Influence of the P$_5$ Residue on $\alpha_1$-Proteinase Inhibitor Mechanism*

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The reactive center loop of native $\alpha_1$-proteinase inhibitor has been reported to be in a helical conformation and in a $\beta$-strand conformation by two different studies. In the $\beta$-strand structure the P$_5$ glutamic acid plays a unique role by stabilizing the loop in the predicted optimal conformation for the interaction with target proteases and insertion into $\beta$-sheet A. We hypothesize here that disrupting the interactions that stabilize the $\beta$-strand conformation of the loop would result in changes in the inhibitory properties of the serpin. In addition, our earlier studies on reactive center loop mutants of $\alpha_1$-proteinase inhibitor suggested that the P$_5$ residue was important in stabilizing the $\alpha_1$-proteinase inhibitor-proteinase complexes. To address these issues we made mutants of $\alpha_1$-proteinase inhibitor with glycine, glutamine, or lysine at the P$_5$ position and measured the rates and stoichiometries of inhibition with trypsin and human neutrophil elastase and the stabilities of the resulting complexes. In most cases the rate of inhibition was reduced by about half and the stoichiometry increased between 2- and 4-fold. The only exception was for trypsin with the lysine variant where the P$_5$ was now the favored site of cleavage. These data show that the P$_5$ Glu is important in maintaining the reactive center loop in a conformation optimal for interaction with the proteinase and for a fast rate of loop insertion. The complexes formed with trypsin and the variant serpins were less stable than that formed with wild-type serpin and resulted in up to 33% regeneration of trypsin activity over a period of 6 days, compared with 17% with wild type. Thus, the P$_5$ residue of $\alpha_1$-proteinase inhibitor is important in all steps of the inhibitory mechanism in a manner consistent with the structural role played by this residue in the $\beta$-strand loop structure of native $\alpha_1$-proteinase inhibitor.

One of the best studied members of the serpin family of serine protease inhibitors is $\alpha_1$-proteinase inhibitor ($\alpha_1$-PI), which is an important inhibitor of neutrophil elastase and is deficient in emphysema (1–3). $\alpha_1$-PI inhibits its target serine proteinases by the branched suicide-substrate pathway (1, 4) characteristic of inhibitory serpins (Fig. 1). The structure of native $\alpha_1$-PI has been solved by two groups (5, 6). From one study (6), the reactive center loop, which contains the scissile P$_1$-P$_9$ bond, was found to be in a helical conformation similar to other native serpins, namely ovalbumin and an $\alpha_1$-antichymotrypsin variant (8, 9). In the other, more detailed, structure of native $\alpha_1$-PI (5) it was found that the reactive center loop is in an extended $\beta$-strand conformation similar to the canonical conformation found for other classes of protein inhibitors of serine proteinases (10). In this structure the P$_5$ glutamic acid residue plays a crucial role in the stabilization of the $\beta$-strand conformation of the reactive center loop by forming hydrogen bonds with Arg-196 and the backbone amide of Met-226. These residues, along with Arg-223, Lys-243, and Arg-281, are part of a basic pocket. It was concluded that these interactions fixed the loop in the optimal conformation for the interaction with proteinases and for rapid loop insertion (5); therefore this structurally important residue also has important mechanistic implications. In this study we disrupted the stabilizing bonds present in native wild-type $\alpha_1$-PI by making P$_5$ Gln to Gly, Gln, and Lys mutations. This allowed us to test the hypothesis regarding the implications of the $\beta$-strand conformation of the reactive center loop of $\alpha_1$-PI on the inhibition mechanism. We assessed the effect of these mutations on the rates and stoichiometries of inhibition with trypsin and human neutrophil elastase.

We showed previously that $\alpha_1$-PI mutants containing the P$_5$-P$_9$ or the P$_{12}$-P$_9$ residues of ovalbumin formed complexes with trypsin which had less stability compared with wild-type $\alpha_1$-PI, as measured by the regeneration of trypsin activity from complexes (11). In these two mutants the P$_5$ Gln was changed to Gly. We suggested that in complexes with wild-type $\alpha_1$-PI either the P$_5$ Gln or the P$_9$ Ile were in direct contact with the trypsin and formed an important bond involved in the stabilization of the complex, or that they interacted with adjacent residues in strand 5A, or the loop connecting helix F with strand 3A, to stabilize the cleaved loop inserted form of the serpin in the complex. As part of this present study we addressed this hypothesis by testing the long term stability of complexes formed with the previously discussed P$_5$ variants.

