Role of the Catalytic Serine in the Interactions of Serine Proteinases with Protein Inhibitors of the Serpin Family

CONTRIBUTION OF A COVALENT INTERACTION TO THE BINDING ENERGY OF SERPIN-PROTEINASE COMPLEXES*

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The contribution of a covalent bond to the stability of complexes of serine proteinases with inhibitors of the serpin family was evaluated by comparing the affinities of β-trypsin and the catalytic serine-modified derivative, β-anhydrotrypsin, for several serpin and non-serpin (Kunitz) inhibitors. Kinetic analyses showed that anhydrotrypsin had little or no ability to compete with trypsin for binding to α1-proteinase inhibitor (α1PI), plasminogen activator inhibitor 1 (PAI-1), antithrombin (AT), or AT-heparin complex when present at up to a 100-fold molar excess over trypsin. By contrast, equimolar levels of anhydrotrypsin blocked trypsin binding to non-serpin inhibitors. Equilibrium binding studies of inhibitor–enzyme interactions monitored by inhibitor displacement of the fluorescence probe, p-aminobenzamidine, from the enzyme active site, confirmed that the binding of serpins to anhydrotrypsin was undetectable in the case of α1PI or AT (K_i > 10^{-5} M), of low affinity in the case of AT-heparin complex (K_i = 7.9 × 10^{-6} M), and of moderate affinity in the case of PAI-1 (K_i = 2 × 10^{-7} M). This contrasted with the stoichiometric high affinity binding of the serpins to trypsin as well as of the non-serpin inhibitors to both trypsin and anhydrotrypsin. Maximal K_i values for serpin-trypsin interactions of 1 to 8 × 10^{-11} M, obtained from kinetic analyses of association and dissociation rate constants, indicated that the affinity of serpins for trypsin was minimally 4 to 6 orders of magnitude greater than that of anhydrotrypsin. Anhydrotrypsin, unlike trypsin, failed to induce the characteristic fluorescence changes in a P9 Ser → Cys PAI-1 variant labeled with a nitrobenzofuran fluorescent probe (NBD) which were shown previously to report the serpin conformational change associated with active enzyme binding. These results demonstrate that a covalent interaction involving the proteinase catalytic serine contributes a major fraction of the binding energy to serpin-trypsin interactions and is essential for inducing the serpin conformational change involved in the trapping of enzyme in stable complexes.

Protein serine proteinase inhibitors of the serpin superfamily play important roles in regulating the serine proteinases of blood coagulation, fibrinolysis, inflammation, and many other physiologic processes (1, 2). These inhibitors are single polypeptide chain proteins of ~400 amino acid residues and are therefore considerably larger than other non-serpin serine proteinase inhibitors which contain from 29 to 190 residues (3, 4). The serpins nevertheless share certain mechanistic features with non-serpin inhibitor families. Both serpin and non-serpin inhibitors thus inhibit proteinases by forming stable equimolar complexes in which a substrate-like interaction is made between an exposed inhibitor binding loop and the enzyme active site (1–4). However, serpins differ from non-serpin inhibitors in requiring a large inhibitor conformational change to trap proteinases in such complexes (5–10). In this conformational change, the inhibitor binding loop is thought to collapse from its exposed position on the protein surface and become inserted into the center of a major β-sheet comprising the core of the protein. By contrast, in the non-serpin inhibitors, the binding loop is rigidly fixed in an optimal substrate binding, “canonical” conformation capable of tight interaction with the proteinase with minimal conformational adjustments (3, 4).

An additional important difference between the serpins and non-serpin inhibitors is that serpin-proteinase complexes are SDS-stable, whereas non-serpin inhibitor-proteinase complexes are dissociated in SDS. This behavior implies that a covalent interaction stabilizes the serpin complexes, whereas noncovalent interactions stabilize the non-serpin inhibitor complexes (1, 2). The observation that serpin-proteinase complexes are dissociated by alkaline pH and nucleophiles or spontaneously at a slow rate, producing in all cases reactive-site cleaved inhibitor and active enzyme, has suggested that these complexes are unusually stable tetrahedral or acyl intermediates of an otherwise normal proteolysis reaction (11–16). NMR data support such a stabilization of serpin-proteinase complexes as covalent tetrahedral or acyl intermediates (17). In contrast, the stabilization of non-serpin inhibitor-proteinase complexes predominantly by noncovalent interactions is indi-

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cated from the finding that anhydropseudoenzymes, in which the catalytic serine has been converted to dehydroalanine, bind these inhibitors with high affinities similar to those of native proteinases (18, 19). The absence of a covalent bond between the proteinase active site serine and the inhibitor scissile bond in non-serpin inhibitor-proteinase complexes has been shown clearly from high resolution x-ray crystallographic structures as well as NMR studies of these complexes (4, 20, 21).

While available evidence supports the idea that serpin-proteinase complexes are stable covalent intermediates linked via the proteinase catalytic serine, the role of this covalent interaction in the inhibitory mechanism and its contribution to high affinity complex formation have not been elucidated. To address these questions, we have compared the affinities of three serpin inhibitors for trypsin and anhydrotrypsin by direct equilibrium binding studies or by kinetic analyses of association and dissociation rate constants for inhibitor-proteinase complex formation. The affinities of two Kunitz inhibitors for native and modified trypsins have also been examined to provide representative examples of non-serpin inhibitors, whose interactions are known not to be significantly affected by the serine modification (18, 19). Additionally, the involvement of the serpin conformational change in these interactions has been examined with a serpin variant containing a specific fluorescent label which reports this conformational change (22). Our results reveal that the high affinity interaction of trypsin with the serpin inhibitors and the ability to induce the serpin conformational change is greatly reduced or abolished by the catalytic serine modification in anhydrotrypsin, in marked contrast to the comparable high affinities of trypsin and anhydrotrypsin for the non-serpin inhibitors. These results establish that a covalent interaction involving the proteinase catalytic serine residue is critical for the high affinity interactions of serpin inhibitors with proteinases and for triggering the serpin conformational change.

EXPERIMENTAL PROCEDURES

Materials—Bovine β-trypsin was purified from a commercial N-tosyl-L-phenylalanine chloromethyl ketone-treated product (Sigma Type XIII) by chromatography on SBTI1–agarose with pH gradient elution to separate α- and β-forms of the enzyme (23, 24). β-Anhydrotrypsin was prepared from unpurified trypsin by modifying the active site serine residue with phenylmethanesulfonyl fluoride followed by base elimination of the phenylmethanesulfonyl moeity essentially as described (18, 25). α- and β-forms of the anhydroenzyme were separated by SBTI–agarose affinity chromatography (24). Residual activity detectable in the anhydrotrypsin with the chromatographic substrate, S-2222 (Chromogenix), ranged between 0.004 and 0.007%. Purified β-trypsin and β-anhydrotrypsin were stored in 1 M HCl plus 10 mM CaCl₂.

