The pH Dependence of Serpin-Proteinase Complex Dissociation Reveals a Mechanism of Complex Stabilization Involving Inactive and Active Conformational States of the Proteinase Which Are Perturbable by Calcium*

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Serpin family protein proteinase inhibitors trap protonetases at the acyl-intermediate stage of cleavage of the serpin as a proteinase intermediate substrate by undergoing a dramatic conformational change, which is thought to distort the proteinase active site and slow deacetylation. To investigate the extent to which proteinase catalytic function is defective in the serpin-proteinase complex, we compared the pH dependence of dissociation of several serpin-proteinase acyl-complexes with that of normal guanidinobenzoyl-proteinase acyl-intermediate complexes. Whereas the apparent rate constant for dissociation of guanidinobenzoyl-proteinase complexes (k_{diss, app}) showed a pH dependence characteristic of His-57 catalysis of complex deacylation, the pH dependence of k_{diss, app} for the serpin-proteinase complexes showed no evidence for His-57 involvement in complex deacylation and was instead characteristic of a hydroxide-mediated deacylation similar to that observed for the hydrolysis of tosylarginine methyl ester. Hydroxylamine enhanced the rate of serpin-proteinase complex dissociation but with a rate constant for nucleophilic attack on the acyl bond several orders of magnitude slower than that of hydroxide, implying limited accessibility of the acyl bond in the complex. The addition of 10–100 mM Ca^{2+} ions stimulated up to 80-fold the dissociation rate constant of several serpin-trypsin complexes in a saturable manner at neutral pH and altered the pH dependence to a pattern characteristic of His-57-catalyzed complex deacylation. These results support a mechanism of kinetic stabilization of serpin-proteinase complexes wherein the complex is trapped as an acyl-intermediate by a serpin conformational change—induced inactivation of the proteinase catalytic function, but suggest that the inactive proteinase conformation in the complex is in equilibrium with an active proteinase conformation that can be stabilized by the preferential binding of an allosteric ligand such as Ca^{2+}.

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Serpins comprise a large superfamily of proteins, the majority of which function as inhibitors of serine or cysteine proteinases (1, 2). Inhibitory serpins inactivate their target proteinases by a novel kinetic trapping mechanism whose ultimate outcome is the formation of a stable, covalently-linked 1:1 serpin-proteinase complex. The trapping mechanism appears to involve an initial binding of the proteinase to an exposed reactive center loop of the proteinase followed by attack of the proteinase catalytic serine on this loop as in a regular substrate reaction (3, 4). However, the substrate reaction becomes arrested following the proteinase attack due to the triggering of a large-scale conformational change in the serpin (4–6), which traps the proteinase at the acyl-intermediate stage of this reaction (7–9). The stabilization of the final inhibited complex is believed to be kinetic, because the complexes irreversibly dissociate to cleaved serpin and active proteinase with a half-life ranging from several days to months (4, 10–12).

The mechanism by which the serpin conformational change traps the acyl-intermediate complex is thought to involve an induced distortion of the proteinase active site, which slows the deacylation of the complex (13–21). The distortion is believed to occur as a result of the cleavage of the serpin-reactive loop, which frees the loop and acyl-linked proteinase to insert into the center of serpin β-sheet A. This insertion causes the proteinase to be translocated to the opposite end of the serpin and undergo conformational distortion through its compression against the serpin body (19, 21–25). Evidence for a distortion of the catalytic residues in the serpin-proteinase complex has come from NMR studies, which have shown that the pK_{a} value of the catalytic His-57 is significantly perturbed in the complex (17, 26). Moreover, studies of a P1 Met → Trp variant of α1-proteinase inhibitor have shown that the serpin P1 residue undergoes a repositioning in the proteinase active site upon transforming the noncovalent serpin-proteinase encounter complex to a covalent complex (18). Other studies have shown that mutations of serpin reactive loop residues involved in proteinase recognition minimally affect the stability of the trapped acyl complex (11, 27, 28), whereas mutation of reactive loop residues, which insert into sheet A greatly affect this stability (12, 29). Such findings are consistent with reactive center loop interactions with the proteinase being disrupted in the complex due to the reactive loop inserting into sheet A. The increased susceptibility of the complexed proteinase to proteolysis by free proteinase has provided compelling evidence for substantial conformational changes induced in the proteinase through complex formation with a serpin (14, 16, 20). The recent structure of a serpin-proteinase complex has validated these findings by revealing that the reactive loop is fully inserted into sheet A in the complex with translocation of the acyl-linked proteinase to the distal end of the serpin where it is physically held to the serpin body by the buried loop with a...
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Major distortion of its structure (19). NMR studies of the same complex in which N\textsuperscript{15}Ala labeling of the serpin or the proteinase was used to detect conformational change similarly concluded that the serpin-reactive loop was fully inserted in the complex with the proteinase at the distal end and grossly distorted in its complex (21).

The present studies were undertaken to further understand the mechanism of kinetic trapping of serpin-proteinase complexes by assessing the functional state of the proteinase catalytic residues in the complex under physiologic conditions in solution and to correlate this function with the structure of these residues observed in the complex crystallized at 4°C (19). In particular, we were interested in determining whether the catalytic triad residues contributed at all to the observable slow deacylation of serpin-proteinase complexes (4, 10, 12) or whether the deacylation was completely noncatalytic and resulted from hydrolysis by solution nucleophiles. A signature for the functioning of the catalytic residues is provided by the p\textsubscript{i}H dependence of deacylation (30, 31). We have therefore investigated the p\textsubscript{i}H dependence of the rate of dissociation of several serpin-proteinase complexes and compared this with the p\textsubscript{i}H dependence of deacylation of normal acyl-intermediate complexes and of a model ester of the serpin-proteinase acyl link.

The effect of the strong nucleophile, hydroxylamine, on the dissociation of serpin-proteinase complexes was also investigated to distinguish between catalytic versus noncatalytic mechanisms of complex deacylation and assess the solvent accessibility of the acyl bond (32). Our results provide evidence that the catalytic residues are largely nonfunctional in the serpin-proteinase complex and support a mechanism of kinetic trapping of the complex in which the proteinase is induced into an inactive conformational state different from that of azymogen (19). However, the existence of an active proteinase conformational state in equilibrium with the inactive state is also suggested by our finding that a ligand capable of allosteric interactions with the proteinase can reactivate the function of the catalytic residues. The mechanism of kinetic trapping of serpin-proteinase complexes thus appears to be dynamic and dependent on the extent to which the serpin stabilizes the proteinase in an inactive state. Such a mechanism may explain the observed dependence of complex stability on reactive loop sequence (12, 29).

