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# The Inhibitory Specificity of Human Proteinase Inhibitor 8 Is Expanded through the Use of Multiple Reactive Site Residues

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**Serine proteinase inhibitors function as regulators of serine proteinase activity in a variety of physiological processes. Proteinase inhibitor 8 (PI8) is a 45 kDa member of the ovalbumin family of serpins that is an inhibitor of trypsin-like proteinases through the use of $Arg^{339}$ as the inhibitory $P_1$ amino acid residue in its reactive site loop. In this study, we have described the inhibitory mechanism of recombinant human PI8 towards chymotrypsin. PI8 formed an SDS-stable complex with and inhibited the amidolytic activity of chymotrypsin via a two-step mechanism with an overall equilibrium inhibition constant of 1.7 nM and an overall second-order association rate constant of $1.0 \times 10^4$ $M^{-1}s^{-1}$, utilizing $Ser^{341}$ as the $P_1$ residue. The use of separate reactive site loop residues by PI8 to inhibit distinctly different classes of proteinases not only supports the hypothesis of the existence of the serpin reactive site as a highly mobile and flexible loop, but also suggests an evolved function in which separate amino acid residues can be used to broaden the inhibitory specificity of PI8.** © 1998 Academic Press

Serine proteinase inhibitors, or serpins, are a superfamily of proteins whose function is to regulate proteinase activity in a wide variety of physiological processes (1). Serpins range from 40 to 60 kDa in molecular mass, resemble $\alpha_1$-proteinase inhibitor in overall structure and include a number of homologous proteins such as $\alpha_1$-antichymotrypsin, $\alpha_2$-antiplasmin, antithrombin III, and plasminogen activator inhibitors 1 and 2 (2). Previous studies have shown that serpins are composed of three $\beta$-sheets surrounded by eight $\alpha$-helices. Their inhibitory specificity is determined by the amino acid residues located within the reactive site loop (RSL)[2], which is highly divergent among serpins and mimics the substrate sequence recognized by the target proteinase (2-4). The reactive site loop exists as a highly mobile stressed loop with a canonical conformation that confers the optimal conformation for high-affinity association with the substrate binding cleft of the cognate proteinase, with the $P_1$-$P_1'$ residues[3] determining the inhibitory specificity and acting as a pseudosubstrate for the target proteinase (5). Thus, the serpin acts as a "suicide substrate" for its target proteinase. Unlike a typical substrate, however, the serpin has the ability to form a tight complex with the proteinase that may be essential for its inactivation (3, 6-8). Inhibition occurs when the $P_1$ residue, or reactive center, of the serpin is recognized by the active site of the target proteinase and an initial loose complex is formed. This is followed by an isomerization of the initial loose complex to a tight 1:1 stoichiometric inhibitory complex between the serpin and the proteinase which is, in most cases, resistant to denaturants (9). Most of the known serpins are secreted, but there are some serpins which can be found intracellularly. The ovalbumin family of serpins is a unique family of serpins that lack a typical cleavable amino-terminal signal sequence, but have been found

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[2] The abbreviations used are: RSL, reactive site loop; PAI-2, plasminogen activator inhibitor-2; EI, elastase inhibitor; SCCA, squamous cell carcinoma antigen; PI6, proteinase inhibitor 6; PI8, proteinase inhibitor 8; PI9, proteinase inhibitor 9; SCCA2, squamous cell carcinoma antigen 2; kDa, kilodalton; Suc-AAPF-*p*NA, Succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide; TLCK, N$\alpha$-p-tosyl-L-lysine chloromethyl ketone; *p*-NPA, *p*-nitrophenyl acetate; DMF, dimethyl formamide; DMSO, dimethyl sulfoxide; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TBS, tris-buffered saline; PBS, phosphate-buffered saline.

[3] Residues within the reactive site loop are numbered analogous to substrates as follows: $P_n \ldots P_3 - P_2 - P_1 - P_1' - P_2' - P_3' \ldots P_n'$, with cleavage occurring at the $P_1 - P_1'$ bond.