SBTI (type 1S) and BPTI (from bovine lung) were purchased from Sigma. Human antithrombin was purified from outdated plasma by heparin-Sepharose, DEAE-Sepharose, and Sephacryl S-200 chromatography as described (26). Human α-1-proteinase inhibitor was isolated from plasma by ammonium sulfate precipitation, followed by zinc chelate and DEAE-Sepharose chromatography (27). Recombinant PAI-1 (>90% active) was expressed in E. coli, purified, and titrated with active site–tetratorukase as described (28). Storage in 5 mM Mes, 0.3 M NaCl, pH 6.0, stabilized the active form. Latent PAI-1 was made by incubating 0.25 μM active inhibitor in 0.1 M Hepes, 0.1 M NaCl, pH 7.4 buffer at 37°C for 17 h, after which time residual inhibitor activity was undetectable (<1%). Variant PAI-1s with site-directed mutations in the P14 residue (Thr → Arg) and in the P9 residue (Ser → Cys) were expressed and purified as described previously (22, 29). Labeling of the P9 Cys variant with iodoacacetamido-NB2 (Molecular Probes) was done as in prior studies (22). Reactive site–cleaved antithrombin was prepared as described (30). A size–fractionated heparin of Mr = 7900 with high affinity for antithrombin was purified as in previous studies (31). Synthetic heparin pentasaccharide corresponding to the antithrombin binding sequence in heparin (32) was generously provided by Dr. Maurice Petittou of Sanofi Recherche. Heparin concentrations were determined by discontinuous assay of residual enzyme activity as described (26). Concentrations of native proteins were determined from the 280 nm absorbance with the use of the following published absorption coefficients (liter·g⁻¹·cm⁻¹) and molecular weights: BPTI, 0.83 and 6513 (33); native and cleaved antithrombin, 0.65 and 58,000 (34); α₁-proteinase inhibitor, 0.48 and 53,000 (35); and BPTI, 0.43 and 43,000 (22). The concentration of NBD-labeled PAI-1 was determined from the 280 nm absorbance after correction for the NBD absorbance at this wavelength (22). β–Trypsin concentrations were determined by active site titration with fluorescein mono-p-guanidinobenzoate (30). Comparison with concentrations determined from the absorbance at 280 nm and an absorption coefficient of 1.54 liter·g⁻¹·cm⁻¹ and molecular weight of 23,900 (23) indicated 77–86% active enzyme. Fluorescein mono-p-guanidinobenzoate was used similarly to determine the active concentration of β-anhydrotrypsin from the amount of anhydroenzyme necessary to fully liberate trypsin from its complex with SBTI (24). Preparations were >80% active by this assay. SBTI concentrations were determined by titration of active site–tetratorukase (10 mM) with an equimolar concentration of antithrombin, and all kinetic analyses were in reasonable agreement with those determined from the 280 nm absorbance with an extinction coefficient calculated from the amino acid sequence (37).

Experimental Conditions—All experiments were conducted at 25°C in a pH 7.4 Hepes buffer consisting of 0.1 M Hepes, 0.1 M NaCl, 10 mM CaCl₂, 0.1% PEG 8000, unless otherwise indicated. All proteins were dialyzed or extensively diluted into this buffer before use, except for trypsin and anhydrotrypsin. The latter enzymes in 10 mM CaCl₂, pH 3 (above) were mixed with an equal volume of 2-fold concentrated Hepes buffer (except for the CaCl₂, which was kept at 10 mM) to give the same final buffer composition. Equilibrium binding was measured at 10⁻¹⁰ M enzyme and all kinetic analyses were done in polyethylene glycol-coated polystyrene cuvettes to minimize protein adsorption artifacts (38).

Kinetic Analysis of Trypsin- and Anhydrotrypsin-Inhibitor Interactions—The effect of anhydrotrypsin on the kinetics of trypsin-inhibitor reactions was analyzed by incubating β–trypsin (10 mM) with 1 eq of inhibitor (based on the results of inhibitor–enzyme titrations) in the absence and presence of 10, 100, and 1,000 nM β-anhydrotrypsin in a 100-μl reaction volume. Inhibitor and anhydrotrypsin (when present) were preincubated for 5 (serpins), 20 (SBTI), or 60 (BPTI) min prior to initiating reactions with trypsin. Reactions were quenched at varying times by adding 0.9 ml of 100 μM S-2222 substrate, and the residual enzyme activity was determined from the initial rate of p-nitroanilide formation by continuous assay (27). Kinetic parameters for the reaction with free inhibitor and then divided by the inhibitor–heparin complex concentration to obtain kₐ (31). Association rate constants (kₐ) for inhibitor–trypsin reactions in the absence of anhydrotrypsin were measured by fitting reaction progress curves by the second order kinetic equation for equimolar reactants. In cases where the assumption of equimolar reactants appeared to be slightly in error, data were fit by the general second order equation for nonequimolar reactants with the inhibitor concentration as an adjustable parameter (39). kₐ values were also measured under pseudo first order conditions with at least a 10-fold molar excess of inhibitor over enzyme by discontinuous assay of residual enzyme activity as described (26). Heparin was present for the reaction with free inhibitor and then divided by the inhibitor–heparin complex concentration to obtain kₐ (31). Association rate constants for anhydrotrypsin–Kunitz inhibitor interactions were measured from the competitive effect of anhydrotrypsin on the rate of association of trypsin with the inhibitors as described (19).

1 The abbreviations used are: SBTI, soybean trypsin inhibitor; Mes, 4-morpholineethanesulfonic acid; BPTI, bovine pancreatic trypsin inhibitor; PAI-1, plasminogen activator inhibitor 1; α₁PI, α₁-proteinase inhibitor; AT, antithrombin; NBD, N,N′-dimethyl-N-(acyetyl)-N′-(7-nitrobenz-3-oxa-1,3-diazol-4-yl)enediamini; S-2222, N-benzoyl-Ile-Glu-Gly-Agr p-nitroanilide.