MATERIALS AND METHODS

**Proteins**—Human plasma antithrombin was purified from outdated plasma by heparin-Sepharose, DEAE-Sepharose, and Sephadex S-200 chromatography steps as described previously (33). Human \( \alpha \_1 \)-proteinase inhibitor (\( \alpha \_1 \Pi \_1 \)) was purified as previously described (34) or purchased from Athens Research Technology (Athens, GA). The M\textsubscript{55R} variant of human \( \alpha \_1 \)-proteinase inhibitor was a recombinant protein expressed in *Escherichia coli* as inclusion bodies, refolded and purified as described previously (23). Purified \( \alpha \_1 \)-proteinase inhibitor-1 (PAI-1) was generously provided by Dr. Daniel Lawrence of the American Red Cross, Rockville, MD. Inhibitor concentrations were determined from measurements of absorbance at 280 nm together with published absorption coefficients (6, 35, 36). Human \( \alpha \)-thrombin was generously provided by Dr. John Fenton of the New York State Department of Public Health, Albany, NY. Human factor Xa was obtained in predominantly the \( \alpha \)-form by activating purified factor Xa with purified Russell’s viper venom proteinase and then purifying the activated enzyme by soybean trypsin inhibitor-agarose chromatography as described (37). Bovine or porcine \( \beta \)-trypsins were purified from commercial trypsin (Sigma Chemical Co.) by soybean trypsin inhibitor-agarose chromatography (38). Human neutrophil elastase (HNE) was purchased from Athens Research. Thrombin, factor Xa and bovine trypsin were active site titrated and found to be 70–100% active based on comparisons with the concentrations measured from the 280-nm absorbance and published absorption coefficients (4, 39). The concentrations of HNE and porcine trypsin were based on the stoichiometric titration of the enzyme with \( \alpha \_1 \Pi \_1 \) as described (40). Proteinase concentrations were routinely determined from initial rates of protein hydrolisis, monitored from the absorbance change at 405 nm, of the substrates, S-2238 (Chromogenix) for thrombin, Spectrozyme FXa (American Diagnostica) for factor Xa, S-2222 (Chromogenix) for trypsin, chromogenic substrates (Sigma), folA-Pro-Val-p-nitroanilide (FVAN) for HNE under standard conditions after calibrating these rates using known concentrations of active site titrated proteinase. The stoichiometric reactions of the inhibitors with proteinases were verified in all cases by titrations of fixed levels of enzyme with inhibitor over molar ratios of inhibitor/enzyme ranging from ~0.2 to 1.2 as described previously (33).

**SDS-PAGE**—The purity of proteins, the formation of serpin-proteinase complexes, and the products of dissociation of these complexes were analyzed by SDS-polyacrylamide gel electrophoresis using the Laemmli discontinuous buffer system (41) and with staining of protein bands with Coomassie Blue R-250. Complex formation and dissociation was analyzed for antithrombin, \( \alpha \_1 \Pi \_1 \), and PAI-1 complexes with trypsin and for antithrombin complexes with thrombin and factor Xa by incubating 2 \( \mu \)M serpin with 3–5 \( \mu \)M proteinase for 3–5 min in ionic strength (I) 0.15 Hepes buffer, pH 7.5 when not further specified. All samples were then freeze-dried and subsequently used for the experiments described here. The effect of calcium on proteolysis of trypsin complexed with antithrombin or M\textsubscript{55R} \( \alpha \_1 \Pi \_1 \) was analyzed by mixing 2 \( \mu \)M serpin with 1.5-fold molar excess of enzyme in I 0.15 Hepes buffer, pH 7.4, with or without 50 mM CaCl\(_2\) and incubating at 25°C for 30 to 5 min. Samples were then quenched with 10 \( \mu \)M Phe-Phe-Arg-chloromethyl ketone and, after 5-min incubation, denatured with SDS sample buffer and boiling prior to electrophoresis.

**Kinetics of Dissociation of Serpin-Proteinase and Guanidinobenzoyl-Proteinase Complexes**—First-order rate constants for the dissociation of serpin-proteinase complexes or guanidinobenzoyl-proteinase complexes were measured by continuous monitoring of the initial rate of proteinase dissociation from the complexes in the presence of reporter chromogenic or fluorogenic substrates as previously described (4, 10, 37). Serpin-proteinase complexes were prepared by incubating 1.1–5 \( \mu \)M serpin and 1 \( \mu \)M proteinase and in the case of antithrombin complexes with thrombin and factor Xa, also 1 \( \mu \)M high affinity heparin (Marlin, \( \approx 8000 \) purified from commercial heparin as described (33)), for 10–60 min at 25°C in 0.1 \( \mu \)M Hepes, 0.1 \( \mu \)M NaCl, 0.1% PEG 8000, pH 7.4, buffer (10.15) except for HNE complexes, which were prepared in 0.1 \( \mu \)M Hepes, 0.5 \( \mu \)M NaCl, 0.1% PEG 8000, pH 8, with a 5-min incubation. SDS-PAGE confirmed that complexes formed under these conditions were intact and minimally degraded. Complexes were then extensively diluted into buffer containing 200–400 \( \mu \)M chromogenic substrate or 50 \( \mu \)M fluorogenic substrate at 37°C to result in complex concentrations of ~10 pm to 10 nm. The substrates used were the same as those indicated above for standard proteinase activity assays, except in some cases, N-p-tosyl-\( \text{Gly-Pro-Arg-} \)7-amido-4-methylcoumarin (Sigma) was used also for monitoring thrombin or trypsin activity with equivalent results. Complex dissociation was measured in buffers of 20 mM sodium phosphate or 0.1 \( \mu \)M HEPES in the pH range 7–8 and 20 mM glycine in the pH range 8.5–9.3. Alternatively, mixed buffers containing 30 mM TAPS, 30 mM Hepes, 15 mM MES in the pH range of 5–9 or 30 mM TAPS, 30 mM CAPS in the pH range 9–11 were used. All buffers additionally contained 0.1 \( \mu \)M NaCl, 0.1 or 1 mM EDTA, 0.1% PEG 8000 with the pH adjusted at

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1 The abbreviations used are: \( \alpha \_1 \Pi \_1 \), \( \alpha \_1 \)-proteinase inhibitor; PAI-1, plasminogen activator inhibitor-1; HNE, human neutrophil elastase; PAGE, polyacrylamide gel electrophoresis; PEG, polyethylene glycol; TAPS, 3-[\( \text{N-2-hydroxyethylaminoethyl} \)-amino]-1-propanesulfonic acid; MIES, 4-morpholinethanesulfonic acid; TAME, p-tosyl-L-arginine methyl ester; CAPS, 3-(cyclohexylamino)propanesulfonic acid.

