Inhibitory Mechanism of Serpins. Interaction of Thrombin with Antithrombin and Protease Nexin 1[†]

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ABSTRACT: The mechanism for the inhibition of thrombin by the serpins antithrombin and protease nexin 1 has been investigated using several kinetic techniques at pH 7.9 and 37 °C with an ionic strength of 0.3 M. Rapid kinetic studies demonstrated that a two-step mechanism for the formation of the stable thrombin−serpin complex applied to both serpins. The inhibition constant for the initial thrombin−antithrombin complex was 265 μM, and the rate constant for the conversion of this complex to the final one was 3.9 s^{-1}; the corresponding values for PN1 were 3.4 μM and 6.0 s^{-1}. By using slow-binding kinetics, it was possible to obtain estimates of the second-order rate constants for the formation of the stable thrombin−serpin complexes (1.2×10^4 and 1.5×10^6 M^{-1} s^{-1} for antithrombin and protease nexin 1, respectively) and the dissociation constants for these complexes (<1 nM for both serpins). The influence of viscosity on the reactions indicated that the rate of interaction of both serpins with thrombin was diffusion-controlled. Moreover, the results indicated that the initial complex reacted more rapidly to form the stable complex than it dissociated to free enzyme and inhibitor; i.e., the behavior of the serpins was analogous to that of "sticky" substrates. By using the results from slow-binding, viscosity, and rapid kinetic studies, it was possible to set values for all of the rate constants for the interactions of antithrombin and protease nexin 1 with thrombin. The effect of pH on the reactions indicated an essential role for the catalytic histidine of thrombin in the formation of complexes with the serpins; complexes were able to form only when this residue was in its active (unprotonated) form.

Serine protease inhibitors (serpins) are a family of structurally homologous proteins whose members are involved in the control of the activity of proteases involved in various processes, including blood coagulation, fibrinolysis, complement activation, and inflammation (Travis & Salvesen, 1983; Huber & Carrell, 1989; Potempa et al., 1994). Mammalian serpins are relatively large proteins (>400 amino acids) composed of three β-sheets surrounded by α-helices. The target protease of the serpin interacts with a reactive-site loop of about 15 amino acids that connects two of the β-sheets (Huber & Carrell, 1989). Crystal structures of serpins indicate that this loop can exist in a number of conformations. In serpins that have been cleaved within the reactive-site loop, the residues on the N-terminal side of the P_1 residue (Schechter & Berger, 1967) are incorporated as an additional strand into the A β-sheet (Löbermann et al., 1984; Baumann et al., 1991, 1992; Mourey et al., 1993). The latent form of plasmin activator inhibitor 1, which has lost its inhibitory activity, has an intact loop incorporated into the same position (Mottonen et al., 1992). Recent X-ray crystallographic studies suggest that the reactive-site loops of active serpins exist in an extended conformation with the insertion of only a few residues at the N-terminus of the loop into the A β-sheet (Schreuder et al., 1994; Carrell et al., 1994; Wei et al., 1994).

Kinetic studies indicate that the formation of the stable protease−serpin complex involves at least two steps (Olson & Shore, 1982; Stone et al., 1987; Bruch & Bieth, 1989; Longstaff & Gaffney, 1991; Faller et al., 1993; Morgenstern et al., 1994). The formation of an initial loose complex is followed by isomerization to yield a tight complex. The active-site serine of the protease appears to be involved in stabilizing the tight complex. Results from a number of studies suggest that the stable complex exists as an acyl-enzyme in which the P_1-P_1' bond of the serpin is cleaved (Rosenberg & Damus, 1973; Owen, 1975; Jesty, 1979; Fish et al., 1979; Ferguson & Finlay, 1983), but recent NMR studies suggest that it may take the form of a tetrahedral intermediate (Matheson et al., 1991). In either case, it is unlikely that the reactive-site loop has been completely inserted into the A β-sheet since this would preclude the observed release of active inhibitor from the complex (Griffith & Lundblad, 1981; Beatty et al., 1982; Shieh et al., 1989).

In the present study, several kinetic techniques have been employed to investigate the steps involved in the formation of stable complexes between thrombin and the serpins, antithrombin and protease nexin 1 (PN1).[1] Slow-binding kinetics was used to estimate the effective association rate constants for the serpins and inhibition constants for the stable complexes. Stopped-flow kinetics demonstrated a two-step mechanism for both serpins. The effect of viscosity on the reactions suggested that the generation of the stable complex was diffusion-controlled, and pH studies indicated

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[1] Abbreviations: PN1, protease nexin 1; Pip, pipecolyl; Bz, benzoyl; pNA, *p*-nitroanilide, AMC, 7-amido-4-methylcoumarin; Z, benzyloxycarbonyl.

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an essential role for catalytic histidine in the inhibitory mechanism. By using data from these experiments, it was possible to estimate values for all of the rate constants for the formation of the thrombin−serpin complexes.

EXPERIMENTAL PROCEDURES

Materials. The substrates D-Phe-Pip-Arg-pNA and D-Val-Leu-Arg-pNA were obtained from Chromgenix (Mölndal, Sweden), and Z-Gly-Pro-Arg-AMC was from Bachem (Bubendorf, Switzerland). Human α-thrombin was prepared and characterized as described previously (Stone & Hofsteenge, 1986). Recombinant protease nexin 1 (PN1) was kindly provided by Prof. Denis Monard (Friedrich Miescher-Institut, Basel, Switzerland). Antithrombin was purified from human plasma as described by MacKay (1981).

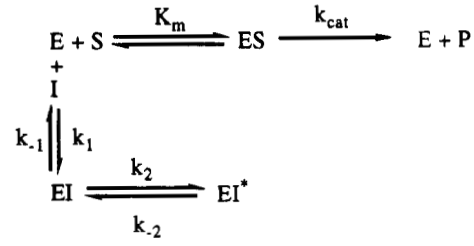
Chromogenic assays were performed at 37 °C in 0.1 M MES/0.05 M Tris/0.05 M ethanolamine buffer containing 0.2 M NaCl and 0.2% (w/v) poly(ethylene glycol) M_r 6000 (assay buffer) using the substrate D-Phe-Pip-Arg-pNA as previously described (Stone et al., 1991). This buffer maintains a constant ionic strength of 0.3 M over the pH range used. In all studies except those investigating pH effects, the pH of the assay buffer was 7.9. For experiments examining the effect of viscosity, the relative viscosity (η_{rel}) was varied by adding 15−32% (w/v) sucrose, and the value of η_{rel} was determined at 37 °C by using an Ubbelodhe viscometer. The release of *p*-nitroaniline due to the cleavage of the substrate was monitored at 400−410 nm with a Hewlett-Packard 8452A spectrophotometer. The concentration of D-Phe-Pip-Arg-pNA was determined from its absorbance at 342 nm (Lottenberg & Jackson, 1983).

In slow-binding inhibition experiments using the chromogenic assay, the concentrations of D-Phe-Pip-Arg-pNA and thrombin were 100−200 μM and 20−200 pM, respectively. The duration of these progress curve experiments varied from 20 min to 15 h. The concentrations of serpins were adjusted such that the steady-state velocity of the progress curves was achieved over this period. Data points were excluded from the analyses when the level of substrate utilization was greater than 10%. In the absence of inhibitor, the decrease in the activity of thrombin was less than 5% over the time period used. Incubation of PN1 and antithrombin at the pH of the assay for the time used did not result in significant decreases in the serpins' activity.