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n benzamidine interaction were fit after dilution correction (≈5%) by the quadratic equation for equilibrium binding

$$\Delta F_{\text{obs}} = \frac{\Delta F_{\text{max}}}{2D} \times \left( [E]_0 + [P]_0 + K_P - \sqrt{[E]_0 + [P]_0 + K_P + \Delta F_{\text{max}}/2D} \right)$$

(Eq. 1)

where $\Delta F_{\text{max}}$ is the maximal fluorescence change, $[E]_0$ and $[P]_0$ are the total enzyme and p-aminobenzamidine concentrations, respectively, and $K_P$ is the dissociation constant for the enzyme-probe interaction. This equation assumes a binding stoichiometry of 1:1 as established previously for the trypsin-probe interaction (40). $\Delta F_{\text{max}}$ and $K_P$ were fitted parameters. Emission spectra of enzyme-probe complexes and free probe were measured with excitation at 355 nm and excitation and emission bandwidths of 4 and 2 nm, respectively. After correcting for the appropriate background spectrum (buffer ± enzyme), spectra of enzyme-probe complexes were obtained by subtracting the contribution of the free probe spectrum calculated from the measured $K_P$.

Fluorescence Titrations of Trypsin and Anhydrotrypsin with Inhibitors—Sequential additions of a concentrated inhibitor solution were made to solutions of 1 μM β-trypsin and 10 μM p-aminobenzamidine probe or of 5 μM β-anhydrotrypsin and 100 μM probe. Fluorescence changes were monitored after each addition following a 1-min equilibration time, at excitation and emission wavelengths of 325 nm and 345 nm, respectively, which yielded the maximum difference between bound and free probe fluorescence. Equilibration times for titrations of 0.1 μM trypsin and 10 μM probe with inhibitors were extended to 10 min for αPI, AT, and BPTI or to 2 min with all other inhibitors. Such times were sufficient to achieve a stable fluorescence. Control titrations of just the probe with inhibitor in the absence of enzyme were done to correct for any background fluorescence changes and to establish the end point of enzyme titrations. These corrections were typically small (1-11%) and approached 17% only for titrations with cleaved antithrombin.

Inhibitor–enzyme titrations were fit by linear regression analysis except for serpin-anhydrotrypsin titrations. The latter were fit by an equation for competitive binding which assumes that the stoichiometries for probe and for inhibitor binding to enzyme are both 1:1,

$$\Delta F_{\text{obs}} = \frac{\Delta F_{\text{max}}}{2D} \times \left( K_P(I + [P]/K_P) + [I]_0 - \sqrt{[E]_0 + [P]_0 + K_P + \Delta F_{\text{max}}/2D} \right)$$

(Eq. 2)

$\Delta F_{\text{obs}}$ in this equation is the observed fluorescence, corrected for background and dilution, minus the starting fluorescence. $[I]_0$ is the total inhibitor concentration, $K_P$ is the dissociation constant for the inhibitor–enzyme interaction, and other parameters are as defined above. This equation follows from the more general cubic equation for competitive binding under the condition where $[P]\text{max} = [P]_0$, which was closely approximated in the titrations (27), (39), (41).

Interactions of NBD-labeled PAI-1 with Trypsin and Anhydrotrypsin—Emission spectra of NBD-labeled 9 Ser → Cys PAI-1 variant in the absence and presence of trypsin and anhydrotrypsin were measured with an excitation wavelength of 480 nm and 4 nm excitation and emission bandpasses. Kinetic analyses of trypsin inhibition by native and PAI 9 Cys-labeled PAI-1 in the absence and presence of anhydrotrypsin were done under pseudo-first order conditions with 10 nM inhibitor, 1 nM enzyme, and 1 μM anhydroenzyme in 100 μl reaction volumes. After varying times, reactions were quenched with 0.9 ml of 50 μl of 50 μM tosyl-Gly-Pro-Arg-7-amido-4-methylcoumarin (Sigma) and the residual trypsin activity measured from the rate of the linear fluorescence increase at excitation and emission wavelengths of 380 and 440 nm.

Effect of anhydrotrypsin on the Active to Latent Conversion of PAI-1—PAI-1 (0.27 μM) was incubated at 37 °C in the absence or presence of β-anhydrotrypsin (1 or 10 μM) for up to 5 h. Samples (100-250 μl) were taken at varying times and mixed with S-2222 substrate to a final concentration of 100 μM in 0.95 ml at 25 °C. Residual inhibitor activity was then assayed by adding 50 μl of 25 nM β-trypsin. The rate of enzyme inactivation was continuously monitored from the exponential decrease in the rate of substrate hydrolysis at 405 nm for at least 10 half-lives. Progress curves were computer-fit by an exponential function with an added linear term to obtain the pseudo-first order inhibition rate constant ($k_{\text{inhib}}$) (26). The decrease in the apparent second order inhibition rate constant, calculated by dividing $k_{\text{inhib}}$ by the inhibitor concentration, was fit by a single exponential decay function to obtain the first order rate constant for PAI-1 inactivation.

Dissociation of Serpin-Trypsin Complexes—Serpin-trypsin complexes prepared by incubating 0.1-1 μM enzyme with a 2- to 5-fold molar excess of inhibitor for 1 to 10 min were dissociated by 50–2000-fold dilution into 100 or 200 μM tosyl-Gly-Pro-Arg-7-amido-4-methylcoumarin substrate. This level of substrate exceeded the measured $K_m$ of 8.3 ± 0.9 μM by 12- to 24-fold and thus ensured the trapping of released enzyme by substrate. Complex dissociation was continuously monitored for 1000 s from the parabolic increase in fluorescence at excitation and emission wavelengths of 380 and 440 nm, respectively, due to the reappearance of enzyme activity. Under these experimental conditions, less than 1% substrate was converted to product and less than 3% of the complex was dissociated, so that an initial linear rate of complex dissociation was measured (26, 42). Data were satisfactorily fit by the equation describing the initial rate of enzyme generation (42),

$$F_{\text{obs}} = F_0 + \frac{k_{\text{off}}([E]_0 + [I]_0)TN}{2}$$

(Eq. 3)

where $F_{\text{obs}}$ and $F_0$ are the fluorescence at time and time 0, respectively, $v_0$ is the rate of change in fluorescence at time 0, $k_{\text{off}}$ is the first order rate constant for complex dissociation, $[E]_0 + [I]_0$ is the starting concentration of complex, and TN is the turnover number for hydrolysis of substrate by enzyme under the conditions of the experiment, expressed as the rate of change in fluorescence per unit of enzyme concentration. The coefficients of the second order polynomial were the fitted parameters, and $k_{\text{off}}$ was calculated from the fitted coefficient of the $t^2$ term to the rate of complex dissociation and the estimated turnover number. The above analysis assumes complex dissociation is an essentially irreversible process with no significant contribution due to residual inhibitor association during the initial rate measurement. Irreversibility of complex dissociation was demonstrated by showing that the inhibitor is released from the complex in an inactive, cleaved form rather than in the native intact form (see "Results"). The contribution of the association process to the apparent $k_{\text{off}}$ measured from the initial rate of complex dissociation was calculated from the expression,

$$\left( \frac{k_{\text{off}}([I]_0 + ([E]_0 + [I]_0)/K_P)}{(1 + ([E]_0 + [I]_0)/K_P)} \times \frac{([E]_0 + [I]_0 + K_P)/D}{[E]_0} \right)$$