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37 °C. Protein adsorption at the low concentrations of complex was avoided by employing plastic cuvettes coated with PEG 20,000 (42). In some cases, 0.1 mg/ml bovine serum albumin was also included without detectable effect on measured rate constants. For antithrombin complexes prepared with the activator, heparin, 100 μg/ml Polybrene was included in the association reaction. Comparison with the kinetics of dissociation of antithrombin complexes prepared in the absence of heparin and measured without Polybrene present yielded indistinguishable results. The effects of hydroxylamine (hydrochloride salt from Sigma), the dipeptide, Ile-Val (Bachem), or calcium chloride on complex dissociation rates was determined by adding these components to the dissociation buffer. EDTA was absent but calcium containing calcium. Calcium chloride effects were measured either in 0.1 mM Hepes buffer at a fixed pH of 7.2 or at varying pH levels in the presence of fixed calcium chloride concentrations of 10 or 100 mM in the mixed buffers indicated above. Complex dissociation was continuously monitored by recording the increase in absorbance at 405 nm or the increase in fluorescence at 380-nm excitation and 440-nm emission for 30–90 min, with less than 10% of the substrate being consumed over this time.

Dissociation of guanidinobenzoyl acyl-intermediate complexes with the same proteinases was measured similarly. The acyl-intermediate complexes were prepared by incubating 100 nM proteinase with fluorescein mono-p-guanidinobenzoate at 140 mM for 15 min in the case of trypsin, at 5 mM for 8 min in the case of thrombin, and at 5 mM for 100 min in the case of factor Xa, all at 25 °C in 30 mM TAPS, 30 mM Hepes, 15 mM MES, 0.1 M NaCl, 1 mM EDTA, 0.1% PEG 8000, pH 7.4. These times were sufficient to maximally form the acyl-enzyme complex in keeping with reported rate constants (37, 43). Complexes were then extensively diluted into 400 μM substrates in the same mixed buffer adjusted to the desired pH at 37 °C and the 405-nm absorbance was continuously measured as for the serpin-proteinase complex dissociation experiments. Indistinguishable results were obtained when p-nitrophenyl-p’-guanidinobenzoate was used as the acylating reagent (44).

To calibrate the relationship between the rate of absorbance or fluorescence change and proteinase concentration under the conditions of measurement, the initial velocities of hydrolysis of substrate under identical reaction conditions, i.e. 37 °C in the same buffer and at the same rate of proteinase concentration, were measured for a range of proteinase concentrations. Progress curves for serpin-proteinase and guanidinobenzoyl-proteinase complex dissociation were fitted by the parabolic function (4),

\[ S_0(0) = S_{0,0} + v_0 \times t + k_{diss,app} \times \left[ E - I_0 \right] \times T N \times t^2/2 \]  

where \( S_0,0 \) and \( S_0,0 \) are the absorbance or fluorescence at time 0 and time zero, respectively, \( v_0 \) is the initial rate of absorbance/fluorescence change at time zero, \( k_{diss,app} \) is the apparent first order rate constant for complex dissociation, TN is the turnover number determined from the slope of the calibration curve relating the initial velocity of substrate cleavage to proteinase concentration and \( E - I_0 \) is the concentration of serpin-proteinase or guanidinobenzoyl-proteinase complex. Computer fitting of progress curves by a second order polynomial function provided the coefficient of the \( t^2 \) term. These fitted coefficients were plotted as a function of the serpin-proteinase complex concentration, which yielded the expected linear dependence in all cases. \( k_{diss,app} \) was then calculated from the linear least-squares slope of these plots by multiplying by 2/TN using the measured value of TN. The independence of measured dissociation rate constants on the concentrations of inhibitor or substrate at the extensive dilutions of complexes employed verified that true dissociation rate constants were measured with no contribution from the rate of association with any excess inhibitor present (44).

In this regard, the linearity of the plots of the initial rate of complex dissociation against complex concentration provided a diagnostic test that reassociation with any excess inhibitor was not influencing the measured rate constant as was verified by simulations of the dissociation kinetics.

Full progress curves of the dissociation of the antithrombin-trypsin complex in the presence of calcium were measured by preparing complex in the absence of calcium for 15 min, followed by the addition of 10 mM p-aminobenzamidine to trap dissociated trypsin and block its reassociation with excess inhibitor (44). The rate of complex dissociation was monitored continuously. Either 100 mM calcium chloride or 1 mM EDTA were then added to fully a further 100-fold into 50 μM N-p-tosyl-Gly-Pro-Arg-7-amido-4-methylcoumarin substrate in 0.1 M Hepes buffer, pH 7.4. The amount of trypsin dissociated from the complex was measured from the initial rate of hydrolysis of substrate monitored from the linear increase in fluorescence using excitation and emission wavelengths of 380 nm and 440 nm, respectively. Rates of dissociation were expressed relative to the average control hydrolysis rates, which were stable over the course of the reaction.

**Tosylarginine Methy Ester Hydrolysis—** Tosylarginine methyl ester hydrolysis was measured in the mixed buffers described above as a function of pH at 37 °C at 0.1–1 mM ester. The hydrolysis was monitored continuously by a decrease in absorbance at 240 nm and by differences in extinction coefficients between the ester and the product of hydrolysis at these wavelengths at the pH of the reaction. For pH values ≥ 8, full progress curves of the hydrolysis measured over reaction times up to ~100 h were fitted by a single-exponential function with a single kinetic constant and removal of buffer serving as reference and following the spectral changes at 250 nm using the measured difference in extinction coefficients between the ester and the acid product of hydrolysis at these wavelengths at the pH of the reaction.

**RESULTS**

**pH Dependence of the Rate of Dissociation of Serpin-Proteinase Complexes**—To determine whether a dysfunctional proteinase catalytic triad was responsible for the kinetic stabilization of serpin-proteinase complexes, we measured the pH dependence of the rate of dissociation of several serpin-proteinase complexes (30, 31). Fig. 1 shows the apparent first order rate constants (\( k_{diss,app} \)) for dissociation of complexes of two serpins, antithrombin and α1-proteinase inhibitor, with the non-target proteinase, trypsin, and of antithrombin with the two target proteinases, thrombin and factor Xa, as a function of pH. As a positive control for the normal functioning of the catalytic triad, Fig. 1 also shows the pH dependence of the first order rate constants for dissociation of the relatively stable guanidinobenzoyl acyl-intermediates generated by reaction of the proteinases with fluorescein mono-p-guanidinobenzoate. Dissociation rate constants were measured by continuous spectrophotometric assays of the initial rate of generation of proteinase activity from inhibited serpin-proteinase or guanidinobenzoyl-proteinase complexes after their extensive dilution into substrate, as in past studies (4, 11, 27). SDS-PAGE analysis of the products of dissociation of the serpin-proteinase complexes, after incubating the complexes for one to several half-lives in the presence of the active site-directed inhibitor, p-aminobenzamidine, to prevent autohydrolysis of free or complexed proteinases, revealed that cleaved serpin and intact proteinase were the only detectable products in all cases. These findings are in agreement with previous reports (4, 10) and consistent with the evidence that serpin-proteinase complexes are kinetically stable acyl-intermediates (7–9), which are slowly dissociated by a deacylation reaction similar to that involved during the reaction of proteinases with regular substrates (4, 10, 12).

