITC-elastase for Suc-Ala₃-pNA versus pH.

![Graphs of fluorescence vs time](image)

**Fig. 2.** Two examples of kinetics of the fluorescence resonance energy transfer between FITC-elastase and TMR-α₁PI: upper trace, pH 5.5; lower trace, pH 8.5. The elastase and α₁PI concentrations were 0.8 and 8 μM, respectively. The excitation and emission wavelengths were 450 and 515 nm, respectively.
rate of disappearance of free elastase, was measured by recording the release of product in mixtures of FITC-elastase, α₁PI, and Suc-Ala₃-pNA as described under “Experimental Procedures.” As expected for irreversible inhibition, all progress curves were simple exponentials from which the pseudo-first-order rate constants \( k \) were calculated by nonlinear regression analysis. At each pH value, \( k \) was determined using a series of \( \alpha₁\)PI concentrations. All \( k \) versus \([\alpha₁\)PI]₀ plots were hyperbolic (see two examples in Fig. 5), strongly suggesting two-step irreversible inhibition (22) as illustrated in Scheme 2.

\[
\text{SCHEME 2}
\]

\[
K_i E + 1 \rightarrow E\text{IM} \rightarrow E\text{I}_2
\]

\( K_i \) is the equilibrium dissociation constant for the rapidly-forming \( E\text{IM} \) Michaelis-type complex, and \( k_{2,e} \) is the rate constant for the irreversible or pseudo-irreversible transformation of \( E\text{IM} \) into \( E\text{I}_2 \), regardless of its nature. The possible subsequent transformation of \( E\text{I}_2 \) into one or several other species does not interfere with the mathematical treatment of the rate of inhibition of the free enzyme in the presence of a substrate. Thus, Equation 1 predicts the observed hyperbolic variation of \( k \) with \([I]_0 \) (22).

\[
k = \frac{k_{2,e}[I]}{[I]_0 + K_i[1 + [S]/K_m]}
\]  

(Eq. 1)

\( k_{2,e} \) and \( K_i(1+[S]/K_m) \) were calculated by nonlinear regression analysis. \( K_i \) was calculated using the \( K_m \) values previously determined at each pH value. Fig. 6 shows that \( k_{2,e} \) does not significantly vary with pH except at pH 5.5, where it is about twice as high as at pH 6.

The kinetics of reaction of FITC-anhydro-elastase with TMR-α₁PI as followed by fluorescence transfer—FITC-labeled anhydro-elastase was mixed with TMR-α₁PI in the stopped-flow apparatus at pH 7.5, and the reaction was followed by fluorescence. The trace shown in Fig. 6A is biphasic as for active elastase (Fig. 2) suggesting that anhydro-elastase reacts with α₁PI via a two-step mechanism similar to that previously demonstrated with active elastase and illustrated in Scheme 1. Moreover, the rate constant for the translocation of anhydro-elastase (0.11 ± 0.02 s⁻¹) is very close to that for the translocation of active elastase (0.13 ± 0.03 s⁻¹).

To confirm that the second exponential represents translocation of \( E\text{IM} \) into \( E\text{I}_2 \) and not an artifact, we reacted the same sample of FITC-anhydro-elastase with TMR-eglin c, a canonical reversible inhibitor that reacts with active elastase in only
one step. Fig. 7B confirms that eglin c also reacts in one step with anhydro-elastase. Thus, translocation of elastase from the first binding site to another site does not require the enzyme’s catalytic machinery.

**Recovery of α1PI following Dissociation of the Anhydro-elastase-α1PI Complex**—A mixture of 30 μM FITC-anhydro-elastase and 30 μM α1PI was chromatographed at pH 7.5 on a Sepharose-eglin c column. FITC-anhydro-elastase was retained at the top of the column, as evidenced by its color while α1PI was collected in the flow-through volume. Titration of the dissociated α1PI with neutrophil elastase confirmed that all of the inhibitor could be recovered. This implies that eglin c was able to fully dissociate the complex. The rate of inhibition of active elastase by the recovered α1PI was measured in the presence of substrate as described under “Experimental Procedures.” Using two different concentrations of α1PI, we got a second-order association rate constant of 5 × 10^5 ± 0.8 s⁻¹ M⁻¹ at pH 7.5. This figure agrees with the rate constant usually found in the buffer conditions used in our laboratory for unreacted α1PI. The α1PI sample recovered from the complex was also analyzed by differential scanning calorimetry. The thermogram revealed a thermal transition at 60.5 °C for both the control α1PI and the sample recovered from the complex with anhydro-elastase. Thus, the translocated α1PI-anhydro-elastase complex is in true thermodynamic equilibrium with free anhydro-elastase and α1PI, since its dissociation is quantitative and yields α1PI molecules whose inhibitory capacity and thermal stability are identical to those of the native inhibitor.