(Eq. 4)

where $[S]_0$ is the substrate concentration, $([E]_0 + [I]_0)/K_P$ is the ratio of un inhibited enzyme (free and complexed with substrate) to inhibited enzyme at time 0, and other parameters are as defined above. This contribution was <2%, confirming that it could be neglected.

Analysis of Enzyme-Serpin Complex Dissociation by SDS-Gel Electrophoresis—Complements were prepared by incubating equimolar concentrations of enzyme and serpin (5 μM) for 1 min in 0.15 M 7.4 Hepes buffer containing 0.02% sodium azide, followed by quenching any unreacted trypsin with 1/100 to 2/200 volume of 5 mM Pho-Phe-Arg-chloromethyl ketone for an additional 5 min. Complex dissociation was then induced by adding 1/90 volume of 100 μM p-aminobenzamidine in Hepes/azide buffer to trap dissociated enzyme. Samples were then immediately after initiating the reaction and at later times up to 72 h and quick-frozen in dry ice/ethanol. All samples were analyzed for complex dissociation by SDS-gel electrophoresis under reducing or non-reducing conditions according to Laemmli (43) in a 10% gel.

Equilibrium Measurement of Ki for Kunitz Inhibitor Interactions with Trypsin and Anhydrotrypsin—To a series of cuvettes was added 10 mM β-trypsin and varying amounts of SBTI or BPTI ranging from 0 to 2 mol of inhibitor/mol of enzyme in 1 ml final volumes. Reactions were allowed to equilibrate for 16–24 h for SBTI or 72 h for BPTI. Residual enzyme activity was then measured by adding 20 μl of 2 mM tosyl-Gly-Pro-Arg-7-amido-4-methylcoumarin fluorescent substrate and following the initial rate of substrate cleavage fluorimetrically as indicated above. Initial rates measured as a function of inhibitor concentration were computer-fit by the equilibrium binding equation,

$$v_{\text{obs}} = v_0 - v_0 \sqrt{\frac{[E]_0 + n[I]_0 + K_I}{[E]_0 + n[I]_0 + K_I - K_P}}$$

(Eq. 5)

where $v_0$ and $v_{\text{obs}}$ are the initial substrate hydrolysis rates measured in the absence and presence of inhibitor, respectively, $n$ is the inhibitor binding stoichiometry, and the other parameters are as defined above. The $K_I$ for the anhydrotrypsin-SBTI interaction was measured by equilibrium competition with trypsin, whereby equilibrium was approached by either adding trypsin to anhydrotrypsin-inhibitor complex or anhydrotrypsin to trypsin-inhibitor complex (18). Separate experiments were conducted with 10 nM trypsin, 9 nM SBTI, and either 50,
**RESULTS**

Effect of Anhydrotrypsin on the Kinetics of Trypsin Inactivation by Serpin and Non-serpin Inhibitors—Binding of the catalytically inactive serine 195<sup>2</sup> → dehydroalanine derivative, β-anhydrotrypsin, to inhibitors of both serpin and non-serpin families was evaluated from the ability of the anhydroenzyme to decrease the rate of the reaction between equimolar trypsin and inhibitor. When present, anhydrotrypsin was preincubated with the inhibitors for a time over which trypsin was mostly inactivated in the absence of anhydroenzyme to allow complex formation (see “Experimental Procedures” and Fig. 1). An equimolar level of anhydrotrypsin blocked or greatly decreased the rate of inactivation of trypsin by the Kunitz family inhibitors, bovine pancreatic trypsin inhibitor (BPTI), and soybean trypsin inhibitor (SBTI) (Fig. 1). Measurement of the rate constants for anhydrotrypsin association with these inhibitors by allowing mixtures of trypsin and anhydrotrypsin to compete for limiting inhibitor (see “Experimental Procedures”) yielded values of $2.0 \pm 0.5 \times 10^{5} \text{M}^{-1} \text{s}^{-1}$ and $2.9 \pm 0.4 \times 10^{6} \text{M}^{-1} \text{s}^{-1}$ for BPTI and SBTI, respectively, which were only modestly reduced from those of trypsin (Table I). Contrasting these results, anhydrotrypsin had little or no detectable effect on the rate of inactivation of trypsin by the serpin inhibitors, α<sub>1</sub>-proteinase inhibitor (α<sub>1</sub>PI), plasminogen activator inhibitor-1 (PAI-1), antithrombin (AT), or antithrombin-heparin complex, when the anhydroenzyme was added at levels as high as a 100-fold molar excess over trypsin and inhibitor (Fig. 1). These results indicated that anhydrotrypsin effectively competed with trypsin for binding to Kunitz inhibitors but showed little or no ability to compete for binding to serpin inhibitors.

Characterization of p-Aminobenzamidine as a Probe of Inhibitor Interactions with Trypsin and Anhydrotrypsin—Binding of the fluorescent arginine analog, p-aminobenzamidine, to the S1 specificity sites of β-trypsin and β-anhydrotrypsin was investigated to determine whether this compound could serve as a probe of inhibitor interactions with both native and modified enzymes (40). Titrations of β-trypsin and β-anhydrotrypsin with p-aminobenzamidine both resulted in saturable enhancements of p-aminobenzamidine fluorescence (Fig. 2). Nonlinear least squares fitting of these data by an equation previously shown to describe the 1:1 equilibrium binding interaction between trypsin and p-aminobenzamidine (40), indicated $K_D$ values of $8.5 \pm 0.6 \mu M$ for the trypsin interaction and $95 \pm 6 \mu M$ for the anhydrotrypsin interaction, demonstrating an 11-fold weaker affinity of the probe for the anhydroenzyme.