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to reside intracellularly (10, 11), or both intracellularly and extracellularly (12-15). The lack of N- and C-terminal extensions and the presence of a serine residue at the penultimate position in the amino acid sequence are also distinguishing characteristics of the ovalbumin-type serpins, whose members include plasminogen activator inhibitor-2 (PAI-2) (15), an elastase inhibitor (EI) isolated from monocyte-like cells (16, 17), squamous cell carcinoma antigen (SCCA) (13), maspin (18), proteinase inhibitor 6 (PI6) (19-21), proteinase inhibitor 8 (PI8) (22), proteinase inhibitor 9 (PI9) (23), squamous cell carcinoma antigen 2 (SCCA2) (24), and bomapin (25). It has been demonstrated that individual mammalian ovalbumin-type serpins can inhibit a variety of prototypic serine proteinases (19, 20, 22, 24) and, in some cases, can exhibit cross-class specificity and inhibit cysteine proteinases (26). Interestingly, PI6, a serpin with which PI8 shares 68% sequence identity, has also been demonstrated to be a potent inhibitor of chymotrypsin through the use of an alternate amino acid residue in the reactive site loop as the $P_1$ residue, which supports the hypothesis that the conformation of the serpin reactive site loop is highly mobile and flexible (27).

PI8 is a widely expressed 45 kDa serpin that has been demonstrated to inhibit trypsin, the trypsin-like proteinases thrombin and coagulation factor Xa, the bacterial endoproteinase subtilisin A, and the prototypical mammalian convertase furin through a variety of kinetic mechanisms (22, 28). The amino acid sequence alignment of PI8 with other serpins predicts $Arg^{339}$ as the $P_1$ residue in the reactive site loop, consistent with the previously identified inhibitory specificity of PI8. In this report, we present a kinetic analysis of the inactivation of chymotrypsin by PI8. Additionally, this inhibition occurs through the use of $Ser^{341}$ as the inhibitory reactive center residue in this interaction, suggesting that PI8, like PI6, may have evolved to utilize distinct reactive site residues to broaden its inhibitory specificity.

## MATERIALS AND METHODS

Recombinant human PI8 (22) and human thrombin were prepared as described (29). Succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (Suc-AAPF-*p*NA) was obtained from Calbiochem-Novabiochem International (San Diego, CA). N$\alpha$-p-tosyl-L-lysine chloromethyl ketone (TLCK)-treated bovine $\alpha$-chymotrypsin, Hepes buffer, bovine serum albumin (BSA), dimethlyl sulfoxide (DMSO), dimethyl formamide (DMF) and *p*-nitrophenyl acetate (*p*-NPA) were purchased from Sigma Chemical Co. (St. Louis, MO). UltraFit 3.0 software was purchased from Biosoft (Ferguson, MO).

*General kinetic methods.* The concentration of catalytically active bovine $\alpha$-chymotrypsin was determined by titration with *p*-NPA as previously described (30). Active site-titrated chymotrypsin was used to determine the partitioning ratio and the amount of PI8 necessary for a 1:1 molar binding stoichiometry for the determination of kinetic constants. This was accomplished by incubating increasing amounts of PI8 with enzyme (10 nM) in a total volume of 150 $\mu$l of 20 mM Hepes (pH 7.5)/0.15 M NaCl/0.01% BSA in individual wells of a microtitration plate previously blocked with buffer containing 0.1% BSA. The reactants were incubated for 30 min at 37 °C and residual amidolytic activity was measured by adding 50 $\mu$l of 2 mM Suc-AAPF-*p*NA in 0.1 M Hepes/0.5 M NaCl/20% DMSO and monitoring $\Delta A_{405}$/min using a UVmax microplate reader (Molecular Devices, Sunnyvale, CA). The data were used to plot the enzymatic rate of substrate hydrolysis as a function of the amount of PI8 added to the reaction well. Linear regression to the *x*-axis was used to calculate the precise amount of PI8 required to form a 1:1 stoichiometric complex. Enzyme-substrate catalytic constants were measured in 20 mM Hepes (pH 7.5)/0.15 M NaCl/0.01% BSA/2% DMSO/1% DMF at 25 °C. Data were fitted to the Michaelis-Menton equation using UltraFit software to determine values for $K_m$ and $k_{cat}$. The catalytic constants for chymotrypsin and the chromogenic substrate Suc-AAPF-*p*NA were $K_m$ = 0.120 mM and $k_{cat}$ = 67 $s^{-1}$.