We found that the variants had reduced rates of enzyme inhibition and increased stoichiometries of inhibition (2–3-fold) showing that mutations of the P$_5$ residue influenced steps both before, and after, the branch point in the mechanism, i.e. rates of proteinase interaction and of loop insertion. This is in agreement with the hypothesis of the importance of the reactive center loop conformation on serpin inhibitory activity as pre-

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1 The abbreviations used are: $\alpha_1$-PI, $\alpha_1$-proteinase inhibitor; P$_i$G$_j$-PI, P$_i$-G$_j$-PI, and P$_i$K$_j$-PI, $\alpha_1$-PI mutants with the P$_i$ residue changed to glycine, glutamine, or lysine, respectively; SI, stoichiometry of inhibition; HNE, human neutrophil elastase; PEG, polyethylene glycol.

2 Using the nomenclature of Scehchter and Berger (7) the P$_i$-P$_j$ bond is that which is cleaved by the proteinase. Residues NH$_2$-terminal to this bond are designated P$_2$, P$_5$, and so on, and those COOH-terminal are designated P$_s'$, P$_j'$, and so on.
Activity of α1-Proteinase Inhibitor P5 Mutants

The suicide-substrate mechanism of serpin action. The serpin, I, reacts with the serine proteinase, E, to form a Michaelis complex, EI. This then reacts to give an intermediate, [EI], as part of the normal proteolytic process. This complex can be converted to a stable proteinase-serpin complex, E-1*, with a rate constant of k1. This complex dissociates only slowly (over many days) to give serpin cleaved at the P1-P1' bond, I*, and regenerated free enzyme. The proteinase component of the complex may also be cleaved (at one or more sites) by free proteinase, P (either the same proteinase as E or a different one) to give E*-1* which cannot dissociate at all. This step has only been well characterized in one case (26, 27), and is included for completeness, but with the caveat that there is much to be learned about this step (see also Ref. 50). The intermediate [EI] can also be cleaved as in a normal subtilisin reaction to give cleaved serpin, P*, and free enzyme, with a rate constant of k2.

Fig. 1. The suicide-substrate mechanism of serpin action. The serpin, I, reacts with the serine proteinase, E, to form a Michaelis complex, EI. This then reacts to give an intermediate, [EI], as part of the normal proteolytic process. This complex can be converted to a stable proteinase-serpin complex, E-1*, with a rate constant of k1. This complex dissociates only slowly (over many days) to give serpin cleaved at the P1-P1' bond, I*, and regenerated free enzyme. The proteinase component of the complex may also be cleaved (at one or more sites) by free proteinase, P (either the same proteinase as E or a different one) to give E*-1* which cannot dissociate at all. This step has only been well characterized in one case (26, 27), and is included for completeness, but with the caveat that there is much to be learned about this step (see also Ref. 50). The intermediate [EI] can also be cleaved as in a normal subtilisin reaction to give cleaved serpin, P*, and free enzyme, with a rate constant of k2.

Directed from the β-strand loop structure (5). We also found that complexes between the variants and trypsin showed regeneration of trypsin faster than with wild-type α1-PI, in agreement with our hypothesis. Thus, we have identified that the P5 Glu is an important residue in the mechanism of proteinase inhibition of α1-PI, in a predictable manner consistent with its important stabilizing role known from the β-strand structure of native α1-PI.

Experimental Procedures

Materials—Restriction enzymes were obtained from Pharmacia Biotech Inc., oligonucleotides were synthesized by DNA Agency (Malvern, PA), Sequenase was from U. S. Biochemical Corp., the pET16b vector was from Novagen (Madison, WI), the Quick Change site-directed mutagenesis kit was from Stratagene (La Jolla, Ca), human neutrophil elastase (HNE) was from Athens Research (Athens, GA), methoxysuccinyl-(Ala)2-Pro-Val-p-nitroanilide and phenylmethylsulfonyl fluoride were from Sigma, and S-2222 was from Chromogenix (Franklin, OH). β-Trypsin was purified from 1-t-lysylamido-2-phenethyl chloromethyl ketone-treated trypsin (Sigma) on soybean trypsin inhibitor-agarose as reported previously (12).