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**Table I**

Association and dissociation rate constants for trypsin-inhibitor complexes

| Inhibitor         | $k_{on}$ | $k_{off}$ |
|-------------------|----------|-----------|
| SBTI              | $4.9 \pm 0.1 \times 10^{6}$ | $3.3 \pm 0.1 \times 10^{6}$ |
| BPTI              | $3.7 \pm 0.1 \times 10^{6}$ | $3.5 \pm 0.1 \times 10^{6}$ |
| AT                | $2.3 \pm 0.1 \times 10^{6}$ | $1.8 \pm 0.1 \times 10^{5}$ |
| AT-heparin        | $1.5 \pm 0.3 \times 10^{5}$ | $1.8 \pm 0.6 \times 10^{5}$ |
| α<sub>1</sub>PI    | $1.7 \pm 0.2 \times 10^{5}$ | $2.4 \pm 0.6 \times 10^{5}$ |
| PAI-1             | $1.7 \pm 0.2 \times 10^{5}$ | $2.6 \pm 0.5 \times 10^{5}$ |

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<sup>2</sup> Chymotrypsin numbering. The catalytic serine is residue number 183 in the trypsin sequence.

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**Fig. 1.** Anhydrotrypsin competition with trypsin for binding to Kunitz and serpin inhibitors. Inhibitors (10 nM) were preincubated in the absence (●) or presence of 10 nM (□), 100 nM (○) or 1000 nM (□□) anhydrotrypsin (AHTrypsin) prior to initiating reactions with 10 nM trypsin. Residual enzyme activity was then measured at the indicated reaction times as described under “Experimental Procedures.” Solid lines are fits of kinetic curves in the absence of anhydrotrypsin by a second order process.
than for the active enzyme. Emission spectra of free and enzyme-complexed \( p \)-aminobenzamidine indicated that binding of the probe to active and inactive enzymes produced similar 13-15 nm blue shifts of the free probe spectrum, but different enhancements of the probe fluorescence of 66-fold for trypsin and 25-fold for anhydrotrypsin at the emission maximum of 365 nm for the bound probe (Fig. 2).

**Equilibrium Binding of Trypsin and Anhydrotrypsin to Serpin and Non-serpin Inhibitors—** To compare the affinities of trypsin and anhydrotrypsin for serpin and non-serpin inhibitors, complexes of native and modified enzymes with \( p \)-aminobenzamidine were titrated with the inhibitors, and binding was monitored from the decrease in fluorescence accompanying the displacement of the bound probe from the enzyme active site as the inhibitor is bound. Titrations of \( \sim 1 \ \mu M \) \( \beta \)-trypsin or \( 5 \ \mu M \) \( \beta \)-anhydrotrypsin with BPTI or SBTI showed essentially linear decreases in probe fluorescence with added inhibitor up to an end point corresponding to the free probe fluorescence. This end point was reached in all cases at approximately 1 molar equivalent of inhibitor added per mol of enzyme (Fig. 3). Such behavior indicated a high affinity stoichiometric binding of the two inhibitors to both active and inactive enzymes. Similar behavior was observed when titrations were conducted at 10-fold lower enzyme concentrations. Further decreases in enzyme concentration were not possible due to an insufficient enhancement of the free probe fluorescence at the probe concentrations required for binding to the enzyme. These results indicated that dissociation constants (\( K_d \)) for non-serpin inhibitor interactions with trypsin and anhydrotrypsin were much lower than the concentration of enzyme titrated, i.e. \( \ll 10^{-7} \) M, in agreement with past studies (18, 19, 44).

A similar stoichiometric displacement of \( p \)-aminobenzamidine from \( \sim 1 \ \mu M \) \( \beta \)-trypsin was observed when the enzyme-probe complex was titrated with the serpin inhibitors, \( \alpha \_1 \) PI, antithrombin, antithrombin-heparin complex, and PAI-1. This was again evidenced from the linear quenching of trypsin-bound \( p \)-aminobenzamidine fluorescence by added inhibitor up to an end point corresponding to the free probe fluorescence and the addition of \( \sim 1 \) mol of inhibitor per mol of enzyme (Fig. 4). As with the non-serpin inhibitors, stoichiometric binding of the serpin inhibitors to trypsin was also observed when titrations were conducted at 10-fold lower enzyme concentrations,
implying high affinity interactions also of the serpin family inhibitors with β-trypsin characterized by $K_I$ values $\ll 10^{-7}$ M. By contrast, titrations of 5 μM β-anhydrotrypsin with the same serpin inhibitors produced no or a comparatively smaller quenching of anhydroenzyme-bound p-aminobenzamidine fluorescence even after several molar equivalents of inhibitor were added (Fig. 4). Thus, no significant decreases in probe fluorescence were observed in titrations with $\alpha_1$PI or antithrombin, a modest quenching of probe fluorescence was produced by titrating with antithrombin-heparin complex, and a more substantial but less than stoichiometric decrease in fluorescence occurred when the anhydroenzyme was titrated with PAI-1. The reduced or undetectable fluorescence changes produced in titrations of anhydrotrypsin with serpin inhibitors did not appear to be due to slow binding, since such changes were found to be stable for at least 3 h in the case of titrations with antithrombin or antithrombin-heparin complex.

Titrations of anhydrotrypsin with antithrombin complexed with full-length or pentasaccharide high affinity heparins over an extended range of inhibitor concentrations revealed a more substantial quenching of the bound probe fluorescence. Heparin or antithrombin alone produced no significant decline in probe fluorescence, indicating that a specific interaction of the inhibitor-heparin complex with anhydrotrypsin was responsible for the fluorescence changes. Addition of a molar excess of SBTI at the end of these titrations reduced the fluorescence to that of the free probe, indicating that the fluorescence quenching was due to displacement of the bound probe from the anhydroenzyme active site. These titrations were fit well by an equation for competitive equilibrium binding (see “Experimental Procedures”) (Fig. 5) which indicated end points indistinguishable from the free probe fluorescence and similar $K_I$ values of 6.7 ± 0.5 μM and 9.0 ± 0.3 μM for titrations with antithrombin-full-length heparin and antithrombin-pentasaccharide complexes, respectively. Reactive site-cleaved antithrombin in the absence or presence of saturating heparin (45) produced minimal displacement of p-aminobenzamidine from anhydrotrypsin, indicating that an intact reactive center loop was required for interaction with the anhydroenzyme.