The pH dependence of the deacylation rate constant for the control guanidinobenzoyl acyl-intermediate complexes showed the expected transition from a linear increase in log \( k_{diss,app} \) with a slope of 1 from pH 5 to 7 to a plateau value for log \( k_{diss,app} \) at pH values greater than 7. Such behavior is diagnostic of the participation of the unprotonated form of His-57 in catalysis of the deacylation reaction and is in keeping with the established pH dependence for deacylation of substrates of chymotrypsin family serine proteinases (30, 31). The data were fit well in all cases by a single residue protonation model in which only the unprotonated form of the enzyme is active, this model being described by the following equation,

\[ k_{diss,app} = k_{diss}/(1 + [H^+]K_H) \]  

where \( K_H \) is the dissociation constant for protonation of His-57 and \( k_{diss} \) is the pH-independent deacylation rate constant for...
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The unprotonated form of the acyl-enzyme complex. The fits yielded indistinguishable pK_a values for His-57 of 6.3 ± 0.1 for trypsin, 6.4 ± 0.1 for factor Xa, and 5.8 ± 0.5 for thrombin, the latter value being associated with a greater error due to the less stable thrombin-guanidinobenzoyl complex. These derived pK_a values are similar to the values of 6.4–6.5 previously reported for the pH dependence of deacylation of the chymotrypsin-guanidinobenzoyl complex (45) and are in keeping with the expected perturbation of the free enzyme pK_a of 6.8 for His-57 by the acyl-linked substrate (46).

Contrasting this behavior, log k_{diss, app} for the serpin-proteinase complexes showed a very different pH dependence, with a low pH plateau at pH levels ≤ 6 being followed by progressive increases at higher pH values without any indication of a plateau at pH levels up to 10–11. For antithrombin complexes with thrombin and factor Xa, the data were well fit by a hydroxide-mediated dissociation of the complex except in the low pH range where the plateau signified an approach to a pH-independent dissociation by water (47). The equation governing this model is,

\[ k_{diss, app} = k_{HOH} + k_{OH} \times [OH^-] \]  

(Eq. 3)

where \( k_{HOH} \) is the pH-independent first order rate constant for dissociation by water and \( k_{OH} \) is the pH-dependent second order rate constant for dissociation by hydroxide. No significant contribution of buffer nucleophiles to the dissociation was suggested by the indistinguishable rates of dissociation observed in different buffers (Fig. 1) (44). For the serpin complexes with trypsin, an inflection in the curve at pH 8–9 suggested that the dissociation of the complex was affected by the protonation state of a residue in the complex. The data were fit well in this case by a model involving both a hydroxide-mediated dissociation and a dissociation process dependent on a base in the complex, the equation for which is given by,

\[ k_{diss, app} = k_{HOH} + k_{OH} \times [OH^-] + k_{EI} \times [\text{EI}] / [H^+] / K_{EI} \]  

(Eq. 4)

The first two terms in this equation reflect the water and hydroxide-dependent dissociation of the major fraction of the complex as in Eq. 3, whereas in the third term, \( k_{EI} \) represents the fraction of complex whose dissociation with a first order dissociation rate constant, \( k_{EI} \), is dependent on a base with dissociation constant, \( K_{EI} \). Nonlinear regression fits of the pH dependence data to Eqs. 3 and 4 provided values for \( k_{HOH} \), \( k_{OH} \), \( K_{EI} \), and the product of \( k_{EI} \) and \( [\text{EI}] / [H^+] / K_{EI} \), which are given in Table I. A single ionizable group with a pK_a value between 8 and 9 appeared to modulate the hydroxide-dependent dissociation of the serpin-trypsin complexes. Our inability to measure the dissociation rates of antithrombin complexes with thrombin and factor Xa in the high pH range due to limited stability of these enzymes made it difficult to determine whether a similar group modulated the hydroxide-dependent dissociation of these complexes.

Also shown in Fig. 1 is the pH dependence of the apparent first order rate constant for hydrolysis of tosylarginine methyl ester, a model for the acyl ester in antithrombin-proteinase complexes, measured under the same conditions used to measure serpin-proteinase complex dissociation rates. The \( k_{diss, app} \) for hydrolysis of the ester was comparable in magnitude to that measured for the serpin-proteinase complexes and showed a pH dependence that was fit well by a hydroxide-mediated dissociation (Eq. 3) at pH values well below the pK_a of arginine. These results suggest that the catalytic His-57 is not competent to catalyze the deacylation of the acyl bond in the serpin-proteinase complexes, except possibly for a minor fraction of the complex. Hydrolysis of the acyl bond instead appears to be largely accounted for by a hydroxide ion-mediated noncatalytic process resembling that of a small ester.

The accessibility of the putative acyl bond in the serpin-proteinase complex to nucleophiles was examined by studying the effect of the potent nucleophile, hydroxylamine, on the kinetics of complex dissociation (32). Hydroxylamine at 1.5 mM considerably enhanced the dissociation rate constant 10- to 60-fold for antithrombin-trypsin, M358R α-PI-trypsin and antithrombin-thrombin complexes at pH 7.4–7.6 (Table II). The rate enhancement was proportional to the hydroxylamine con-
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Table I

Kinetic parameters for the hydroxide-mediated dissociation of serpin-proteinase complexes at 37 °C

| Serpin-proteinase complex | $k_{diss}^{\text{app}}$ | $k_{diss}^{\text{app}}$ | $pK_{diss}^{\text{app}}$ | $f_{diss} \times k_{diss}^{\text{app}}$ |
|---------------------------|-------------------------|-------------------------|-------------------------|-------------------------|
| AT-thrombin               | $1.5 \pm 0.3 \times 10^{-7}$ | $1.3 \pm 0.1$ | $8.5 \pm 0.2$ | $2.2 \pm 0.3 \times 10^{-5}$ |
| AT-factor Xα              | $2.6 \pm 0.5 \times 10^{-7}$ | $1.8 \pm 0.2$ | $8.1 \pm 0.7$ | $3.4 \pm 1.4 \times 10^{-6}$ |
| AT-trypsin                | $5.1 \pm 0.6 \times 10^{-7}$ | $0.42 \pm 0.06$ | $0.7 \pm 0.0$ | $3.7 \pm 0.8$ |
| $\alpha_1$PI-trypsin      | $3.2 \pm 0.7 \times 10^{-7}$ | $0.4 \pm 0.6$ | $0.7 \pm 0.0$ | $3.7 \pm 0.8$ |
| $\alpha_1$PI-trypsin      | $7 \pm 4 \times 10^{-8}$ | $0.8 \pm 0.0$ | $0.7 \pm 0.0$ | $3.7 \pm 0.8$ |

*Data obtained in the pH 6–10.7 range in the mixed buffer system were fit by a hydroxide-mediated dissociation but allowing for the perturbation of $k_{diss}^{\text{app}}$ by the ionization of the arginine side chain at high pH levels. Because the kinetic parameters for hydrolysis of the neutral arginine side chain were poorly determined in this fit, only the kinetic parameters pertaining to the hydrolysis of the charged arginine side chain are given.