Mutagenesis, Expression, Refolding, and Purification of Recombinant α1-Proteinase Inhibitor—The wild-type α1-PI DNA was deleted with deletion of the first five codons was contained in a Novol-XbaI fragment in the pET16b vector (11). Site-directed mutagenesis of the P5 residue from glutamic acid to glutamine, glycine, or lysine was performed using the Quick Change site-directed mutagenesis kit following the manufacturer’s protocol. The oligonucleotides were 19 bases in length and spanned the P5 residue codon to the first base of the P2 codon. The codon for glutamic acid was GAG and the mutated codons were CAG, GGG, and AAG, respectively.

The recombinant proteins (wild-type and mutants) were produced as inclusion bodies in Escherichia coli. The recovery of the active proteins from the inclusion bodies was done as described previously (11). The purified monomeric proteins were stored at -70 °C; however, the process of freezing and thawing can induce some aggregation of the monomers. Consequently the samples used for the studies described here were analyzed to determine the percentage of active monomer in each thawed sample. This was assessed after separation on a Mono Q column using a linear gradient of 0–2 M NaCl in 10 mM sodium phosphate, pH 6.5, 1 mM EDTA, 1 mM β-mercaptoethanol. Proteins were detected using a Shimadzu RF 535 fluorescence detector (with λex = 280 nm and λem = 340 nm). The samples were quantified according to their fluorescence intensity and the percentage of active monomer determined. Such an analysis is possible due to the different elution of monomeric and polymeric α1-PI on Mono Q (13, 14). The percentages of active monomers in the thawed aliquots were as follows: wild-type α1-PI, 92%; P5G-α1-PI, 67%; P5Q-α1-PI, 68%; and P5K-α1-PI, 94%.

Determination of the Stoichiometry of Inhibition for Trypsin and Elastase—Stoichiometry of inhibition (SI) values for the inhibition of trypsin were determined by incubating different concentrations of wild-type and the P5 variants with 12.5 nM trypsin in 0.1 M Hepes, pH 7.4, 0.1 M NaCl, 10 mM CaCl2, and 0.1% PEG 8000. The residual amidolytic activity was determined by the addition of 100 μM S-2222. Linear regression analysis of the decrease in proteinase activity with increasing concentration of α1-PI yielded the estimates for the stoichiometry of inhibition as the intercept on the abscissa. SI values for the inhibition of HNE were determined in a similar fashion by incubating different concentrations of wild-type and the P5 variants of α1-PI for 30 min or 1 h at 25 °C, with 5 nM HNE in 0.1 M Hepes, pH 8.0, 0.5 M NaCl, and 0.1% PEG 8000. The residual amidolytic activity was determined by the addition of 200 μM methoxysuccinyl-(Ala)2-Pro-Val-p-nitroanilide. The obtained values for SI were adjusted by a correcting factor corresponding to the percentage of monomeric species present for the α1-PI species tested.

Rates of Inhibition of Trypsin and Elastase by Wild-type and Variant α1-PI—The rate of inhibition of trypsin by recombinant α1-PI was determined at 25 °C by use of a discontinuous assay procedure. Under pseudo-first order conditions, 150 or 200 nM wild-type α1-PI, 300 nM or 1 μM P5G-α1-PI, 600 nM or 1.54 μM P5Q-α1-PI, and 735 nM or 1.12 μM P5K-α1-PI were incubated with 12.5 nM trypsin in 0.1 M Hepes, pH 7.4, 0.1 M NaCl, 10 mM CaCl2, and 0.1% PEG 8000. The residual trypsin activity was determined at various time by diluting the reaction mixture into the assay buffer containing 100 μM S-2222. The pseudo-first order constant, kapp, for the reaction was obtained from the slope of a semi-log plot of the residual trypsin activity against time, and the second order rate constant, kapp, was determined by kapp([I]/[I]), where [I] is the initial serpin concentration.

The rate of HNE inhibition by recombinant α1-PI was determined at 25 °C by use of a continuous assay procedure. Under pseudo-first order conditions, 0.5 nM HNE was incubated with 2.5 or 5 nM wild-type α1-PI, 15 nM P5G-α1-PI, 15 nM P5Q-α1-PI, or 5 nM P5K-α1-PI in the presence of 200 μM methoxysuccinyl-(Ala)2-Pro-Val-p-nitroanilide in 0.1 M Hepes, pH 8.0, 0.5 M NaCl, and 0.1% PEG 8000. The reaction was continuously recorded at 405 nm. The rate constant for the reaction was obtained after fitting the reaction curve to the equation kapp = (kapp/[I]) + (S/Ka), where K is the value for HNE with the substrate.