Titrations of 2.5 or 5 μM anhydrotrypsin with several molar equivalents of PAI-1 resulted in nearly complete quenching of the bound probe fluorescence. Global fitting of these titrations showed that a single $K_I$ of 0.23 ± 0.05 μM for the PAI-1-anhydroenzyme interaction satisfactorily described the binding data (Fig. 5). Latent PAI-1, in which the reactive center loop is inserted into $\beta$-sheet A (46), was considerably less effective in displacing the probe from the anhydroenzyme. The estimated $K_I$ of 7 ± 1 μM for the latent PAI-1-anhydroenzyme interaction indicated at least a 30-fold reduced affinity compared to the native inhibitor interaction, suggesting that optimal anhydrotrypsin binding required the reactive center loop to be in the

![Figure 4](http://www.jbc.org/)

**Fig. 4.** Binding of serpin inhibitors to trypsin and anhydrotrypsin. Titrations of 0.82 μM trypsin and 10 μM p-aminobenzamidine (top panel) or of 5 μM anhydrotrypsin and 100 μM p-aminobenzamidine (bottom panel) with $\alpha_1$PI (○), AT (◇), AT-heparin complex (◆), and PAI-1 (●), as in Fig. 3. AT-heparin complex was formed by including 3 μM heparin in the enzyme-probe solution for titration of trypsin and 25 μM heparin for titration of anhydrotrypsin. Solid lines in the upper panel represent the expected dependence for 1:1 stoichiometric binding.

![Figure 5](http://www.jbc.org/)

**Fig. 5.** Binding of native and modified serpins to anhydrotrypsin. Top panel, titrations of 5 μM anhydrotrypsin and 100 μM p-aminobenzamidine with antithrombin (◇), antithrombin complexed with full-length (●) and pentasaccharide (◆) heparins, and reactive site-cleaved antithrombin with (●) and without (◇) complexed heparin, as in Fig. 4. Antithrombin-heparin complexes were formed by including heparin in the enzyme-probe solution at 25 μM (native AT) or 100 μM (cleaved AT) (45). Relative fluorescence changes are normalized to the maximum change obtained by addition of a molar excess of SBTI at the end of the titrations. Bottom panel, titrations of 2.5 μM (○) or 5 μM (●) anhydrotrypsin with PAI-1, 2.5 μM anhydrotrypsin with PI4 Thr → Arg variant PAI-1 (●) (taken from Ref. 29), and 2.5 μM anhydrotrypsin with latent PAI-1 ( ◇), all in the presence of 100 μM p-aminobenzamidine, as in the upper panel. Solid lines in both panels are fits by the competitive binding equation given under “Experimental Procedures.” Native PAI-1 titrations were globally fit by a single $K_I$. 

![Image](http://www.jbc.org/)
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Figure 6. Fluorescence changes accompanying the binding of trypsin and anhydrotrypsin to NBD-labeled P9 Ser → Cys PAI-1 variant. Emission spectra of 0.1 μM NBD-labeled P9 variant alone or after addition of 1 mol eq of trypsin or 16 mol eq of anhydrotrypsin. See “Experimental Procedures” for experimental details.

native exposed conformation. Together, these results indicated that non-serpin inhibitors exhibited high affinity interactions with both trypsin and anhydrotrypsin, whereas serpins showed a high affinity interaction with just trypsin and a comparatively weak affinity interaction with anhydrotrypsin.

Role of the Serpin Conformational Change in Anhydrotrypsin-Serin Interactions—To determine whether the interaction of serpins with anhydrotrypsin induced the serpin conformational change thought to accompany the interaction with active enzymes, i.e. the insertion of the reactive center loop into β-sheet A of the inhibitor, we examined the interaction of anhydrotrypsin with a P9 Ser → Cys variant of PAI-1 fluorescently labeled with the reporter group, NBD, which senses this conformational change (22). The labeled PAI-1 variant inactivated trypsin with about a 2-fold reduced association rate constant of 1 × 10^6 M⁻¹ s⁻¹ and was observed to form an SDS-stable complex with the enzyme by SDS-gel electrophoresis, confirming that it behaved similarly to wild-type PAI-1. The fluorescence emission of the NBD-labeled PAI-1 was blue-shifted from 542 to 525 nm and enhanced 6.7-fold at the latter wavelength when reacted with equimolar β-trypsin (Fig. 6). No further increase in fluorescence occurred when additional trypsin was added. These fluorescence changes resembled those previously shown to be induced in the labeled PAI-1 by the binding of target proteinases, cleavage in the reactive center loop, or conversion from an active to a latent form (22). In contrast, anhydrotrypsin produced no apparent shift in emission maximum and only minimal increases in fluorescence (1.2-fold) of the reporter group when 5 mol eq of anhydrotrypsin over serpin were added, and no further changes when up to 16 eq were added; i.e., levels equivalent to ~10 times the Kᵣ for the wild-type PAI-1 interaction (Fig. 6). Binding of anhydrotrypsin to the labeled PAI-1 variant at such concentrations was confirmed by showing that 1 μM anhydrotrypsin decreased the pseudo-first order rate constant for inactivation of 1 nM trypsin by 10 nM variant to an extent (4- to 5-fold) which was similar to that of wild-type PAI-1 and consistent with the Kᵣ values measured for the wild-type PAI-1 interaction (Table II). These results indicated that anhydrotrypsin binding to PAI-1 failed to induce reactive center loop insertion.

Further supporting this conclusion, anhydrotrypsin bound to a P14 Thr → Arg hinge region mutant of PAI-1 which was defective in reactive center loop insertion (29), with a Kᵣ of 0.04 ± 0.07 μM, fairly similar to that of active wild-type PAI-1 (Fig. 5). This result implied that optimal anhydrotrypsin binding does not require loop insertion. To obtain additional evidence for this conclusion, the effect of anhydrotrypsin binding to PAI-1 on the rate of spontaneous insertion of the reactive center loop into β-sheet A was evaluated from the rate of conversion of active PAI-1 to the inactive, latent form. The half-life for this inactivation measured at 37°C (see “Experimental Procedures”) was increased in the presence of nearly saturating anhydrotrypsin (1 μM) from 1.8 ± 0.1 h to 3.2 ± 0.6 h (average of 3 to 5 experiments). Increasing the anhydrotrypsin concentration to 10 μM did not produce any further increase in half-life (2.5 ± 0.1 h), indicating that anhydrotrypsin binding antagonizes rather than promotes the spontaneous insertion of the reactive center loop into β-sheet A.