centrations, consistent with a second order kinetic process, and SDS-PAGE confirmed that hydroxylamine accelerated the dissociation of these complexes in comparison to complexes dissociated in the absence of nucleophile. The calculated second order rate constants for aminolysis of the acyl bond by hydroxylamine varied substantially from $–5 \times 10^{-6} \text{ M}^{-1} \text{s}^{-1}$ to $–2 \times 10^{-7} \text{ M}^{-1} \text{s}^{-1}$ for the different complexes and indicated that the aminolysis reaction was much less efficient than the hydroxide-mediated hydrolysis reaction whose rate constants ranged from 0.4 to $–2 \times 10^{-7} \text{ M}^{-1} \text{s}^{-1}$. Moreover, the aminolysis of the serpin-trypsin acyl-complexes was >10-fold less efficient than that of the guanidinobenzoyl-trypsin acyl complex (Table I). These results suggest that the acyl bond in the serpin-proteinase complexes is much less accessible to hydroxylamine than to hydroxide ion.

To test whether an inactive zymogen-like conformation of trypsin in the serpin-trypsin complexes involving disruption of the Ile-16 to Asp-194 salt bridge could be induced into an active conformation, we determined the effect of adding the dipetide, Ile-Val, on the dissociation rate constants for serpin-trypsin complexes (48, 49). Addition of up to 0.1 mM Ile-Val, levels sufficient to induce guanidinobenzoyl-trypsinogen into a trypsin-like conformation, produced no significant effect on the dissociation rate constants for any of the serpin-trypsin complexes.

Stimulation of the Rate of Serpin-Proteinase Complex Dissociation by Calcium Ions—Fig. 2 shows that the rate of dissociation of the antithrombin-trypsin complex at pH 7.4, as monitored from the increased rate of hydrolysis of a chromogenic substrate as active enzyme is generated from the complex, was greatly stimulated in the presence of 10 mM calcium ions. Calculation of the first order rate constant for complex dissociation from this and other data indicated a 40- to 50-fold enhancement of the dissociation rate constant. Full progress curves of the dissociation confirmed the apparent first order dissociation rate constants measured from the initial rates of complex dissociation and verified the large stimulatory effect of calcium ions on this dissociation (Fig. 2B). Fig. 3 compares the rate constants for dissociation of several serpin-proteinase complexes in the absence and presence of either 10 mM or 100 mM calcium ions. Calcium produced significant stimulatory effects of $–6$-fold, $–10$-fold, $–20$-fold, and $–50$-fold on the dissociation rate constants for bovine trypsin complexes with $\alpha_1$PI, PAI-1, M355R $\alpha_1$PI, and antithrombin, respectively, and a $–10$-fold stimulation of the dissociation rate constant for the porcine trypsin-antithrombin complex. Considerably smaller stimulatory effects of $–4$-fold on dissociation rate constants were observed for serpin complexes with other proteinases. That these effects were specific for calcium ion and did not involve a nonspecific ionic strength effect was indicated from the insignificant enhancements of antithrombin-trypsin and M355R $\alpha_1$PI-trypsin complex dissociation rates ($–2$-fold) produced by increasing the NaCl concentration by 0.3 M, i.e. equivalent to the increase in ionic strength produced by 100 mM CaCl$_2$. These results suggested that calcium binding to a specific site on trypsin might be involved in the stimulatory effect. Addition of Ile-Val dipetide at 0.1 mM had no significant effect on the calcium-stimulated dissociation rate constants.

Fig. 4 shows the dependence of the calcium-stimulated dissociation rate constant on calcium concentration for the anti-thrombin-trypsin and M355R $\alpha_1$PI-trypsin complexes measured at pH 7.2. The calcium stimulation of the dissociation rate constant showed a saturable dependence on the calcium ion concentration over the range of 10 µM to 100 mM, which was well fit by a simple equilibrium binding process given by:

$$k_{diss, app} = k_{diss, app, 0} \times \frac{K_{Ca, app}}{[Ca^{2+}]} + k_{diss, app, Ca} \times [Ca^{2+}]$$

where $k_{diss, app, 0}$ and $k_{diss, app, Ca}$ are the apparent dissociation rate constants of the calcium-free and calcium-bound complexes at pH 7.2, respectively, and $K_{Ca, app}$ is the apparent dissociation equilibrium constant for calcium binding to the complex. The fits yielded apparent dissociation constants of 1.2 ± 0.2 and 0.49 ± 0.07 mM and maximal rate enhancements of 80- and 20-fold for calcium-stimulated dissociation of antithrombin-trypsin and M355R $\alpha_1$PI-trypsin complexes, respectively. Hydroxylamine was found to more effectively stimulate the rate of dissociation of the M355R $\alpha_1$PI-trypsin complex in the calcium-bound state as compared with the calcium-free state, the second order rate constant for aminolysis of the complex increasing $>50$-fold from 1.8 × 10$^{-4}$ M$^{-1}$ s$^{-1}$ to $>1 \times 10^{-2}$ M$^{-1}$ s$^{-1}$ (Table II), suggesting that calcium binding increases the susceptibility of the acyl bond to nucleophilic attack. SDS-PAGE analysis showed that calcium partly protected trypsin in antithrombin and M355R $\alpha_1$PI complexes with trypsin from proteolysis when the complex was formed with a molar excess of free trypsin (not shown).

Fig. 5 shows the effect of calcium ions on the pH dependence of the dissociation rate constant for the antithrombin-trypsin complex. The pH dependence of log $k_{diss, app}$ was progressively altered in the presence of 10 or 100 mM calcium ions from a dependence reflecting primarily a hydroxide-mediated dissociation in the absence of calcium ions to a pH dependence resembling that of the guanidinobenzoyl acyl-intermediate and characteristic of His-57-catalyzed deacylation of the acyl bond. Calcium ions had no effect on the rate constant for deacylation of the trypsin-guanidinobenzoyl acyl-intermediate over this pH range (Fig. 1), indicating that their stimulatory effect on complex dissociation and its pH dependence was specific for the serpin-proteinase complex. Such data suggested that the dissociation of the antithrombin-trypsin complex was controlled by inactive and active proteinase conformational states whose
**Table II**

**Effect of hydroxylamine on the rate constants for dissociation of serpin-proteinase complexes at pH 7.4–7.6, 37 °C**

Dissociation rate constants were measured in 0.1 M Hepes buffer at 37 °C under the indicated conditions from the slope of plots of the initial rate of complex dissociation versus complex concentration as described under “Materials and Methods” for at least four complex concentrations within error of <10%. The pH was 7.4 for trypsin-GB and AT-thrombin complexes, 7.5 for M358R α1 PI-trypsin complexes, and 7.6 for AT-trypsin complexes.