*Slow-binding inhibition kinetics.* Inhibition progress curves were obtained under pseudo-first-order conditions by incubating the reactants in 0.5 mL of the buffer used to determine the catalytic constants at 25 °C. Polystyrene cuvettes were previously blocked with 20 mM Hepes (pH 7.5)/0.15 M NaCl/0.1% BSA, and reactions were started by the addition of enzyme to a solution containing the chromogenic substrate and appropriate inhibitor concentration. Reactions for each experiment were started within 30 s and the cuvettes were placed in a Beckman DU-65 spectrophotometer equipped with a six-cell cuvette holder to allow simultaneous monitoring of multiple reactions at 405 nm. The final concentrations of the reactants were 0.16 nM chymotrypsin, 1.0 mM Suc-AAPF-*p*NA and 64, 96, 128, and 160 nM PI8. Spontaneous substrate hydrolysis was measured in separate experiments and determined to be negligible. The reactions were allowed to proceed until steady-state velocity was attained and the data were fitted to the integrated rate equation for slow-binding inhibition (31),

$$A = \nu_s t + (\nu_o - \nu_s)\frac{1 - e^{-k't}}{k'} + A_o, \qquad [1]$$

by nonlinear regression using UltraFit software to obtain values for the initial velocity ($\nu_o$), the steady-state velocity ($\nu_s$), the initial absorbance ($A_o$) and the apparent first order rate constant ($k'$) for the establishment of steady-state equilibrium of the proteinase-inhibitor complex. The data obtained from nonlinear regression analysis were then used in various graphical transformations (31-36) to obtain the inhibition and rate constants for the interaction of PI8 with chymotrypsin.

*Amino acid sequencing.* Separate reaction mixtures of PI8 (2 $\mu$M) and chymotrypsin (400 nM), and PI8 (2 $\mu$M) and human thrombin (400 nM) were incubated in PBS for 1 hour at 25 °C. PMSF was added to 1 mM, and the reaction mixture was desalted and washed by centrifugation on a ProSpin sample preparation cartridge (Perkin Elmer/ABI, Foster City, CA). The reaction mixtures were subsequently sequenced on a Beckman LF3000 protein sequencer.

*Detection of SDS-stable PI8/chymotrypsin complexes.* Chymotrypsin was radiolabeled with $Na^{125}I$ using the IODO-GEN transfer method (37) to an average specific radioactivity of 3.0 $\mu$Ci/$\mu$g. $^{125}$I-chymotrypsin (0.7 pmol) was incubated with PI8 (0.5 nmol) for 30 minutes at 37 °C. Samples were subsequently boiled in the presence of SDS and subjected to SDS-PAGE under reducing conditions (38). Following electrophoresis, proteins were transferred to PVDF by electroblotting for 45 min at 500 mA in 10 mM CAPS (pH 11) containing 10% methanol. The membrane was washed in TBS, dried, and subjected to autoradiography.

## RESULTS

The partitioning ratio for the interaction of PI8 with chymotrypsin was 20:1. Preliminary studies indicated that the interaction between PI8 and chymotrypsin obeyed slow-binding inhibition kinetics, as the amidolytic activity of chymotrypsin inhibited by PI8 attained steady-state equilibrium and the data were successfully fitted to Eq. 1. There are three mechanisms that can describe the slow onset of inhibition (31):

$$P \underset{k_{-1}}{\overset{k_1[I]}{\rightleftharpoons}} \qquad [A]$$

$$P \underset{k_{-1}}{\overset{k_1[I]}{\rightleftharpoons}} PI \underset{k_{-2}}{\overset{k_2(slow)}{\rightleftharpoons}} PI' \qquad [B]$$

$$P \underset{k_{-1}}{\overset{k_1(slow)}{\rightleftharpoons}} P' \underset{k_{-2}}{\overset{k_2[I]}{\rightleftharpoons}} PI'. \qquad [C]$$