Evaluation of the Affinities of Inhibitor-Trypsin Interactions—Kinetic analyses of on-rate constants (k₄) and off-rate constants (k₅) for serpin-trypsin interactions were used to estimate maximal Kᵣ values for these interactions. Kᵣ was determined by measuring the rate of trypsin inactivation by inhibitors both under second order (Fig. 1) and pseudo-first order conditions (see “Experimental Procedures”). Table I summarizes the values obtained. k₄ was analyzed by diluting serpin-trypsin complexes into a fluorogenic substrate at 12–24 × Kᵣ and continuously monitoring the initial rate of appearance of enzyme activity as the complex dissociates (42). Progress curves were well fit by the expected parabolic equation (Fig. 7) which yielded k₅ values given in Table I. Analysis of the products of complex dissociation by SDS-gel electrophoresis revealed that for all serpin-trypsin complexes, the inhibitor was released in a cleaved, inactive form rather than in the intact form (Fig. 8), indicating that the measured k₅ represented the rate constant for irreversible turnover of the complex. This implied that the rate constant for dissociation of complex back to native inhibitor must be considerably smaller than the measured k₅ (see “Discussion”). Kᵣ values determined for serpin-trypsin interactions from the ratios of k₄/k₅, thus reflect upper limits for actual equilibrium Kᵣ values. These maximal Kᵣ values were found to range from 1 to 8 × 10⁻¹¹ M (Table II). Comparison with the Kᵣ values determined for serpin-anhydrotrypsin interactions indicated that the anhydroenzyme bound serpins with at least a 4–6 order of magnitude reduced affinity compared to trypsin. In contrast to the SDS-stable complexes formed between serpins and trypsin, no such complexes were observed when serpins were mixed with anhydrotrypsin and then visualized by SDS-gel electrophoresis (Fig. 8), consistent with the noncovalent nature of serpin-anhydrotrypsin interactions.

Table II also compares the affinities of β-trypsin and β-anhydrotrypsin for the two non-serpin inhibitors. Kᵣ values were determined by equilibrium titrations of 10⁻¹⁰ M β-trypsin with BPTI or SBTI, monitored by the loss of enzyme activity after attainment of equilibrium. The measured Kᵣ values were in fair agreement with literature values (19, 44). Kᵣ values for anhydrotrypsin-inhibitor interactions were measured by equilibrium competition of trypsin and anhydrotrypsin for limiting inhibitor in the case of SBTI (18) or obtained by assuming the ratio of Kᵣ values for trypsin and anhydrotrypsin interactions previously determined in the case of BPTI (19). The Kᵣ obtained for the SBTI-anhydrotrypsin interaction agreed well with the previously reported value (18). The results shown in Table II confirm past studies that the anhydro modification of trypsin

3 If reactive center loop insertion facilitates the reversible equilibrium binding of anhydrotrypsin to PAI-1, then the P14 PAI-1 variant which is defective in loop insertion would be expected to show a decrease in binding affinity for the anhydroenzyme.
produces a relatively modest reduction in the affinity of Kunitz inhibitor interactions for the enzyme (18), as compared to the substantial affinity reduction of the serpin inhibitor interactions. Kunitz inhibitor complexes with trypsin or anhydrotrypsin were completely dissociated upon SDS-gel electrophoresis and showed no evidence for inhibitor cleavage under the conditions used to dissociate the serpin-trypsin complexes.

**DISCUSSION**

The role of the catalytic serine residue of serine proteinases in their high affinity interactions with protein inhibitors of serpin and non-serpin families has been investigated by comparing the binding of inhibitors of the different families to trypsin and its catalytic serine-modified derivative, anhydrotrypsin. In agreement with past studies (18, 19), the non-serpin inhibitors examined were found to bind both trypsin and anhydrotrypsin with high affinities (K<sub>i</sub> = 0.6–230 μM), confirming that a functional proteinase active site serine residue is not required to achieve a high affinity interaction. By contrast, several serpin inhibitors were shown to bind trypsin with high affinity (K<sub>i</sub> < 12–78 μM), whereas their binding to anhydrotrypsin was at least 4 to 6 orders of magnitude weaker (K<sub>i</sub> = 0.2 μM to >20 μM). Such results indicate that the proteinase catalytic serine residue is critical for generating a high affinity interaction with serpin-type inhibitors.

The differences in the affinities of trypsin and anhydrotrypsin for the two non-serpin inhibitors span the range of differences in affinities that have been measured for a number of such inhibitors with native and anhydroenzymes (2- to 130-
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(39, 55, 56). The contribution of such active site independent interactions to serpin-proteinase complex formation does not conflict with the substantial active site-dependent contribution of the catalytic serine residue to the affinity of serpin-proteinase interactions shown in this study, since none of the previous studies compared the relative affinities of serpins for native and active site-modified proteinases. A similar large differential affinity of PAI-1 and antiplasmin for native and active site-modified forms of their target enzymes would thus be predicted from our results. While our findings confirm past observations that noncovalent interactions can make a significant contribution to the association of serpins with proteinases, they also show that the contribution of such noncovalent interactions can vary greatly from serpin to serpin. The covalent interaction mediated by the active site serine may thus be sufficient to produce a high affinity association in many cases.

Our finding that the proteinase catalytic serine residue makes a substantial contribution to the high affinity interactions of serpins with their target proteinases is consistent with previous evidence that serpin-proteinase complexes are stabilized by a covalent bond between the proteinase catalytic serine and inhibitor P1 residue (11–13, 17). The formation of this covalent bond may enhance the affinity of serpin-proteinase complexes by triggering the conformational change thought to be required for stable complex formation in which the reactive center loop is inserted into β-sheet A of the inhibitor (5, 7–10). This proposal would be in keeping with the observations in this study that the noncovalent interaction between serpins and anhydrotrypsin does not appear to induce this conformational change. Thus, the interaction of a fluorescent-labeled PAI-1 variant with anhydrotrypsin is not accompanied by the characteristic fluorescence changes which accompany its interaction with trypsin and which have been shown to report insertion of the reactive center loop into β-sheet A in proteinase-complexed, cleaved, or latent forms of PAI-1 (22). Moreover, a P14 Thr → Arg PAI-1 variant defective in its ability to undergo the reactive center loop conformational change (29) bound anhydrotrypsin with an affinity similar to that of native PAI-1, consistent with the interaction not being dependent on the conformational change. Additionally, binding of anhydrotrypsin to PAI-1 diminished the rate of spontaneous loop insertion involved in converting active PAI-1 to its latent form, indicating that bound anhydrotrypsin interferes with the serpin conformational change, in agreement with the stabilizing effects of other catalytic serine-modified enzymes on the active PAI-1 conformation (57). These findings conflict with the suggestion of a previous study that the interaction of serpins with catalytic serine-modified enzymes requires the serpin conformational change (52). This suggestion was based on the observation that the binding of serpins to dichloroisocoumarin-inactivated proteinases was abolished when these serpins were complexed with reactive center loop peptides which block the conformational change (6, 9). While the reason for these discrepant findings is uncertain, the regeneration of active proteinase from dichloroisocoumarin-inactivated proteinases (58) and efficiency of cleavage of serpin-peptide complexes by catalytic proteinase (59) could be the basis for the different findings. In the present studies, the stably inactivated anhydrotrypsin has clearly been shown to bind PAI-1 without inducing the serpin conformational change, consistent with the catalytic serine being required to induce this change.