Stopped-flow fluorogenic assays were performed essentially as described by Gan et al. (1994) in the assay buffer at 37 °C. Thrombin activity was monitored after mixing a solution containing 12.5−25 nM thrombin with one containing PN1 or antithrombin and 50 μM Z-Gly-Pro-Arg-AMC. Experiments were performed using an Applied Photophysics SF17.MV stopped-flow fluorometer. The excitation wavelength was 380 nm with a bandwidth of 10 nm, and a 400 nm cutoff filter was used between the cell and the fluorescence detector. For data analysis, the average of at least three individual traces was used. Rate constants and amplitudes of the averaged traces were obtained by fitting the data to the equation describing a single-exponential decay using the SF17.MV nonlinear regression software.

Determination of the Active Concentrations of Proteins. The concentration of thrombin was determined by active-site titration with *p*-nitrophenyl *p'*-guanidinobenzoate in 0.1 M sodium barbitone and 0.02 M $CaCl_2$ (pH 8.3) at 25 °C (Chase & Shaw, 1970). Aliquots of different amounts of serpins were incubated with a known concentration of thrombin for a period of time sufficient to ensure that the formation of complex was complete. The residual thrombin concentration was then determined after the addition of 100 μM D-Val-Leu-Arg-pNA, as described earlier. Regression analysis of the dependence of residual activity on the amount of serpin yielded the concentration of active serpin. The kinetic constants were calculated on the basis of active serpin concentrations.

Scheme 1



Dissociation of Thrombin−Serpin Complexes. Thrombin (1 μM) was incubated alone and with equimolar concentrations of the serpins in assay buffer for 10 min at 37 °C. Duplicate 10 μL aliquots of each incubation were then taken, and the thrombin activity was measured using 200 μM D-Val-Leu-Arg-pNA as described earlier. Further duplicate aliquots were removed, and the thrombin activity was determined at seven different times over a period of 2 days. The activity of thrombin alone decreased by about 20% over this period. The increase in the activity of thrombin in the thrombin−antithrombin sample (A) relative to the activity of thrombin alone (A_o) was then fitted by nonlinear regression to the equation for an exponential decay ($A/A_o = 1 - e^{-kt}$) in order to estimate the rate constant (k) for the dissociation of the complex.

THEORY AND DATA ANALYSIS

General Kinetic Mechanism. There is kinetic evidence that the formation of the stable protease−serpin complex involves two steps (Olson & Shore, 1982; Stone et al., 1987; Bruch & Bieth, 1989; Longstaff & Gaffney, 1991; Faller et al., 1993; Morgenstern et al., 1994), and thus, Scheme 1 presents the most general inhibitory mechanism (Morrison & Walsh, 1988).

In Scheme 1, E, I, S, and P represent thrombin, serpin, substrate, and substrate cleavage products, respectively. For this mechanism, the progress curve of the formation of product will be described by the following equation (Morrison, 1982):

$$[P] = v_s t + \frac{v_i - v_s}{k'}(1 - \exp(-k't)) \qquad (1)$$

where [P] is the concentration of product at time t, k' is an apparent first-order rate constant, and v_i and v_s are the initial and steady-state velocities, respectively. When the concentration of inhibitor is much greater than its apparent overall inhibition constant (K_i'), v_s will be negligible and eq 1 simplifies to

$$[P] = (v_i/k')(1 - \exp(-k't)) \qquad (2)$$

For the two-step mechanism presented in Scheme 1, both v_i and v_s will decrease with inhibitor concentration, and k' will show a hyperbolic dependence on the concentration of inhibitor. The following relationships for v_i, v_s, and k' can be derived on the basis that the initial complex (EI) is in rapid equilibrium with the free enzyme (E) (Cha, 1975; Morrison, 1982; Morrison & Walsh, 1988). The rapid-equilibrium assumption will be valid if $k_{-1} \gg k_2$:

$$k' = \frac{k_2}{1 + K_{init}(1 + [S]/K_m)/[I]} + k_{-2} \quad (3)$$

$$v_i = \frac{v_0}{1 + [I]/(K_{init}(1 + [S]/K_m))} \quad (4)$$

$$v_s = \frac{v_0}{1 + [I]/(K_i(1 + [S]/K_m))} \quad (5)$$

where

$$K_{init} = k_{-1}/k_1 \quad (6)$$

$$K_i = \frac{K_{init}k_{-2}}{k_2 + k_{-2}} \quad (7)$$

The parameter v_o is the enzyme's velocity in the absence of inhibitor, K_i is the overall inhibition constant derived from the amount of inhibited enzyme at infinite time, and K_{init} is the inhibition constant for the initial complex (EI). Data obtained in experiments in which the effect of viscosity on the rate of association of the serpins was examined suggested that the rapid-equilibrium assumption was not appropriate for thrombin–serpin interactions; i.e., the condition $k_{-1} \gg k_2$ did not apply (see the following and Results). If a steady-state concentration of EI forms rapidly, and if k_1[E][I] is approximately equal to $(k_{-1} + k_2)$[EI] during the course of the progress-curve experiment, the following expression for K_{init} can be derived:

$$K_{init} = \frac{k_{-1} + k_2}{k_1} \quad (8)$$

For the two-step mechanism in which a steady-state concentration of EI forms, the preceding equations for v_i, v_s, and k' will apply, except that K_{init} will be defined by eq 8; i.e., v_i and v_s decrease with serpin concentration, while k' shows a hyperbolic concentration dependence.

Stopped-Flow Kinetic Studies. Stopped-flow kinetic data were fitted to the equation describing a single-exponential decay to obtain estimates for k' and the amplitude of the decay. The concentrations of antithrombin and PN1 used were much greater than K_i and, thus, v_s was negligible. The value of v_i could be calculated from the amplitude multiplied by k' (see eq 2). The concentration dependence of the k' values was fitted to eq 3 by nonlinear regression to give estimates of K_{init} and k_2, while the variation of v_i with concentration was fitted to eq 4 to yield a second estimate of K_{init}. The value of K_m for Z-Gly-Pro-Arg-AMC required in these calculations was determined to be 287 ± 9 μM.

Slow-Binding Kinetic Studies. Under the conditions of the slow-binding assays, v_i did not vary with the concentration of serpin, and k' displayed a linear dependence on serpin concentration. This will occur when significant concentrations of the initial (EI) complex are not formed at the concentrations of serpin used (Morrison & Walsh, 1988), and in this case the dependence of k' on the concentration of serpin will be given by the following equation:

$$k' = \frac{k_2[I]}{K_{init}(1 + [S]/K_m)} + k_{-2} \quad (9a)$$

$$= k'_{ass}([I] + K_i') \quad (9b)$$

where the effective association rate constant k_{ass} is defined as k_2/K_{init}, and k'_{ass} and K_i' are apparent constants equal to $k_{ass}/(1 + [S]/K_m)$ and $K_i(1 + [S]/K_m)$, respectively. Equations 5 and 9b were substituted into eq 1, and the data from a slow-binding inhibition experiment consisting of six or seven progress curves with different concentrations of serpin were fitted to this overall equation by nonlinear regression (Stone & Hofsteenge, 1986). From this analysis, estimates of K_i and k_{ass} were obtained; the K_m value required for these calculations was obtained in a previous study (Stone et al., 1991). Data were also fitted to an equation that assumed that the inhibition was irreversible (v_s in eq 1 was set to zero); in all cases, a better fit was obtained when reversible inhibition was assumed (see Figure 2).