In mechanism A, the proteinase (P) binds to the inhibitor (I) in a single step to form an PI complex. In mechanism B, a loose PI complex is rapidly formed, followed by the slow isomerization to the tight PI′ complex. In mechanism C, the proteinase is assumed to exist in two forms, and active form P and an inactive form P′, which is accessible to the inhibitor. The isomerization of P to P′ is followed by the rapid formation of a tight PI′ complex. These different mechanisms can be distinguished by graphical transformations of the values obtained from the slow-binding kinetic approach (30). The kinetic characterization of the inhibition of chymotrypsin by PI8 was performed with PI8 concentrations ranging from 400 to 1000 times the molar concentration of chymotrypsin. A family of inhibition progress curves representative of the reaction between chymotrypsin and PI8 at its selected concentrations is shown in Fig. 1, and data obtained from the progress curves were fitted to Eq. 1 by nonlinear regression analysis. The results indicated that the initial velocity, $\nu_o$, was inversely proportional to the inhibitor concentration for each set of progress curves, suggesting that the inhibition of chymotrypsin by PI8 follows a two-step mechanism described by mechanism B (30). This observation was confirmed by plotting $\nu_{max}/\nu_o$ against the PI8 concentration (data not shown). The graph was linear with a positive slope, from which the $K_i$ for this interaction was calculated according to the relationship $\nu_{max}/\nu_o = K_m[I]/[S]K_i + 1 + K_m/[S]$, and was estimated to be 190 (±19) nM ($n = 4$). Additionally, the apparent first order rate constant $k'$ was found to increase as PI8 concentration increases, which is consistent with only mechanisms A and B. On the basis of these observations, the kinetic constants for the inhibition of chymotrypsin by PI8 were determined by treating the data according to mechanism B.

The overall inhibition constant $K_i'$ was determined

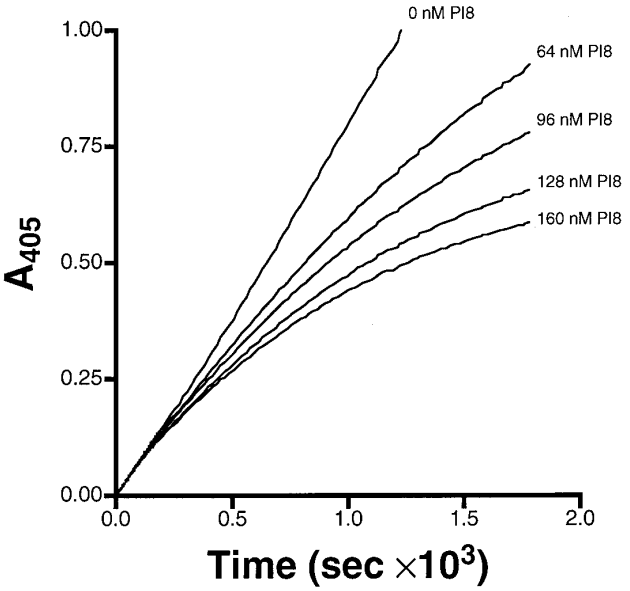


**FIG. 1.** Progress curves from slow-binding kinetics for the inhibition of chymotrypsin by PI8. Chymotrypsin (0.16 nM) was reacted with 64, 96, 128, and 160 nM PI8 in 20 mM Hepes (pH 7.5)/0.15 M NaCl/0.01% BSA/2% DMSO/1% DMF at 25 °C in the presence of 1.0 mM Suc-AAPF-*p*NA. The reactions were monitored continuously for 5 hours and the data were fitted to Eq. 1 to generate values for the variables $\nu_o$, $\nu_s$, $A_o$ and $k'$.

from plots of both $\nu_{max}/\nu_s$ and $(\nu_o - \nu_s)/\nu_s$ versus PI8 concentration (data not shown). The slopes of these plots are equal to $K_m/[S]K_i'$, from which $K_i'$ was calculated to be 1.7 (±0.2) nM ($n = 4$). The overall second-order association rate constant $k_{assoc}$ was determined by plotting $\log([P]_\infty - [P]_t)$ versus time, where $[P]_\infty = \nu_o/k'$ and $[P]_t$ is the absorbance measured at various times between 0 and 15 min for individual progress curves (data not shown) (34). The slope of the lines obtained is equal to $-0.43[I]\{ k_{assoc}/(1 + [S]/K_m)\}$, from which $k_{assoc}$ was calculated to be 1.0 (±0.4) × $10^4$ $M^{-1}s^{-1}$ ($n = 4$). A linear plot of $k'$ against $\nu_o/\nu_s$ was produced to determine the rate constant for the reverse isomerization step $k_{-2}$ of the chymotrypsin-PI8 tight complex (data not shown). $k_{-2}$ was calculated directly from the slope of the line to be 1.05 (±0.04) × $10^{-5}$ $s^{-1}$ ($n = 4$). Using the relationship $T_{1/2} = 0.693/k_{-2}$, a half-life of 18.33 hours was estimated for the reverse isomerization of the tight complex to the loose complex. The value of the rate constant for the formation of the tight complex, $k_2$, was determined by fitting a plot of $k'$ versus PI8 concentration (data not shown) to the equation (30)