| Complex          | Condition          | \( k_{\text{diss, app}} \) \( \text{s}^{-1} \) | \( k_{\text{NH2OH}} \) \( \text{M}^{-1} \text{s}^{-1} \) |
|------------------|--------------------|---------------------------------|------------------|
| AT-trypsin       | 1.5 mM NaCl        | 4.2 \times 10^{-6}              | 5.9 \times 10^{-5} |
|                  | 0.75 mM NH₂OH      | 1.5 \times 10^{-4}              | 1 \times 10^{-4}  |
| AT-thrombin      | 1.5 mM NaCl        | 1.3 \times 10^{-7}              | 7.6 \times 10^{-6} |
| M358R α1 PI-trypsin | 1.5 mM NaCl    | 2.9 \times 10^{-5}              | 5.1 \times 10^{-4} |
|                  | 1.5 mM NH₂OH      | 2.9 \times 10^{-4}              | 1.8 \times 10^{-4} |
|                  | 20 mM Ca²⁺        | 5.1 \times 10^{-4}              | 1.3 \times 10^{-3} |
|                  | 20 mM Ca²⁺ + 0.1 mM NH₂OH | 2.4 \times 10^{-3}       | 1 \times 10^{-2}  |
| Trypsin-GB       | 0                  | 9.1 \times 10^{-5}              | 2.8 \times 10^{-4} |
|                  | 0.1 mM NH₂OH      | 2.8 \times 10^{-4}              | 5.2 \times 10^{-4} |
|                  | 0.2 mM NH₂OH      | 2 \times 10^{-3}                | 2 \times 10^{-3}   |

**Fig. 2.** Calcium stimulates the dissociation of the antithrombin-trypsin complex. A, comparison of the initial rates of generation of protease activity due to the dissociation of 0.7 mM antithrombin-trypsin complex in 0.1 M Hepes buffer in the presence of 1 mM EDTA (○) or 10 mM CaCl₂ (●) at 37 °C. The increase in protease activity is reflected by the increase in the rate of the absorbance change at 405 nm due to cleavage of the chromogenic substrate, S2222, with time. Solid lines are fits by the parabolic equation 1 given under “Materials and Methods” from which the initial rate of complex dissociation was derived. B, progress curves for the dissociation of ~1 μM antithrombin-trypsin complex in the presence of either 100 mM CaCl₂ or 1 mM EDTA, with the reversible active site inhibitor, α-amino benzamidine, also present at 10 mM (●) to block enzyme reassociation with excess inhibitor. The generation of enzyme from the complex was measured by assaying the activity of aliquots at varying times, after 10,000-fold dilution of the enzyme controls lacking the protein inhibitor assayed in tandem to calculate the % enzyme recovered, as described under “Materials and Methods.” Solid lines are fits of dissociation reactions by a single-exponential function or linear function with calcium or EDTA present, respectively.

relative stabilities were dependent on the linked calcium and hydrogen ion binding equilibria depicted in Scheme 1. The equation which describes this model is as follows,

\[
\begin{align*}
\text{k}_{\text{diss, app}} &= \frac{(1 + [\text{H}^+]\alpha K_\alpha) \times (k_{\text{OH}} + k_{\text{GH}} \times [\text{OH}^-]) + k_2 \times K_E \times (1 + [\text{Ca}^{2+}] / K_{\text{Ca}})}{(1 + [\text{H}^+]\alpha K_\alpha) + K_E \times (1 + [\text{H}^+] / K_\alpha) \times (1 + [\text{Ca}^{2+}] / K_{\text{Ca}})} \\
\end{align*}
\]

(Eq. 6)

in which \( K_\alpha \) and \( K_{\text{Ca}} \) are dissociation constants for hydrogen ion and calcium ion binding, respectively, to the active form of the complex, \( K_E \) represents the equilibrium constant for the interconversion between inactive and active complex states, \( \alpha \) is the linkage factor by which the conformational equilibrium is affected by hydrogen ion binding and vice versa, \( K_E \) is the intrinsic rate constant for His-57-catalyzed dissociation of the active form of the complex, and \( k_{\text{diss}} \) and \( k_{\text{OH}} \) represent the rate constants for the hydroxide-mediated dissociation of the inactive complex form as given in Eq. 3. This equation reduces to Eq. 4 when [Ca²⁺] = 0 if one neglects the term, \( K_E \).
Mechanism of Serpin-Proteinase Complex Stabilization

Fig. 4. Calcium concentration dependence of the rate constants for dissociation of serpin-trypsin complexes. Rate constants for the dissociation of antithrombin-trypsin complex (panel A) or M358R α1PI-trypsin complex (panel B) were measured in 0.1 M Hepes buffer, pH 7.2, at 37 °C in the presence of varying concentrations of CaCl2 or 100 mM CaCl2 (■) or 10 mM CaCl2 (▲) at 37 °C and the indicated pH values as described under "Materials and Methods." Data obtained in the absence of calcium (1 mM EDTA, ○) represent a global fit of the antithrombin-trypsin complex data together with the pH dependence data of Fig. 5 to the model of Scheme 1, whereas the M358R α1PI-trypsin complex data were fit by the hyperbolic binding equation 5 in the text.

Fig. 5. pH dependence of the rate constant for dissociation of the antithrombin-trypsin complex in the absence or presence of calcium. First order rate constants for the dissociation of the antithrombin-trypsin complex were measured in 0.1 M Hepes or a mixed MES/Hepes/TAPS buffer containing either 10 mM CaCl2 (▲) or 100 mM CaCl2 (■) at 37 °C and the indicated pH values as described under “Materials and Methods.” Data obtained in the absence of calcium (1 mM EDTA, ○) are reproduced from Fig. 1 for comparison. Solid lines represent a global fit of this data together with the data of Fig. 4 by Eq. 6, with $k_{\text{H}^+}$ and $k_{\text{OH}^-}$ fixed at values determined from the data in the absence of calcium (Table I).

\[
\frac{1 + [\text{H}^+]/K_{\text{H}^+}}{K_{\text{E}}} \times (1 + [\text{Ca}^{2+}]/K_{\text{Ca}^{2+}}),
\]

in the denominator due to it being small relative to other terms, with $K_{\text{E}}$ closely approximating $f_{\text{E}}$ in Eq. 4. The data of Figs. 4 and 5 were globally fit well by Eq. 6 after fixing $k_{\text{H}^+}$ and $k_{\text{OH}^-}$ at the values determined from the fit of data in the absence of calcium to Eq. 4 (Table I). This fitting indicated that a base with $pK_a$ of 8.6 ± 0.2 in the inactive conformational state of the complex was shifted downward to 6.9 ± 0.1 in the active conformational state and that this base promoted the deacylation of the complex in the latter state ($k_{\text{disapp}} = 2.0 ± 0.6 \times 10^{-4} \text{ s}^{-1}$). Moreover, the fit indicated that calcium bound to the active conformational state of the complex with an intrinsic dissociation constant of 0.093 ± 0.011 mM and that 0.2 ± 0.1% of the complex existed in this active state in the protonated base form of the calcium-free complex, with this fraction increasing to 11 ± 3% in the unprotonated base form. The model thus rationalized the >100-fold weaker apparent affinity of the complex for calcium as being due to the small fraction of the complex in a form competent to bind calcium at the pH where the affinity was measured. Together, the data support the conclusion that the binding of calcium ions to the antithrombin-trypsin complex induces the proteinase into an active conformational state in which the function of the catalytic histidine is restored, although with the $pK_a$ of the histidine perturbed slightly upward and with an inefficient rate of deacylation similar to that of the guanidinobenzoyl-trypsin complex.