**Discussion**

**Translocation Precedes Acylation**—In recent years many kinetic, chemical, and crystallographic data have been collected in view of understanding the mechanism by which serpins inhibit serine proteinases. It is commonly accepted that the enzyme and the inhibitor first form a Michaelis-type complex within which the serpin is subsequently cleaved to yield an acyl enzyme linking the serine residue of the proteinase with the P₁ residue of the serpin (5–7). Formation of the acyl enzyme is believed to relax the strained conformation of the serpin, to incorporate the N-terminal part of the reactive site loop into β-sheet A and hence to cause a translocation of the proteinase that stabilizes the acyl enzyme bond. This model is summarized in Scheme 3.

\[
\text{EI}_{\text{M}}, \text{EI}_{\text{ac}}, \text{and EI}_{\text{tr}} \text{ are the Michaelis-type complex, the acyl enzyme, and the translocated acyl enzyme, respectively; } K^* \text{ is the equilibrium dissociation constant of the Michaelis-type complex; } k_{\text{ac}} \text{ and } k_{\text{tr}} \text{ are the acylation and the translocation rate constants, respectively.}
\]

**Scheme 3**

\[
K^* \quad E + I \rightarrow EI_{\text{M}} \rightarrow EI_{\text{ac}} \rightarrow EI_{\text{tr}}
\]

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\[
K^* \quad E + I \rightarrow EI_{\text{M}} \rightarrow EI_{\text{ac}} \rightarrow EI_{\text{tr}}
\]

**Scheme 3**

The catalytic-triad of serine proteinases comprises Asp₁⁰², His₅⁷, and Ser₁⁹⁵. Catalysis involves acylation and deacylation of Ser₁⁹⁵, both of which are sensitive to pH because they depend upon the state of ionization of His₅⁷ (pKₐ ≈ 6.7). As a control, we have shown that k_{cat} for the elastase-catalyzed hydrolysis of the model substrate Suc-Ala₃-pNA increases 18-fold between pH 5.5 and 8.5. Therefore, if EI₅₇ would follow immediately EI₃₇ (Scheme 3), its rate of appearance should strongly increase with pH. We did not measure the kinetics of EI₅₇ formation but we used two ways to measure the kinetics of EI₅₇ decay: fluorescence, which yielded k₂₉ (Scheme 1), and substrate hydrolysis, which gave k₂₉ (Scheme 2). Both k₂₉ and k₂₉ were found to be essentially pH-independent.

k₂₉ is the first-order rate constant for the fluorescently detected decay of the Michaelis-type complex EI₃₇ (Scheme 1). In our experiment fluorescence detects only those species that significantly differ in energy transfer efficiency. It is thus not unlikely that fluorescently silent events such as acylation take place during the fluorescence change that characterizes the translocation of EI₃₇. As a consequence, EI₃₇ is not necessarily a single molecular species and k₂₉ is possibly an apparent rate constant that comprises both the pH-dependent constant, k₂₉, and the pH-independent one, k₁₉. Thus, the pH independence of k₂₉ is, at first sight, compatible with both of the above-mentioned reaction schemes but imposes some restrictions. Either Scheme 4 is valid and k₂₉ = k₃₉ whatever the magnitude of k₂₉,
or Scheme 3 is valid, in which case $k_{tr}$ must necessarily be rate-limiting for the formation of the final complex $E_{I_T}$, which implies that $k_{ac} \gg k_{tr}$. Therefore, $k_{ac}$ should be much larger than $k_{2,e}$, the translocation rate constant measured by fluorescence resonance energy transfer, i.e. $0.13 \text{ s}^{-1}$. In summary, Scheme 3 can only be compatible with the observed pH inde-
The pH independence of the rate-limiting step for the inhibition of chymase by antichymotrypsin was suggested by Schechter et al. (24). However, this study measured the overall inhibition rate constant with no information on the nature of the reaction steps. Serpin-proteinase reaction mechanisms with two consecutive reversible steps have been recently proposed by Kvassman et al. (25) for the inhibition of plasminogen activator by plasminogen activator inhibitor 1, by Nair et al. (26) for the inhibition of chymotrypsin by antichymotrypsin, and by Stone et al. (27) for the inhibition of thrombin by several serpins. However, the methods used in these works were not adapted to determine whether the proteinase was translocated and whether acylation occurred before or after translocation.