$$k' = k_{-2} + k_2 \frac{\dfrac{[I]}{K_i}}{1 + \dfrac{[S]}{K_m} + \dfrac{[I]}{K_i}} \qquad [2]$$

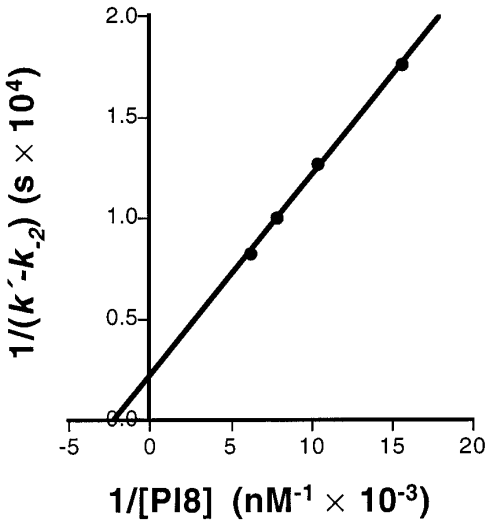


**FIG. 2.** $1/(k' - k_{-1})$ versus 1/[PI8] for the interaction of PI8 with chymotrypsin. Values for $k'$ were generated as described in the legend to Fig. 1. The value of $k_{-2}$ was determined from plot of $k'$ against $\nu_{max}/\nu_s$. The line crosses the positive $y$-axis at a point approximately equal to $1/k_2$, indicative of a two-step binding mechanism that follows mechanism B.

by nonlinear regression analysis. By using this method, $k_2$ was estimated to be 1.6 ($\pm$0.2) $\times$ $10^{-3}$ $s^{-1}$ ($n$ = 4). In order to verify that the interaction of PI8 and chymotrypsin occurs by mechanism B and to justify the use of a hyperbola to describe the relationship between $k'$ and [PI8], a double-reciprocal plot of $1/(k' - k_{-2})$ versus 1/[PI8] was generated, using the values obtained from Eqs. 1 and 2, which is linear for both mechanism A and mechanism B (Fig. 2) (35). Mechanisms A and B can be differentiated by this approach since in mechanism A, the line passes through the origin, whereas in mechanism B, the line crosses the positive $y$-axis at a point approximately equal to $1/k_2$. The $y$-intercept of the plot in Fig. 2 indicated a value for $k_2$ of 2.9 ($\pm$0.9) $\times$ $10^{-3}$ $s^{-1}$ ($n$ = 5), which is reasonably close to the value of $k_2$ determined by Eq. 2. More importantly, the plot in Fig. 2 justifies the manipulation of data and determination of kinetic constants according to mechanism B.

PI8 was incubated separately with chymotrypsin and thrombin, followed by N-terminal amino acid sequencing to determine the cleavage site in PI8 during the interaction with these proteinases and, ultimately, the position of the $P_1$ amino acid used in the interaction (Fig. 3). Sequencing of the reaction between PI8 and chymotrypsin revealed the sequence Arg-Met-Glu-Pro-Arg-Phe, which corresponds to amino acids 342-347 of PI8, and the sequence Ile-Val-Asn-Gly-Glu-Glu, which is identical to the N-terminal sequence of the chymotrypsin B chain. The reaction of PI8 with thrombin yielded the sequences Cys-Ser-Arg-Met-Glu-Pro, Ile-Val-Glu-Gly-Ser-Asp, and Thr-Phe-Gly-Ser-Gly-Glu, which correspond to amino acids 340-345 of PI8, the N-terminal sequence of the thrombin heavy chain, and the N-terminal sequence of the light chain of thrombin, respectively. In both sequence analyses, no PI8 N-terminal sequence was observed, consistent with the results in previous studies that indicated a blocked N-terminus for recombinant PI8 (22). The sequences obtained indicate that the reactive centers for the inhibition of chymotrypsin and thrombin by PI8 are $Arg^{339}$ and $Ser^{341}$, respectively.