DISCUSSION

The mechanism by which protein proteinase inhibitors of the serpin superfamily kinetically trap serine proteinases as stable acyl-intermediate complexes has been investigated by studying the pH dependence of deacylation of several serpin-proteinase complexes. Significantly, the pH dependence of serpin-proteinase complex dissociation was found to strongly deviate from the classical pH dependence for deacylation of acyl-intermediate complexes of serine proteinases with their substrates, indicating an abnormal functioning of the catalytic residues in the serpin-proteinase complexes. Thus, the pH dependence did not show the signature involvement of the unprotonated form of His-57 in the deacylation (45) and instead was characteristic of a nucleophilic attack of hydroxide ion on the acyl bond mediating complex dissociation (47). The similarity of the serpin-proteinase deacylation rate constants and their pH dependence to those for the uncatalyzed hydrolysis of tosylarginine methyl ester, a reasonable model for the arginine-serine acyl ester in antithrombin and M358R α1PI-proteinase complexes, supports the idea that hydroxide ion is largely responsible for the observed dissociation of the complexes. However, the strong nucleophile, hydroxylamine, enhanced the deacylation of the acyl bond through aminolysis with a second order rate constant that was several orders of magnitude smaller than that measured for hydroxide ion attack, despite the comparable reactivity of these nucleophiles toward esters (47). It would thus appear that the acyl bond has limited accessibility to nucleophiles in the complex with the larger hydroxylamine molecule being far less accessible than hydroxide ion. Although the acyl bond appears to be accessible in the crystal structure of the complex, the disordered S1 binding pocket, which is not visible in the...
structure may nevertheless be sufficiently ordered to limit acyl bond accessibility (19). Such limited acyl bond accessibility is supported by the previous observation that the fluorescence of a P1 Trp residue in the complex of M358W α1PI with chymotrypsin is not quenchable by iodide and is characteristic of the P1 Trp being in a hydrophobic environment protected from solvent (18).

Our conclusion that the proteinase catalytic residues are rendered dysfunctional in the serpin-proteinase complex is in keeping with the dramatic reductions in deacylation of the acyl bond, which stabilizes these complexes. Previous rapid kinetic comparisons of inhibitor and substrate reactions of serpins with proteinases (3, 50–52) together with our present findings suggest that the rate constant for deacylation of the serpin-proteinase acyl-intermediate complex is reduced by 7 to 8 orders of magnitude at pH 7.4 when the serpin conformational change traps the proteinase in a stable complex. Such reductions in catalytic power exceed those resulting from engineered mutations in the catalytic residues (53) and are consistent with additional disruptions in the active site, which render the catalytic residues nonfunctional. Based on the serpin-proteinase complex structure, such disruptions include loss of the Ile-16 to Asp-194 salt bridge and oxyanion hole, the separation of Ser-195 and His-57 by >6 Å, and loss of reactive loop interactions with proteinase subsites (19), implying a loss of all the active site elements required to stabilize the transition state for deacylation of the acyl linkage between the serpin and proteinase (47, 53). These structural changes together account for our finding of a complete disabling of the catalytic machinery of the proteinase, which allows only an uncatalyzed rate of deacylation of the trapped acyl intermediate. The comparable losses in catalytic power observed for the complexes of trypsin with different serpins and for a single serpin with different proteinases argues that the proteinase active site distortion mechanism, which requires full insertion of the serpin reactive loop, is a general one used by serpins and that models involving differing extents of reactive loop insertion are less likely (54, 55).

Our findings are in keeping with structural and biochemical evidence that the mechanism by which serpins trap proteinases in stable acyl-intermediate complexes involves more than just a conformational switching of the proteinase into azymogen-like state as previously proposed (17). Previous observations that the complexed proteinase becomes much more susceptible to proteolysis by free proteinase (14, 16, 20) are consistent with the structural findings that the proteinase is partly denatured in the complex due to its compression against the serpin, the energy for this compression being derived from the large stabilization of the serpin structure by reactive loop insertion into β-sheet A (19). The resulting disorder in about one-third of the proteinase in the crystal structure and possibly more in the solution structure based on NMR data (21) represents a much more drastic change in structure than that resulting fromzymogen activation. In keeping with the proteinase not being in a zymogen-like state in the serpin-proteinase complex, the dipeptide, Ile-Val, failed to enhance the deacylation of serpin-trypsin complexes by mimicking the Ile-16 to Asp-194 salt bridge, which triggers the conformational switch to a catalytically active proteinase (48, 49). Moreover, the favored inactive proteinase conformation in the serpin-proteinase complex was found to be incapable of undergoing His-57-catalyzed deacylation, in contrast tozymogens, which undergo acylation and deacylation like that of the active proteinase, albeit at greatly reduced rates, with a pH dependence consistent with His-57 catalysis (45).

Although the proteinase distortion mechanism involves marked changes in proteinase structure, which appear to include partial denaturation of the enzyme (19), our finding that calcium ions are capable of restoring the catalytic function of the proteinase in several serpin-trypsin complexes suggests that calcium binding can reverse these structural changes. The stimulatory effect of calcium ions on the rate of dissociation of serpin-trypsin complexes suggests that high affinity calcium ion binding to a specific calcium binding loop on the proteinase is responsible for these effects (56). The binding of calcium to this site in the proenzyme, trypsinogen, is known to promote the autoactivation of the proenzyme (57), suggesting that bound calcium destabilizes thezymogen conformation and shifts the proenzyme into an active enzyme conformation. Bovine pancreatic trypsin inhibitor also can bind to the proenzyme by driving it into the active enzyme conformation, although with an energetic cost for inducing this unfavorable conformation, which is reflected by a six order of magnitude lower affinity of the inhibitor for thezymogen relative to the active enzyme (48). Similarly, calcium may alter the conformation of trypsin in the serpin-proteinase complex from its inactive partially denatured state to an active native state and account for the enhanced rate of deacylation of the serpin-proteinase complex. This idea is supported by the structure of the serpin-proteinase complex, which reveals that the calcium binding loop comprising residues 70–80 of trypsin (chymotrypsin numbering) (56) is one of the regions disordered in the complex (19). Calcium binding to this loop clearly requires the loop to refold, which may induce the coordinate refolding of the proteinase domain disordered by the serpin conformational change. Experimental evidence for this refolding was provided by the observation that trypsin complexed with a serpin was protected from proteolysis by free trypsin when calcium was bound, similar to the effect of calcium on free trypsin (56). The binding of calcium to a refolded active conformation of the proteinase implies that such a conformation exists in the absence of calcium in equilibrium with the predominant inactive conformation.