Each slow-binding inhibition experiment was performed at least twice, and the values of the parameters given in Table 1 represent the weighted means of these determinations. The standard errors of values reported in Table 1 were obtained from the variance–covariance matrix of the regression analysis. Because of the large number of points used in progress curve kinetics (about 30 points per curve were analyzed), the standard errors are underestimated (Duggleby & Morrison, 1978). It should be noted that while the analysis of the data in terms of slow-binding inhibition yielded values for k_{ass} that were highly reproducible between experiments (a variation of less than 10% in the estimated values was obtained), the estimates for K_i varied by as much as 2–3-fold between experiments. This variation can be attributed to the fact that the inhibitor concentrations were not ideal for the estimation of K_i. In order to achieve inhibition within a reasonable period of time during which the enzyme and inhibitor were stable, it was necessary to use concentrations of inhibitor that were much greater than the K_i value.

For the determination of viscosity and pH effects, data from single progress curves were analyzed according to eq 1. Under these conditions, it is possible to derive the following expression for k_{ass} (Morrison & Stone, 1985):

$$k_{ass} = \frac{k'(1 + [S]/K_m)}{[I](1 - v_s/v_i)} \quad (10)$$

K_m values for D-Phe-Pip-Arg-pNA determined previously under the conditions of the assay were used in these calculations (Stone et al., 1991).

Viscosity Effects on Thrombin–Serpin Interactions. Viscosity will affect all steps involving diffusion (k_1 and k_{-1}), and the rate constants for these steps can be expressed as $k = {}^0k/\eta_{rel}$, where η_{rel} is the relative viscosity of the solution and ${}^0k$ is the value of the rate constant in the absence of a viscogenic agent (Nakatani & Dunford, 1979; Brouwer & Kirsh, 1982). For the two-step reaction of Scheme 1, k_{ass} will equal k_1k_2/k_{-1} and $k_1k_2/(k_{-1} + k_2)$ for the rapid-

Table 1: Kinetic Parameters for the Inhibition of Thrombin by Serpins[a]

	parameter			
	k_{ass} (M^{-1} s^{-1})	K_{init} (μM)	k_2 (s^{-1})	K_i (pM)
antithrombin[b]	$(1.18 \pm 0.10) \times 10^4$			310 ± 10
antithrombin[c]	$(1.47 \pm 0.06) \times 10^4$	265 ± 49	3.9 ± 0.6	
PN1[b]	$(1.51 \pm 0.18) \times 10^6$			11 ± 1
PN1[c]	$(1.77 \pm 0.09) \times 10^6$	3.41 ± 0.48	6.0 ± 0.5	

[a] Assays were performed and data were analyzed as described in Experimental Procedures to yield the estimates of the parameters which are given together with their standard errors. [b] These values were obtained from slow-binding inhibition experiments. [c] These values of K_{init}, k_2, and k_{ass} ($=k_2/K_{init}$) for antithrombin and PN1 were determined from stopped-flow kinetic experiments (Figure 1). Additional estimates for K_{init} of 261 ± 49 and 2.73 ± 0.61 μM were obtained for antithrombin and PN1, respectively, from analyses of the concentration dependence v_i according to eq 4.

equilibrium and steady-state mechanisms, respectively. Thus, no viscosity effect on k_{ass} will be observed for the rapid-equilibrium two-step mechanism, because viscosity will affect the magnitudes of k_1 and k_{-1} to the same extent with the result that k_{ass} will not be changed by viscosity. For the steady-state two-step mechanism, the observed value of k_{ass} for the serpin will depend on the viscosity of the solution according to

$$k_{ass} = \frac{k_1}{\eta_{rel} + k_{-1}/k_2} \quad (11)$$

At least two estimates of k_{ass} were obtained at different relative viscosities by fitting progress curve data to eq 1 and using eq 10 to calculate the values. Viscosity has been shown to alter the K_m for the substrate D-Phe-Pip-Arg-pNA, and previously determined values for this parameter were used in the calculation of k_{ass} (Stone et al., 1991). The estimates of k_{ass} were weighted according the inverse of their variances and fitted to eq 11 to obtain estimates for k_1 and k_2/k_{-1}. In order to compare the k_2/k_{-1} ratio for interactions with widely different values of k_1, the ratio ${}^0k_{ass}/k_{ass}$ can be plotted against η_{rel} (eq 12), where ${}^0k_{ass}$ is the value of k_{ass} in the absence of a viscogenic reagent. For this plot, the horizontal intercept will be given by $-k_{-1}/k_2$, and the vertical intercept will be $k_{-1}/(k_{-1} + k_2)$:

$$\frac{{}^0k_{ass}}{k_{ass}} = \frac{k_{-1} + k_2\eta_{rel}}{k_{-1} + k_2} \quad (12)$$

For these plots (Figure 3), the values of ${}^0k_{ass}$ determined from the analysis of the data according to eq 11 were used.

For interpretation of the effects of viscosity in terms of a reduction in the rates of diffusion, it is important that the structures of the reactants are not perturbed by the viscogenic reagent. Viscosity will not affect the rate of reactions that are not diffusion-controlled, and thus, the absence of an effect of viscosity on slow reactions provides a control for the lack of an effect of the viscogenic reagent on the structures of the reactants (Brouwer & Kirsh, 1982). It has previously been shown that viscosity does not affect the slow reaction of thrombin with Bz-Arg-pNA (Stone et al., 1991). In addition, the slow reaction of antithrombin with activated protein C also was not affected by viscosity. The inactivation rate constant for antithrombin with activated protein C was determined as described previously (Hermans & Stone, 1993). This rate constant was 0.22 ± 0.03 s^{-1} in the absence of sucrose and 0.20 ± 0.02 s^{-1} in the presence of 32% (w/v) sucrose.

pH Effects on Thrombin–Serpin Interactions. Estimates for k_{ass} were obtained at different pH values by fitting progress curve data to eq 1 and using eq 10 to calculate the k_{ass} values. At least two estimates were obtained at each pH value. The pH profiles of k_{ass} for antithrombin and PN1 with thrombin were bell-shaped and could be described by

$$k_{ass} = \frac{k^*_{ass}}{1 + [H]/K_1 + K_2/[H]} \quad (13)$$

where k^*_{ass} is the pH-independent value of k_{ass}, and K_1 and K_2 are acid dissociation constants. Estimates of k_{ass} obtained at different pH values were weighted according the inverse of their variances and fitted to eq 13 by nonlinear regression.