PI8 was also tested for its ability to form an SDS-stable complex with chymotrypsin. After incubation of $^{125}$I-chymotrypsin with PI8, an SDS-stable complex was seen with an apparent molecular mass of 65 kDa, along with additional bands at 55 kDa and 32 kDa (data not shown). The ability of PI8 to form an SDS-stable complex with chymotrypsin is consistent with the two-step inhibitory mechanism observed for this interaction.

## DISCUSSION

In the present study, we have performed a detailed kinetic analysis of the inactivation of chymotrypsin by PI8, which occurred via a two-step mechanism using $Ser^{341}$ as the $P_1$ residue. SCCA (13) and bovine $\alpha_1$-antichymotrypsin (39) also utilize a Ser residue in their RSL in their inhibition of cathepsin L and chymotrypsin, respectively. The overall equilibrium inhibition constant for the inactivation of chymotrypsin by PI8 was 1.7 nM, indicating that PI8 is a fairly potent inhibitor of this proteinase. The $k_{assoc}$ for chymotrypsin and PI8 was determined to be $1.0 \times 10^4$ $M^{-1}s^{-1}$, which is slightly lower than the second-order association rate constants for the inhibition of plasma kallikrein by C1-inhibitor ($6.9 \times 10^4$ $M^{-1}s^{-1}$) (40) and factor Xa by PI8 ($7.5 \times 10^4$ $M^{-1}s^{-1}$) (22). PI8 was also able to form an SDS-stable complex with chymotrypsin, as expected for a two-step mechanism that results in the formation of a 65 kDa tight inhibitory complex. In addition, two bands at 55 kDa and 32 kDa were observed, suggesting that the complex is cleaved by free chymotrypsin during the reaction, possibly at sites that are exposed during isom-

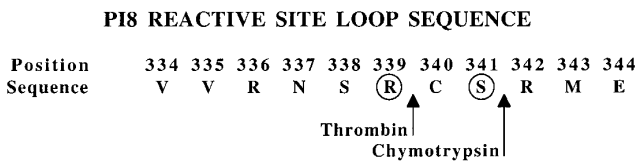


**FIG. 3.** Multiple reactive centers in the PI8 reactive site loop. Cleavage of the $P_1$-$P_1'$ scissile bond by thrombin and chymotrypsin occurs in the positions indicated by the arrows. Amino acid residues in the PI8 RSL which act as reactive centers ($P_1$ amino acids) are circled.

erization. The inhibitory structure of serpins is conformationally unstable and the reactive site loop is highly susceptible to cleavage by proteinases that they do or do not inhibit. The titration of PI8 with chymotrypsin indicated a partitioning ratio of 20:1, indicating that twenty molecules of inhibitor are required to inactivate one molecule of enzyme. The exposed nature of the reactive site loop enables the serpin to act as either a substrate or an inhibitor for a proteinase, or both. This phenomenon has been observed for the interactions of $\alpha_1$-antichymotrypsin and $\alpha_1$-proteinase inhibitor with human mast cell chymase (41), C1-inhibitor with plasma kallikrein (42), and $\alpha_2$-antiplasmin with trypsin and chymotrypsin (43) and is a recognized characteristic of some of the interactions that occur between proteinases and serpins.

The data presented in this report indicates that there is a certain degree of flexibility in the use of amino acid residues as the inhibitory $P_1$ residue in the serpin RSL, and this flexibility may function to increase the serpin's inhibitory specificity. PI8 was able to utilize separate reactive centers to inhibit trypsin- and chymotrypsin-like proteinases through the same two step mechanism that is commonly seen for serpin-proteinase interactions. In addition, PI8 contains two sequences, RNSR and RCSR in its RSL that are identical to the minimal sequence required for efficient recognition and processing by the convertase furin, a serine proteinase of which PI8 is a potent inhibitor, also via a two-step mechanism (28). It is possible that PI8 is able to inhibit furin by utilizing one or both of these dibasic sequences. At this time, this apparent flexibility in the use of separate amino acid residues as reactive centers appears to be unique to two ovalbumin serpins, PI6 and PI8, and may indicate an evolved characteristic of these serpins that allows for a broadened inhibitory specificity.

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