Although the fluorescence change that accompanies translocation is described by a simple exponential, it is possible that a further conformational change takes place after acylation since formation of the acylenzyme is accompanied by liberation of the new N terminus P 1 residue. Such a conformational change is not detected by fluorescence transfer. This either means that each translocated molecule is rapidly acylated so that the accumulated fluorescence changes of the two consecutive movements are included in the fluorescence change accompanying the rate-limiting exponential or that the hypothetical conformational change induced by acylation does not significantly modify the interchromophore distance. In the first assumption, the translocation occurs in two steps: (i) one involving the uncleaved reactive site loop that is brought to a position where acylation can safely occur in a solvent-free environment to prevent deacylation, and (ii) the other resulting from the rearrangement consecutive to loop cleavage. The second assumption implies that the proteinase has reached its final position after the translocation step.

**Is Translocation Reversible?**—Chromatography of the anhydro-elastase–αPI complex on a Sepharose-eglin c column was able to dissociate the complex and to recover functionally active and structurally intact αPI, indicating that the reagents are in true thermodynamic equilibrium as outlined in Scheme 5.

\[ \text{ahE + I} \xrightleftharpoons[k_{1tr}]^{k_{tr}} \text{ahEI}_r \]

**SCHEME 5**

ahE is anhydro-elastase. Since significant translocation of the initial Michaelis-type complex was observed, \( k_{tr} \) must be larger than \( k_{-tr} \). For instance, if \( k_{tr} = 10 k_{-tr} \), the translocation is virtually complete.

It is likely that translocation of active elastase is also reversible or pseudo-reversible. Active and anhydro-elastase differ only in one residue. Both form a reversible complex with eglin c, and a Michaelis-type complex with αPI, which further undergoes translocation. It is difficult to conceive that the serine residue of the active site of elastase prevents reversibility of translocation. The data of Whisstock et al. (28) favor this view. These authors modeled the interaction of αPI with neutrophil elastase and came to the conclusion that insertion of the uncleaved binding loop up to residue P12 into the β-sheet A allowed a possible docking of the proteinase to the translocated site. They reported that the required conformational changes occur with the correct stereochemistry and without steric clashes. Furthermore, the proteinase and the serpin were held together by a large number of van der Waals and hydrogen bonds. There is thus an energetically favorable docking at the translocated site which does not involve the serine residue of the enzyme’s active center and does not require reactive site loop cleavage of the serpin. Further experiments are needed to firmly demonstrate that the translocated αPI-active elastase complex is in equilibrium with the Michaelis-type complex.

**Implications of the New Model**—In our study we show that in the second reversible step the anhydro-proteinase has been translocated. Accordingly, the Michaelis-type complex between an anhydro-proteinase and a serpin, which is rapidly converted into the translocated complex, should no longer be considered as a model for the first encounter complex (10, 11, 29, 30) in which the serpin is probably still close to its native conformation.

Each step described in the minimal Scheme 4 is determinant for the selection of the target proteinase by the serpin and for its fate. The fact that each step may be governed by a special property of the proteinase leads necessarily to a variety of situations. Thus, the ability of the proteinase to form a tight or a loose \( E_{IM} \) complex is probably independent of its ability to
trigger translocation. Similarly, the features that are determinant in these two first steps are certainly not related to the ability of the proteinase to catalyze the formation of a covalent complex with the serpin. This provides a flexibility in the regulation of proteinase inhibition at each step that may be the cause of the selection of serpins in potentially dangerous processes such as the cascade of the coagulation, the complement cascade, or fibrinolysis. The example of the inhibition of proteinase by $\alpha_2$-antiplasmin, which leads to an SDS-stable complex (31) but appears to be reversible for some time (32, 33), illustrates what can be achieved with the flexible mechanism proposed above.