RESULTS

Kinetic Mechanism for Inhibition of Thrombin by PN1 and Antithrombin. The inhibition of thrombin by antithrombin and PN1 was studied by stopped-flow and slow-binding kinetic techniques in order to obtain estimates of the inhibition constants for the initial (K_{init}) and final complexes (K_i) and of the rate constant for the formation of the final complex from the initial one (k_2). Rapid kinetic studies yielded estimates for K_{init} and k_2, while slow-binding inhibition experiments allowed the estimation of K_i.

A two-step mechanism could be demonstrated for antithrombin and PN1 by using stopped-flow kinetics. Rate constants (k') for the inhibition of thrombin by antithrombin and PN1 were determined using the substrate Z-Gly-Pro-Arg-AMC to monitor thrombin's activity in a stopped-flow fluorometer. The observed values of k' displayed a hyperbolic dependence on the concentrations of antithrombin and PN1 (Figure 1). These data were fitted to eq 3, which describes the expected concentration dependence of k' for the two-step mechanism of Scheme 1. The estimates obtained for k_{-2} (the y-intercept of eq 3) were not significantly different from zero. Therefore, this parameter was set to zero and the data were reanalyzed; the results of these analyses are given in Table 1. The inhibition constants for the initial complex (K_{init}) were 265 μM for antithrombin and 3.4 μM for PN1. The rate constants for the formation of the second complex (k_2) were 3.9 s^{-1} for antithrombin and 6.0 s^{-1} for PN1. A second estimate for K_{init} was obtained from an analysis of the variation of v_i with the concentration of serpin according to eq 4 (data not shown). The estimates for K_{init} of 261 ± 49 μM for antithrombin and 2.73 ± 0.61 μM for PN1 obtained from these analyses corresponded well with those from the analyses of the variation of k' (Table 1). Values for the effective association rate constant at low concentrations of serpin ($k_{ass} = k_2/K_{init}$) were 1.47×10^4 and 1.77×10^6 M^{-1} s^{-1} for antithrombin and PN1, respectively (Table 1).

Estimates for k_{ass} were also obtained from slow-binding inhibition experiments. These values agreed with those from the stopped-flow experiments (Table 1) and with those previously published for PN1 and antithrombin (Scott et al., 1985; Stone et al., 1987; Wallace et al., 1989; Olson & Shore, 1989; Jesty, 1979; Hogg et al., 1991). Both serpins formed very stable, reversible complexes with thrombin. Analysis

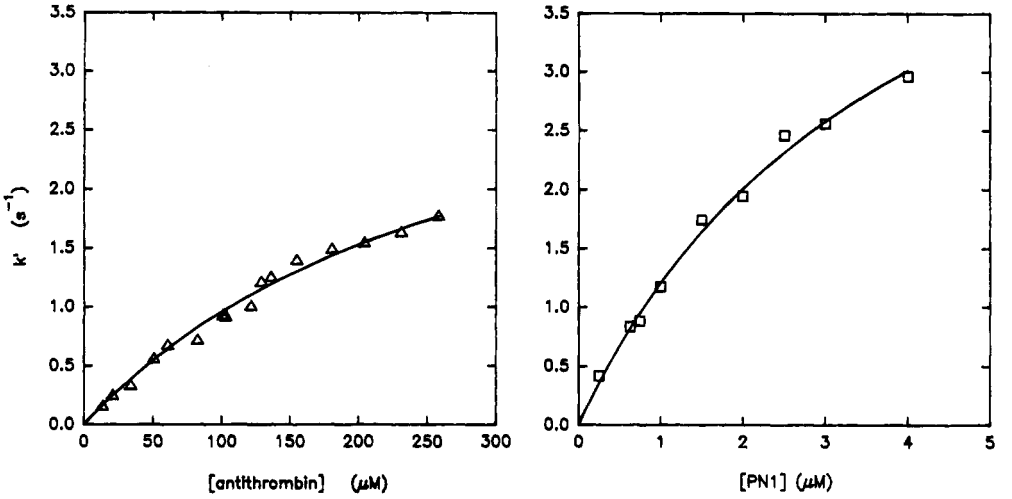


FIGURE 1: Concentration dependence of the observed rate constant for the inhibition of thrombin by antithrombin and PN1. Stopped-flow kinetic assays were performed, and the data were analyzed as described in Experimental Procedures to yield estimates of the observed rate constant (k') at the indicated concentrations of antithrombin and PN1. The concentration dependence of k' was analyzed according to eq 3 with k_{-2} set to zero to give estimates for K_{init} and k_2 (Table 1). The lines show the fit of the data to this equation.

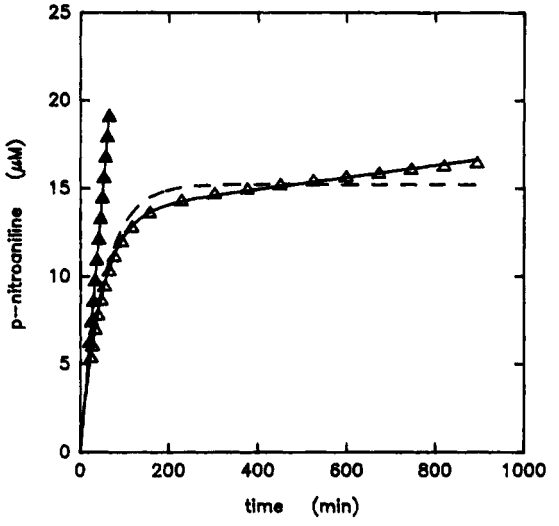


FIGURE 2: Comparison of the fit of slow-binding inhibition data to equations describing reversible and irreversible mechanisms. Slow-binding kinetic assays were performed as described in Experimental Procedures with 25 pM thrombin and 194 μM D-Phe-Pip-Arg-pNA. The assays contained 0 (▲) and 0.5 μM (△) antithrombin. Data points before 20 min are not shown, and only each second thereafter is plotted. The data were fitted to equations describing reversible (solid line) and irreversible inhibition (dotted line) as outlined in Theory and Data Analysis. The better fit to the reversible mechanism is evident.

of the data according to the equation that assumed irreversible inhibition yielded a worse fit, as shown in Figure 2 for antithrombin. The complex with antithrombin, however, was somewhat less stable than the one with PN1; the K_i value for antithrombin was about 30-fold higher than that observed for PN1 (310 versus 11 pM; Table 1). The K_i values for antithrombin and PN1 agreed well with those previously determined (Jesty, 1979; Griffith & Lundblad, 1981; Stone et al., 1987). An estimate of the rate of dissociation of serpin from the final complex can be calculated using the relationship $k_{diss} = K_i k_{ass}$; the estimates for k_{diss} obtained using this formula were 3.7×10^{-6} and 1.7×10^{-5} s^{-1} for antithrombin and PN1, respectively. The estimate of k_{diss} for antithrombin agreed with values previously obtained for this constant using different methods and conditions (Jesty, 1979; Griffith & Lundblad, 1981). The apparent reversibility of the stable complex could be due to either the release of intact inhibitor from the complex, as shown in Scheme 1, or the release of cleaved inhibitor (I′) as shown in Scheme 2.