Evidence for this active conformation was found from the finding of an inflection in the pH dependence for dissociation of antithrombin and α1PI complexes with trypsin in the high pH range, which implied that a base in the complex affected the dissociation rate. This data, together with the data showing that calcium progressively reverts the pH dependence of antithrombin-trypsin complex dissociation to one resembling that of the deacylation of the guanidinobenzoyl acyl-enzyme complexes, could be quantitatively fit by the model of Scheme 1, wherein the proteinase exists in both inactive and active conformational states in the calcium-free complex and calcium shifts the proteinase into an active state by selectively binding to the active conformation. The model explains the inflection in the pH dependence curve in the absence of calcium as arising from the small fraction of functionally active proteinase in equilibrium with the predominant inactive proteinase, this fraction being −0.2% for the antithrombin-trypsin complex at pH 7.2. The active fraction increases at higher pH values in parallel with the deprotonation of an upwardly perturbed His-57 pKₐ in the inactive proteinase conformation. The observed pKₐ perturbation is in agreement with direct NMR measurements of this pKₐ in a serpin-proteinase complex (26). A smaller fraction of the α1PI-trypsin complex existing in the active state is implied by the −10-fold lower apparent rate constant for deacylation determined for the active fraction (Table 1) and is in keeping with the slower rate of dissociation of this complex. The even lower dissociation rate constants observed for antithrombin complexes with thrombin and factor Xa and failure to observe the inflection in the pH dependence suggest that the fraction of active complex may be the lowest in
these complexes, which involve the natural target proteinases of antithrombin. The observed dissociation rate constant of a serpin-proteinase complex may thus depend on the extent to which the inactive proteinase conformation has been stabilized in the complex and the accessibility of hydroxide ion to the acyl bond in this conformation. An alternative explanation for the inflection in the pH dependence data, but such a model fails to account for negatively charged nucleophile could also explain the inflection in the pH dependence data, but such a model fails to account for calcium’s ability to shift the complex into an active state that requires an active state in the absence of calcium.

The intrinsic \( K_D \) of \( \sim 0.1 \) m\( M \) for calcium binding to the antithrombin-trypsin complex obtained from the fitting of data to the model of Scheme 1 is consistent with the proteinase being in a conformation competent to bind calcium with high affinity and is close to the calcium affinity reported for free trypsin (\( K_D \sim 10 \) m\( M \)) (58). The weaker apparent calcium affinity measured for the antithrombin-trypsin complex at pH 7.2 reflects the small fraction of complex in the active conformation at this pH and is an indication of the energetic cost of shifting the proteinase from the favored inactive state to the active state. The higher calcium affinity measured for the M358R \( \omega \)PI-trypsin complex implies that a greater fraction of the proteinase exists in the active conformation for this complex, a conclusion in keeping with the higher dissociation rate constant measured for this complex in the absence of calcium. The calcium-induced change in pH dependence of the rate of antithrombin-trypsin complex dissociation to one resembling the guanidinobenzoyl acyl-intermediate implies a re-engagement of the catalytic apparatus in the calcium-bound complex, although this re-engagement must reflect only a partial restoration of function, because the pK\text{a} \( \omega \)IleVal, was unable to stimulate the dissociation of the calcium-bound complex. The disrupted Ile-16 to Asp-194 salt bridge may thus be restored by calcium binding. The comparable catalytic efficiencies of deacylation of the calcium-bound serpin-proteinase complexes and the guanidinobenzoyl acyl-enzyme complexes (44, 45) suggest that poor positioning of the serpin P1 residue in the S1 site may be responsible for the low catalytic efficiency of the His-57-mediated deacylation for both types of complexes based on the structure of the guanidinobenzoyl complex with trypsin (59). Such an improper positioning of the P1 residue in the proteinase active site in the calcium-bound serpin-proteinase complex would be expected if the proteinase remains physically anchored to the serpin by a taut reactive loop tether even after refolding of the disordered proteinase domain. Although calcium-induced refolding of the proteinase would be expected to cause a steric crowding at the serpin-proteinase interface, this may be relieved by the expulsion of the reactive loop residues adjacent to P1 with the proteinase still anchored (19). The equilibrium between inactive and active proteinase states in the serpin-proteinase complex may thus be concerted with movements of reactive loop residues proximal to the proteinase in and out of sheet A. The importance of proximal loop residue-sheet A interactions in complex stability could explain the different stabilities of variant serpin-proteinase complexes in which serpin reactive loop P2–P6 residues are mutated (12, 29).

Although the small calcium ion effect on antithrombin-thrombin complex dissociation is consistent with the calcium binding residue, Glu-70, being replaced with Lys in thrombin (56), similar small calcium effects on the dissociation of the antithrombin-factor Xa complex which retains a calcium binding site (60) were found. The inability of calcium ions to stimulate the dissociation of the antithrombin-factor Xa complex and variable ability of calcium to stimulate the dissociation of several serpin-trypsin complexes may reflect the different affinities of these proteinases for calcium in the serpin-proteinase complex as well as the different fractions of proteinase in the active conformation competent to bind calcium. Because the calcium affinity is linked to the energetics of refolding of the proteinase, it follows that higher energy barriers may exist for this refolding in different complexes that depend on both the serpin and the proteinase. These findings are thus not incompatible with our proposal that calcium ions can alter the conformation of the proteinase in the serpin-proteinase complex and thereby affect the ability of the catalytic residues to catalyze deacylation of the complex. Such findings are reminiscent of the observation that a variant heparin cofactor II-thrombin complex is stimulated to dissociate by the binding of heparin to a thrombin exosite, which allosterically alters the catalytic residues (61).

In summary, our findings provide direct evidence that serpin-proteinase complexes are kinetically stabilized by a complete inactivation of proteinase catalytic residue function and protection of the acyl bond from solvent nucleophiles and are in keeping with the gross distortions of the proteinase active site observed in the crystal structure of a serpin-proteinase complex and inferred from NMR structural studies of the same complex (19, 21). However, our findings additionally demonstrate that the inactive proteinase conformation in the complex is in a dynamic equilibrium with an active proteinase conformation and that the complex can be shifted into the active conformation by the preferential binding of proteinase ligands to this conformation. They thus reveal a more dynamic mechanism by which serpins regulate the activity of their target proteinases and suggest that proteinase ligands could potentially play a role in this regulation.

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The pH Dependence of Serpin-Proteinase Complex Dissociation Reveals a Mechanism of Complex Stabilization Involving Inactive and Active Conformational States of the Proteinase Which Are Perturbable by Calcium

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