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REFERENCES
1. Potempa, J., Korzus, E., and Travis, J. (1994) J. Biol. Chem. 269, 15957–15960
2. Schick, C., Pemberton, P. A., Shi, G. P., Kamachi, Y., Cataltepe, S., Bartuski, A. J., Gornstein, E. R., Bro¨mme, D., Chapman, H. A., and Silverman, G. A. (1998) Biochemistry 37, 5258–5266
3. Elliott, P. R., Abrahams, J. P., and Lomas, D. A. (1998) J. Mol. Biol. 275, 419–425
4. Lobermann, H., Tokuoka, R., Deisenhofer, J., and Huber, R. (1984) J. Mol. Biol. 177, 531–556
5. Lawrence, D. A., Ginsburg, D., Day, D. E., Berkompas, M. B., Verhamme, I. M., Kvassman, J. O., and Shore, J. D. (1995) J. Biol. Chem. 270, 25309–25312
6. Wilczynska, M., Fa, M., Ohlsson, P. I., and Ny, T. (1995) J. Biol. Chem. 270, 29652–29655
7. Egelund, R., Rodenburg, K. W., Rasmussen, P. A., Rasmussen, M. S., Goldberg, R. E., and Petersen, T. E. (1998) Biochemistry 37, 6375–6379
8. Mellet, P., Boudier, C., Melly, Y., and Bieth, J. G. (1998) J. Biol. Chem. 273, 9119–9123
9. Wilczynska, M., Fa, M., Karol, J., Ohlsson, P. I., Johansson, L. B., and Ny, T. (1997) Nat. Struct. Biol. 4, 354–357
10. Stratikos, E., and Gettins, P. G. W. (1998) J. Biol. Chem. 273, 15582–15589
11. Stratikos, E., and Gettins, P. G. W. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 4808–4813
12. Jin, L., Abrahams, J. P., Skinner, R., Petiotou, M., Pike, R. N., and Carrel, R. W. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 14683–14688
13. Mellet, P., Ermolieff, J., and Bieth, J. G. (1995) Biochemistry 34, 2645–2652
14. Carrel, R. W., and Stein, P. E. (1996) Biol. Chem. Hoppe-Seyer 377, 1–17
15. Duranton, J., Adam, C., and Bieth, J. G. (1998) Biochemistry 37, 11239–11245
16. Shotton, D. M. (1970) Methods Enzymol. 19, 113–140
17. Boudier, C., and Bieth, J. G. (1992) J. Biol. Chem. 267, 4370–4375
18. Faller, B., Dirrig, S., Rahaud, M., and Bieth, J. G. (1990) Biochem. J. 270, 639–644
19. Ako, H., Foster, R. J., and Ryan, C. A. (1974) Biochemistry 13, 132–139
20. Williams, H. R., Lin, T. Y., Naviya, M. A., Springer, J. P., and Hogsteen, K. (1987) Biochim. J. 242, 267–273
21. Schulze, A. J., Frohnert, P. W., Egh, R. A., and Huber, R. (1992) Biochemistry 31, 7560–7565
22. Morrison, J. F., and Walsh, C. T. (1988) Adv. Enzymol. Relat. Areas Mol. Biol. 61, 251–301
23. Verhamme, I., Kvassman, J. O., Day, D., Debrock, S., Vleugels, N., Declerck, P. J., and Shore, J. D. (1999) J. Biol. Chem. 274, 17511–17515
24. Schechter, N. M., Plotnick, M., Sehwood, T., Walter, M., and Rubin, H. (1997) J. Biol. Chem. 272, 24499–24507
25. Kvassman, J. O., Verhamme, I., and Shore, J. D. (1998) Biochemistry 37, 15491–15502
26. Nair, S. A., and Cooperman, B. S. (1998) J. Biol. Chem. 273, 17459–17462
27. Stone, S. R., and Le Bonniec, B. F. (1997) J. Mol. Biol. 265, 346–362
28. Whistock, J., Leak, A. M., and Carrel, R. W. (1996) Proteins 26, 288–303
29. Stratikos, E., and Gettins, P. G. W. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 453–458
30. Futamura, A., Stratikos, E., Olson, S. T., and Gettins, P. G. W. (1998) Biochemistry 37, 13110–13119
31. Stone, S. R., and Le Bonniec, B. F. (1997) J. Mol. Biol. 265, 346–362
32. Shie, B. H., Potempa, J., and Travis, J. (1989) J. Biol. Chem. 264, 13420–13423
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