Scheme 2

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EI^* \xrightarrow{k_3} E + I'$$

The mechanism of Scheme 2 would be consistent with the observed data, provided the rate of cleavage of the inhibitor (k_3) was small, such that significant depletion of the inhibitor did not occur during the course of the experiment. Inhibitor depletion would manifest itself as an upward curvature of the progress curves due to relief of the inhibition, and such curvature was not observed. The contribution of the cleavage pathway to the reversibility of inhibition was examined by incubating thrombin and the serpins at an equimolar concentration of 1 μM and measuring the release of active thrombin over a period of 2 days; during this time period, the activity of 1 μM thrombin alone decreased by about 20%. At the concentrations used, active thrombin will be generated only by the release of cleaved inhibitor since active inhibitor will immediately recombine with the enzyme. The PN1−thrombin complex did not break down to yield any active thrombin, whereas 0.1 μM thrombin was released from the antithrombin−thrombin complex over the 2 days (data not shown). Regression analysis of the time dependence of the release of active thrombin from the complex according to an exponential decay yielded a rate constant of $(5.8 \pm 0.3) \times 10^{-7}$ s^{-1}. This value for the rate of release of cleaved antithrombin is very close to the estimate obtained previously at neutral pH (Griffith & Lundblad, 1981). The rates of release of cleaved antithrombin and PN1 from the complex were significantly lower than the values for k_{diss} calculated from the slow-binding inhibition data. Thus, the release of cleaved inhibitor from the complex did not appear to be a major pathway for dissociation of the complex under the conditions of the assay.

Viscosity Effects on Serpin Inhibition. The effect of viscosity on k_{ass} was investigated by using buffers containing varying concentrations of sucrose. The reactions of both PN1

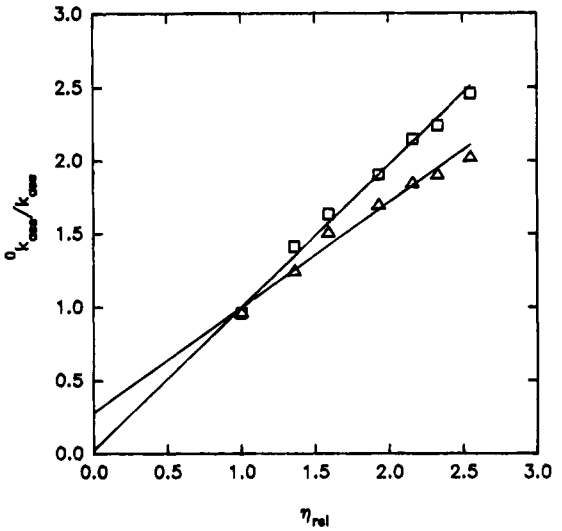


FIGURE 3: Variation of the association rate constants for serpins with viscosity. Assays were performed and data were analyzed as described in Experimental Procedures to give at least two estimates of k_{ass} for PN1 (□) and antithrombin (△) at each viscosity. These values were fitted by weighted nonlinear regression to eq 11, and the lines show the fit of the data to this equation. In order to compare viscosity effects for PN1 and antithrombin, which had widely different values of k_{ass}, the ratio $^0k_{ass}/k_{ass}$ is plotted against η_{rel}, where $^0k_{ass}$ is the value of k_{ass} in the absence of added sucrose. For this plot, the horizontal intercept is $-k_1/k_2$.

Table 2: Effect of Viscosity on the Reaction of Serpins with Thrombin[a]

	k_1 (M^{-1} s^{-1})	k_2/k_{-1}
antithrombin	$(1.83 \pm 0.11) \times 10^4$	2.6 ± 0.8
PN1	$(1.64 \pm 0.09) \times 10^6$	*b*

[a] The data in Figure 3 were analyzed according to eq 11 to yield the estimates of k_1 and k_2/k_{-1}, which are given together with their standard errors. [b] For PN1, the estimate of k_{-1}/k_2 was not different from zero, i.e., $k_2 \gg k_{-1}$.

and antithrombin with thrombin were found to be affected by viscosity (Figure 3). The data were analyzed as outlined in the Theory and Data Analysis section to yield values for k_1 and k_2/k_{-1}, which are given in Table 2. The value of k_2/k_{-1} for PN1 was too large to be measured. For a plot of $^0k_{ass}/k_{ass}$ against η_{rel} (Figure 3), the horizontal intercept will approach zero when k_2/k_{-1} is large (see Theory and Data Analysis). For the thrombin–antithrombin reaction, it was possible to obtain an estimate of 2.6 ± 0.8 for k_2/k_{-1} (Table 2). The values for k_1 determined from an analysis of the data of Figure 3 were 1.8×10^4 and 1.6×10^6 M^{-1} s^{-1} for antithrombin and PN1, respectively (Table 2).

pH Effects on Serpin Inhibition. The values of k_{ass} for antithrombin and PN1 with thrombin were determined over the pH range 6–10. The pH profiles for the k_{ass} values for these reactions were bell-shaped (Figure 4). Analysis of the data according to eq 13 yielded pK_a values of about 7.4 and 8.9–9.0 for both interactions (Table 3). These values are similar to those obtained previously from an analysis of the pH dependence of k_{cat}/K_m for the substrate Bz-Arg-pNA with thrombin (Stone et al., 1991).

DISCUSSION

Inhibitory Mechanism of Serpins. In the present study, several kinetic techniques have been employed to elucidate

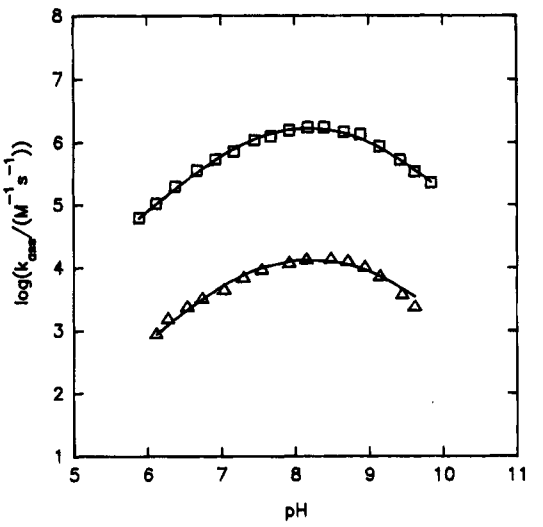


FIGURE 4: pH dependence of k_{ass} values for serpins. Assays were performed and data were analyzed as described in Experimental Procedures to yield k_{ass} values for antithrombin (△) and PN1 (□) at the indicated pH values. At least two estimates for k_{ass} were obtained at each pH value, and the weighted mean value is plotted. The pH dependence of the k_{ass} values was analyzed according to eq 13, and the lines drawn show the fit of the data to this equation.