Insight into how the covalent active site serine interaction and the serpin conformational change stabilize serpin-proteinase complexes comes from a consideration of how Kunitz and other non-serpin inhibitors form stable complexes with proteinases without such a covalent interaction or analogous confor-

4 The alteration from a tetrahedral to a trigonal geometry of the serine 195 α-carbon atom produced by dehydration of the serine side chain does not appear to account for the decreased affinity of anhydrotrypsin for p-amino benzenzamide, since a similar 10-fold decreased binding of the probe has been found in a Ser → Ala variant of thrombin (S. T. Olson and J. E. Sadler, unpublished data).
motional change. High resolution x-ray crystal structures of complexes of non-serpin inhibitors with serine proteinases have shown that these complexes are stabilized by a complementary substrate-like interaction between an inhibitor binding loop and the enzyme active site (4). This lock and key interaction results from the inhibitor binding loop being rigidly fixed in an optimal, canonical substrate binding conformation which is common to non-serpin inhibitor families. These inhibitors thus are substrates which utilize most of their available proteinase binding energy to stabilize a Michaelis complex with proteinase, thereby leaving no additional binding energy to stabilize the transition state necessary for proteolysis to proceed beyond the Michaelis complex (60).

The importance of a covalent interaction and the serpin conformational change to serpin-proteinase interactions supports the previously proposed mechanism of serpin action (Scheme 2) in which a substrate-like reaction between inhibitor (I) and enzyme (E) proceeds beyond a Michaelis complex (EI) to the tetrahedral or acyl intermediate stage of proteolysis (EI*) before the complex is stabilized by the serpin conformational change (EI*) (14, 30, 49, 61, 62). Implicit in such a suicide substrate mechanism is a competition between the conformational change which traps proteinase in a covalent intermediate of the proteolysis pathway and a normal breakdown of the covalent intermediate along the proteolysis pathway to generate reactive-site cleaved inhibitor (I*H) and free proteinase, as depicted in Scheme 2.

$$E + I \rightleftharpoons E \cdot I \rightarrow E \cdot I^* \rightarrow E + I_H$$

**Scheme 2**

The existence of such a competing substrate reaction has been amply documented in native and variant serpin reactions (14, 30, 49, 61, 62). The mechanism of Scheme 2 also indicates that the stabilization of serpin-proteinase complexes by the conformational change does not prevent the substrate reaction but only slows it down, consistent with the findings of present and past studies (16, 48, 49).

There are two possible mechanisms by which the covalent bond and the serpin conformational change may stabilize serpin-proteinase complexes. According to one mechanism, insertion of the reactive center loop into β-sheet A could induce the loop to adopt the canonical conformation characteristic of non-serpin inhibitors (4, 8). Such a mechanism could produce a thermodynamic stabilization of the covalent intermediate as it does the Michaelis complex in the non-serpin inhibitors, i.e. by fully utilizing the binding energy of the reactive center loop interaction with proteinase to stabilize the ground state of the covalent intermediate, leaving no binding energy left to stabilize the transition state for further reaction along the proteolysis pathway. According to the second mechanism, reactive center loop insertion into β-sheet A may produce a disruption of the proteinase catalytic machinery which halts further reaction along the proteolysis pathway (2, 63, 64). This disruption could arise from a loss of the optimal alignment of the catalytic triad, from a dislodging of the reactive bond from its productive mode of binding in the proteinase active site or from the loss of contacts between residues flanking the reactive bond and proteinase subsites. The resulting loss of catalytic power would render the covalent linkage between the proteinase catalytic serine and inhibitor P1 residues kinetically stable by greatly reducing the rate of its breakdown. Such a proposal would be consistent with the rates of turnover of stable serpin-proteinase complexes of $10^{-4}$ to $10^{-6}$ s$^{-1}$ measured in this and presumably other studies (48, 65, 66) and $k_{cat}$ values for cleavage of peptide substrates by serine proteinases with mutations in the catalytic triad residues of $10^{-4}$ to $10^{-5}$ s$^{-1}$ (67, 68). The observation that the limiting rates of formation of stable serpin-proteinase complexes from Michaelis complexes (4–100 s$^{-1}$) (22, 69, 70) also limit the rates of turnover of substrate forms of these serpins (59) further suggests $10^{-5}$ to $10^{-6}$-fold decreases in $k_{cat}$ for proteinase cleavage of serpins as a result of stable complex formation. Similar decreases in $k_{cat}$ of $10^{-5}$-fold result from the disarming of the proteinase catalytic machinery by mutagenesis (67, 68).

The main distinguishing features of these two mechanisms is that the former results in a complex thermodynamically stabilized by ground state interactions between the reactive center loop and proteinase active site, whereas the latter produces a complex kinetically stabilized by the slow cleavage of the covalent linkage. Reactive center-loop interactions thus act in this latter mechanism to promote initial Michaelis complex formation and to stabilize the transition state for subsequent covalent complex formation as with a normal substrate and minimally contribute to stabilizing the final serpin-proteinase complex. Initial support for the latter mechanism has come from studies of a natural P1′ variant of antithrombin, which showed that the P1′ residue stabilizes the transition state leading to the covalent antithrombin-proteinase complex and does not contribute to stabilizing the ground state of this complex (64). Whether other reactive center loop residues similarly contribute to a transition state rather than ground state stabilization of serpin-proteinase complexes will require further investigations of the sources of the proteinase binding defects in serpin variants with mutations in the reactive center loop.

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