Table 3: pK_a and pH-Independent Values for the Interaction of Serpins with Thrombin[a]

	pH-independent values (M^{-1} s^{-1})	pK_1	pK_2
antithrombin	$(1.74 \pm 0.13) \times 10^4$	7.39 ± 0.07	9.01 ± 0.08
PN1	$(2.33 \pm 0.06) \times 10^6$	7.45 ± 0.02	8.88 ± 0.02
Bz-Arg-pNA	$(1.34 \pm 0.07) \times 10^3$	7.57 ± 0.03	9.09 ± 0.05

[a] Estimates of k_{ass} were obtained between pH 6 and 10 as described under Experimental Procedures. The data in Figure 4 were fitted to eq 13 by weighted nonlinear regression. The estimates of the parameters and their standard errors obtained for these analyses are given in the table. The pK_a values obtained for the nonsticky substrate Bz-Arg-pNA are given for comparison (Stone et al., 1991).

the nature of the steps involved in the formation of the stable thrombin–serpin complexes and to determine the relative magnitudes of their rate constants. Stopped-flow kinetic studies demonstrated that two steps are involved in the formation of the stable complex (Figure 1). A two-step mechanism has also been shown to apply to other serpin–protease interactions (Olson & Shore, 1982; Stone et al., 1987; Bruch & Bieth, 1989; Longstaff & Gaffney, 1991; Faller et al., 1993; Morgenstern et al., 1994). In particular, Olson and Shore (1982) have shown that the formation of the stable thrombin–antithrombin complex involves two steps. The values for K_{init} and k_2 obtained by these workers for the reaction of antithrombin at 25 °C (1.4 mM and 10 s^{-1}) do not differ greatly from those obtained at 37 °C in the present study (269 μM and 3.9 s^{-1}; Table 1). For PN1, estimates for K_{init} and k_2 of 3.4 μM and 6 s^{-1} were obtained (Table 1). It is interesting to note that while the difference in the magnitudes of K_{init} for PN1 and antithrombin was 80-fold, the values of k_2 were very similar. This suggests that a similar process may be involved in the second step of the interaction of both serpins with thrombin.

It has been proposed that the stable serpin–protease complex exists as either an acyl or a tetrahedral intermediate (Travis & Salvesen, 1983; Potempa et al., 1994). Data similar to those shown in Figure 2 indicate that the inhibition

of thrombin by antithrombin and PN1 is best described by a reversible mechanism. Moreover, the release of cleaved inhibitor from the complex does not appear to be the principal reason for the reversibility. Results obtained with α_2-antiplasmin and trypsin also indicate that the release of cleaved inhibitor is not the principal pathway for the dissociation this serpin–protease combination (Shieh et al., 1989). The data of Griffith and Lundblad (1981) indicate, however, that with different buffer conditions the release of cleaved antithrombin may become a major pathway for dissociation of the complex. Whereas the antithrombin-thrombin complex was found to dissociate to yield mainly uncleaved inhibitor at neutral pH, the release of cleaved inhibitor became a major pathway at high pH. The release of uncleaved inhibitor from the complex is consistent with the proposal, supported by NMR studies, that the stable complex is mainly in the form of a tetrahedral intermediate (Matheson et al., 1991). Such a complex could break down to yield the intact serpin. In contrast, the formation of an acyl-enzyme involves the cleavage of the reactive-site bond, and the breakdown of the complex would only yield cleaved serpin in this case (Potempa et al., 1994). The reversible nature of the complex is, however, unlikely to be important *in vivo*. The low K_i values obtained (Table 1) indicate that very tight complexes are formed between thrombin and the two serpins. Moreover, from the estimates of k_{diss}, it can be calculated that half-life values for dissociation of the complexes are 52 and 11 h for antithrombin and PN1, respectively; thus, the thrombin–serpin complexes can be considered effectively irreversible.

Viscosity Effects on Serpin Inhibition. The results of the viscosity experiments provided an estimate for the second-order rate constant for the formation of the initial complex (k_1) and yielded information regarding the relative magnitudes of k_2 and k_{-1} for antithrombin and PN1. Viscosity effects have been used previously to determine the values of rate constants for the hydrolysis of substrates by thrombin (Stone et al., 1991; Wells & Di Cera, 1992; De Cristofaro et al., 1993; Picozzi et al., 1993). In such studies, it has been possible to differentiate between "sticky" and "non-sticky" substrates. Upon the formation of the Michaelis complex, sticky substrates undergo catalysis more rapidly than they dissociate from this complex (Cleland, 1986). To our knowledge, viscosity effects have not been previously used to investigate the relative magnitudes of the rate constants for the formation of serpin–protease complexes. The kinetic behavior of antithrombin and PN1 was analogous to that of sticky substrates. They reacted more rapidly to form the stable complex than they dissociated from the initial complex, i.e., k_2 was greater than k_{-1} for both reactions. From the viscosity data, it is also possible to conclude that the initial thrombin–serpin complexes are not in rapid equilibrium with the free enzyme; viscosity effects on k_{ass} are not observed for a rapid-equilibrium mechanism (see Theory and Data Analysis). While the data for antithrombin yielded reliable estimates for k_1 and k_2/k_{-1} (Table 2), it was possible only to conclude that the magnitude of k_2 was much greater than that of k_{-1} for PN1. The association rate constant (k_{ass}) at low inhibitor concentrations is $k_1k_2/(k_{-1} + k_2)$, and this will equal k_1 when $k_2 \gg k_{-1}$, as in the case of PN1, i.e., the association of PN1 with thrombin will be entirely diffusion-controlled at low concentrations.

Scheme 3

$$E + AT \underset{1.5\ s^{-1}}{\overset{1.8 \times 10^4\ M^{-1}s^{-1}}{\rightleftharpoons}} E.AT \underset{4 \times 10^{-6}\ s^{-1}}{\overset{4\ s^{-1}}{\rightleftharpoons}} E.AT^*$$

Scheme 4

$$E + PN1 \underset{< 0.6\ s^{-1}}{\overset{1.6 \times 10^6\ M^{-1}s^{-1}}{\rightleftharpoons}} E.PN1 \underset{2 \times 10^{-5}\ s^{-1}}{\overset{6\ s^{-1}}{\rightleftharpoons}} E.PN1^*$$

Although the estimate of k_1 obtained from the viscosity experiments for PN1 ($1.6 \times 10^6\ M^{-1}\ s^{-1}$) is roughly of the order expected for a diffusion-controlled reaction, the estimate for this parameter with antithrombin is much lower than expected for such a reaction. The simplest explanation for this discrepancy is that only a small fraction of the antithrombin molecules collide with thrombin in an orientation that results in productive complex formation with the active site. If the value of k_1 for PN1 is taken as representative of the true diffusion-controlled rate for serpin-thrombin interactions, then it must be concluded that as little as 1% of the antithrombin molecules collide with thrombin in an appropriate orientation. Heparin accelerates the rate of interaction of antithrombin with thrombin by 10^3-fold by a mechanism that involves the binding of both thrombin and antithrombin to heparin (Olson & Björk, 1992). Thus, a potential mechanism for at least part of the heparin acceleration is that it promotes an orientation of thrombin and antithrombin such that productive complex formation is encouraged.

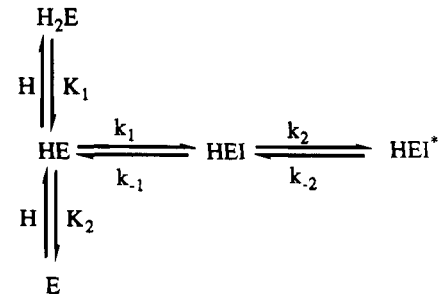
By using the values of k_2/k_{-1} determined in the viscosity experiments, together with those for k_2 obtained from the stopped-flow studies, it is possible to derive estimates for k_{-1}. The viscosity effects observed with PN1 indicated that k_2 is much greater than k_{-1}; the value of k_2 was 6 s^{-1} (Table 1) and, thus, an upper limit of 0.6 s^{-1} can be set for k_{-1}. For the thrombin–antithrombin reaction, an estimate of 3.9 s^{-1} was obtained for k_2 (Table 1), and by using this value together with the k_2/k_{-1} ratio of 2.6 (Table 2), a value of 1.5 s^{-1} can be calculated for k_{-1}. It is also possible to calculate estimates for k_{-2}. For PN1 and antithrombin, the final complex was much tighter than the initial complex ($K_i \ll K_{init}$). This condition will apply when $k_2 \gg k_{-2}$, and in this case, the rate constant for dissociation of the complex (k_{diss}) will equal k_{-2} ($=k_{ass}K_i$); consequently, values of 3.7×10^{-6} and 1.7×10^{-5} s^{-1} can be assigned to k_{-2} for antithrombin and PN1, respectively. Thus, by using a combination of kinetic techniques, it has been possible to estimate values for all of the kinetic constants for the inhibition of thrombin by antithrombin and PN1, and these values are summarized in Schemes 3 and 4, where AT represents antithrombin. It should be noted that for PN1 and antithrombin under the conditions employed, the major pathway for dissociation of the complex was the production of intact inhibitor, as shown in Schemes 3 and 4. With different conditions, however, the release of cleaved antithrombin may become the major pathway (Danielsson & Björk, 1980, 1983; Griffith & Lundblad, 1981). Thus, the mechanism presented in Scheme 2, in which the stable complex can break down to yield both cleaved and uncleaved inhibitor, must be considered the most general mechanism for the thrombin–antithrombin interaction.

The effect of viscosity on the thrombin−antithrombin reaction has been previously studied by Hogg et al. (1991), who interpreted the observed effects in terms of an expanded initial thrombin−antithrombin complex. The data were analyzed on the basis of thermodynamic nonideality. According to this treatment, a plot of $\ln(k_{ass}/^0k_{ass})$ against the molar concentration of sucrose should yield a straight line with the slope of the plot being related to the change in volume observed upon the formation of the initial complex. When the data of Figure 3 were plotted according to this relationship, linear plots were obtained. Thus, a distinction between the two models explaining the viscosity effects (volume change versus diffusion control) could not be made on the basis of the fit of the data to the equations describing the models. Analysis of the data in terms of the effect of viscosity on diffusion-controlled steps, however, does yield data that are consistent with those obtained in other kinetic experiments. The effect of viscosity on the thrombin−PN1 interaction indicated that k_2 was much greater than k_{-1}. In this case, the expression for K_{init} will reduce to k_2/k_1 (see eq 8). The value of k_1 obtained in the viscosity experiments was 1.6×10^6 M^{-1} s^{-1} and analysis of the data in Figure 1 yielded an estimate of 6 s^{-1} for k_2. By using the preceding expression for K_{init} together with these two estimates, it is possible to calculate a value of 3.8 μM for this parameter. The excellent agreement between the calculated and experimentally determined values (3.8 compared with 3.4 μM; Table 1) suggests that the analysis of the data on the basis of viscosity effects on diffusion-controlled steps is appropriate. The observed viscosity effects on the thrombin−antithrombin interaction are also consistent with the data obtained in stopped-flow studies. By substituting the values of k_1 and k_2/k_{-1} acquired in the viscosity experiments (Table 2), together with that obtained for k_2 in the stopped-flow studies (Table 1), into the expression $K_{init} = k_2(1 + k_{-1}/k_2)/k_1$ (see eq 8), an estimate of 300 μM is obtained for K_{init}. This value agrees well with the experimentally determined value of 265 μM (Table 1).

pH Effects on Serpin Inhibition. The pH profiles observed with antithrombin and PN1 were very similar to that observed for Bz-Arg-pNA (a non-sticky substrate), with similar pK_a values being obtained from all profiles (Table 3). In the case of Bz-Arg-pNA, the pK_a values of 7.4 and 9.0 were assigned to His57 and the α-amino group of Ile16, respectively (Stone et al., 1991). In serine proteases, the protonated form of the α-amino group of Ile16 forms a salt bridge with Asp194, which maintains the structure of the active site (Fersht, 1985). The active-site histidine (His57) must be in its unprotonated form for catalysis to occur (Fersht, 1985). Thus, the data are consistent with the proposal that, for the optimal rate of association of the serpins with thrombin, His57 must be in its active (unprotonated) form and the active-site geometry must be maintained by the Ile16−Asp194 salt bridge. The effects of pH on the association of serpins with thrombin can be most simply explained by the mechanism presented in Scheme 5.

In Scheme 5, HE is the active form of thrombin with His57 unprotonated and Ile16 protonated; K_1 and K_2 are the acid dissociation constants for His57 and Ile16, respectively. There are two points that should be noted about the data of Figure 4: (1) The pH profiles for both serpins approach slopes of +1 at low pH values and of −1 at high pH values; no tendency to plateau at high or low pH was apparent. (2)

Scheme 5



The pK_a values determined from the profiles correspond to those determined for the non-sticky substrate Bz-Arg-pNA (Table 3). These two features of the pH profiles indicate that the incorrectly protonated enzyme forms (H_2E and E) do not significantly contribute to the formation of the stable complex. If the enzyme forms with His57 protonated (H_2E) or Ile16 unprotonated (E) react at a significant rate to form stable complexes (H_2EI^* or EI*), a plateau in the pH profile would be observed at low or high pH (Cleland, 1986). Thus, the absence of such plateau values indicates that the k_{ass} values for the serpins with the incorrectly protonated enzyme forms (His57 protonated and Ile16 unprotonated) must be much lower than the k_{ass} values for the correctly protonated enzyme form. Moreover, the data from pH studies and viscosity experiments suggest that, when His57 is protonated and Ile16 is unprotonated, thrombin is not even able to form initial complexes with the serpins. Viscosity effects indicated that k_2 was greater than k_{-1} for both serpins, i.e., the serpins were kinetically sticky. This observation is important for the interpretation of the pH profiles. While the pH profiles of non-sticky reactants yield true pK_a values, the observed pK_a values for sticky reactants are usually displaced from the true values by $\log(1 + k_2/k_{-1})$, if the initial incorrectly protonated complex forms and is able to convert to a correctly protonated final complex; the acidic pK_a value will be $\log(1 + k_2/k_{-1})$ lower than the true value, and the basic value will be $\log(1 + k_2/k_{-1})$ higher (Cleland, 1986). With the serpins, this displacement has not occurred; the same pK_a values were obtained for the serpins as were obtained for the non-sticky substrate Bz-Arg-pNA. The simplest interpretation of the data is that initial complexes are not observed with the incorrectly protonated enzyme forms, as shown in Scheme 5. In this case, no displacement of the pK_a values will be observed. Alternatively, if the incorrectly protonated enzyme is able form an initial complex with the serpins, such complexes must be dead-end ones, i.e., they cannot be converted into the correctly protonated initial complex (HEI) to yield a stable complex (Cleland, 1977, 1986).

The observed dependence of k_{ass} on the protonation state of the active-site histidine is consistent with a tetrahedral intermediate lying on the pathway for the formation of the stable protease−serpin complex; His57 would have to be unprotonated for the generation of this enzyme form. As noted earlier, similar values for k_2 were obtained with antithrombin and PN1, suggesting that a common reaction controls the rate of k_2 for both serpins. This observation would be consistent with a chemical step being the rate-limiting process, and the results from the pH studies suggest that the formation of a tetrahedral intermediate would be a likely possibility.

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REFERENCES

Baumann, U., Huber, R., Bode, W., Grosse, D., Lesjak, M., & Laurell, C. B. (1991) *J. Mol. Biol. 218*, 595−606.
Baumann, U., Bode, W., Huber, R., Travis, J., & Potempa, J. (1992) *J. Mol. Biol. 226*, 1207−1218.
Beatty, K., Travis, J., & Bieth, J. (1982) *Biochim. Biophys. Acta 704*, 221−226.
Brouwer, A., & Kirsh, J. (1982) *Biochemistry 21*, 1302−1307.
Bruch, M., & Bieth, J. G. (1989) *Biochem J. 259*, 929−930.
Carrell, R. W., Stein, P. E., Fermi, G., & Wardell, M. R. (1994) *Structure 2*, 257−270.
Cha, S. (1975) *Biochem. Pharmacol. 24*, 2177−2185.
Chase, T., Jr., & Shaw, E. (1970) *Methods Enzymol. 19*, 20−27.
Cleland, W. W. (1977) *Adv. Enzymol. Relat. Areas Mol. Biol. 45*, 273−387.
Cleland, W. W. (1986) in *Investigations of Rates and Mechanisms of Reactions. Vol. 6* (Bernasconi, C. F., Ed.) pp 791−870, Wiley, New York.
Danielsson, A., & Björk, I. (1980) *FEBS Lett. 119*, 241−244.
Danielsson, A., & Björk, I. (1983) *Biochem. J. 213*, 345−353.
De Cristofaro, R., Rocca, B., Bizzi, B., & Landolfi, R. (1993) *Biochem. J. 289*, 475−480.
Duggleby, R. G., & Morrison, J. F. (1978) *Biochim. Biophys. Acta 526*, 398−409.
Faller, B., Cadéne, M., & Bieth, J. G. (1993) *Biochemistry 32*, 9230−9235.
Ferguson, W. S., & Finlay, T. H. (1983) *Arch. Biochem. Biophys. 220*, 301−308.
Fersht, A. R. (1985) *Enzyme Structure and Mechanism*, Freeman, New York.
Fish, W. W., Orre, K., & Björk, I. (1979) *Eur. J. Biochem. 101*, 39−44.
Gan, Z.-R., Yiping, L., Chen, Z., Lewis, S. D., & Shafer, J. A. (1994) *J. Biol. Chem. 269*, 1301−1305.
Griffith, M. J., & Lundbald, R. L. (1981) *Biochemistry 20*, 105−110.
Hermans, J. M., & Stone, S. R. (1993) *Biochem. J. 295*, 239−245.
Hogg, P. J., Jackson, C. M., & Winzor, D. J. (1991) *Biochim. Biophys. Acta 1073*, 609−613.
Huber, R., & Carrell, R. W. (1989) *Biochemistry 28*, 8951−8966.
Jesty, J. (1979) *J. Biol. Chem. 254*, 1044−1049.
Löbermann, H., Tokuoka, R., Deisenhofer, J., & Huber, R. (1984) *J. Mol. Biol. 177*, 531−556.
Longstaff, C., & Gaffney, P. J. (1991) *Biochemistry 30*, 979−986.
Lottenberg, R., & Jackson, C. M. (1983) *Biochim. Biophys. Acta 742*, 558−564.
MacKay, E. J. (1981) *Thromb. Res. 21*, 375−382.
Matheson, N. R., Halbeck, H. V., & Travis, J. (1991) *J. Biol. Chem. 266*, 13489−13491.
Morgenstern, K. A., Sprecher, C., Holth, L., Foster, D., Grant, F. J., Ching, A., & Kisiel, W. (1994) *Biochemistry 33*, 3432−3441.
Morrison, J. F. (1982) *Trends Biochem. Sci. 7*, 102−105.
Morrison, J. F., & Stone, S. R. (1985) *Comments Mol. Cell. Biophys. 2*, 347−368.
Morrison, J. F., & Walsh, C. T. (1988) *Adv. Enzymol. Relat. Areas Mol. Biol. 61*, 201−301.
Mottonen, J., Strand, A., Symersky, J., Sweet, R. M., Danley, D. E., Geoghegan, K. F., Gerard, R. D., & Goldsmith, E. J. (1992) *Nature 355*, 270−273.
Mourey, L., Samama, J.-P., Delarue, M., Petitou, M., Choay, J., & Moras, D. (1993) *J. Mol. Biol. 232*, 1253−1258.
Olson, S. T., & Shore, J. D. (1982) *J. Biol. Chem. 257*, 14891−14895.
Olson, S. T., & Björk, I. (1992) in *Thrombin Structure and Function* (Berliner, L. J., Ed.) pp 159−218, Plenum Press, New York.
Owen, W. G. (1975) *Biochim. Biophys. Acta 405*, 380−387.
Picozzi, M., & De Cristofaro, R. (1993) *Biochem. J. 294*, 563−567.
Potempa, J., Korzus, E., & Travis, J. (1994) *J. Biol. Chem. 269*, 15957−15960.
Rosenberg, R. D., & Damus, P. S. (1973) *J. Biol. Chem. 248*, 6490−6505.
Schechter, I., & Berger, A. (1967) *Biochem. Biophys. Res. Commun. 27*, 157−162.
Schreuder, H. A., de Boer, B., Dijkema, R., Mulders, J., Theunissen, H. J. M., Grootenhuis, P. D. J., & Hol, W. G. J. (1994) *Nature Struct. Biol. 1*, 48−54.
Scott, R. W., Bergaman, B. L., Bajpai, A., Hersh, R. T., Rodriguez, H., Jones, B. N., Barreda, C., Watts, S., & Barker, J. B. (1985) *J. Biol. Chem. 260*, 7029−7034.
Shieh, B., Potempa, J., & Travis, J. (1989) *J. Biol. Chem. 264*, 13420−13423.
Stone, S. R., & Hofsteenge, J. (1986) *Biochemistry 25*, 4622−4628.
Stone, S. R., Nick, H., Hofsteenge, J., & Monard, D. (1987) *Arch. Biochem. Biophys. 252*, 237−244.
Stone, S. R., Betz, A., & Hofsteenge, J. (1991) *Biochemistry 30*, 9841−9848.
Travis, J., & Salvesen, G. S. (1983) *Annu. Rev. Biochem. 52*, 655−709.
Wallace, A., Rovelli, G., Hofsteenge, J., & Stone, S. R. (1989) *Biochem. J. 257*, 191−196.
Wei, A., Rubin, H., Cooperman, B. S., & Christianson, D. W. (1994) *Nature Struct. Biol. 1*, 251−258.
Wells, C. M., & Di Cera, E. (1992) *Biochemistry 31*, 11721−11730.

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