The constant kapp, determined by both the discontinuous and continuous assays is defined as kapp = (kapp/[I]) + (S/Ka) + b. This term is dependent on the amount of partitioning between the noninhibitory substrates, the inhibitory pathway and the noninhibitory pathway, indicated by the (kapp/[I]) term. The SI is defined as (kapp + b/k app), therefore multiplying kapp by the SI will give the rate constant kapp which represents the rate of formation of [EI] (1). If the proteinase also cleaves within the reactive center at a site other than the P1-P1' bond, this analysis cannot be used, as it is not possible to separate this inactivation of the serpin from the inactivation by cleavage at the P1-P1' bond (kapp).

Stability of Complexes between α1-PI and Trypsin—Inhibition of the Cleavage Sites in Wild-type and Variant α1-PI by HNE and Trypsin—For all of the α1-PI variants, cleavage was done using HNE and trypsin, and characterization of the cleavage site was performed by NH2-terminal sequence analysis of the cleavage reaction mixture after stopping the reaction by addition of phenylmethylsulfonyl fluoride to 5 mM and freezing until analysis. Automated Edman degru-
Inhibition of Trypsin and HNE—The aims of this study were 2-fold. First, to test the effect of disrupting the native conformation of the reactive center loop on the inhibitory activity of $\alpha_1$-PI. From the two known native structures of $\alpha_1$-PI, the one describing a $\beta$-strand conformation predicted it would have direct mechanistic implications. We disrupted this optimal conformation by introducing changes at P5, the crucial residue for stabilization (5). The second aim was to test whether these same changes impair the stability of the resulting complexes with trypsin. Thus, variants of $\alpha_1$-PI with changes at the P5 residue from glutamic acid to glutamine, or lysine were expressed in E. coli. Glutamine was chosen to remove the charge but to keep the same size residue, glycine was observed in ovalbumin and the $\alpha_1$-PI-ovalbumin chimeras studied previously (11), and lysine was chosen to introduce a positive charge at this position. The rates and SI of trypsin and HNE by the $\alpha_1$-PI-P5 variants were measured (Table I). Amino-terminal sequencing of reaction mixtures of the variants with HNE showed that the only detectable cleavage site was at the P5-P4 bond. Therefore multiplication of $k_{\text{app}}$ by SI gives $k'_{\text{app}}$ which corresponds to the rate of formation of [EI], i.e. the steps prior to the branch point (1). In all cases $k_{\text{app}}$ was reduced by about half. Therefore, the P5 residue can affect the initial interaction with the proteinase as judged by the reduction in $k'_{\text{app}}$ and the steps after the branch point, namely loop insertion, as judged by the increase in SI. In the case of trypsin with P5K-1-PI, the SI appeared to be close to 10. However, amino-terminal sequencing of this reaction mixture revealed that the predominant site of cleavage was not at the P5-P4 bond but at the P4-P3 bond, consequently $k'_{\text{app}}$ could not be determined. Amino-terminal sequencing of reaction mixtures with both trypsin and HNE showed that in all other cases the only cleavage site was the P5-P4 bond. Overall, the data show that it is the loss of the glutamic acid that is critical rather than the gain of an unfavorable amino acid. This supports the idea that the P5 Glu is necessary for stabilizing the canonical $\beta$-strand conformation of the reactive center loop through interactions with the basic pocket (see Introduction) and that loss of these interactions alters the conformation of the loop to one that is no longer optimal for interaction with the proteinase and for loop insertion.

The results of SDS-polyacrylamide gel electrophoresis analysis of the reactions were consistent with the kinetic and SI data (Fig. 2). A shows the reaction with HNE. With wild-type $\alpha_1$-PI and P5K-1-PI bands of complex are clearly seen. In the case of P5G-1-PI and P5Q-1-PI most of the complex is in a cleaved form. Cleavage of complex by free proteinase is a normal phenomenon (see below and Fig. 1). The reason this can be seen is that for P5G-1-PI and P5Q-1-PI the SI values for the reaction are 3.8 and 4.2, respectively, hence the proteinase remains active for longer and can cleave the complex. In support of this, active proteinase can be seen in lanes 7 and 8. This is not a problem for the kinetic assays which are carried out under reducing conditions and stained with Coomassie Blue. B: reaction of $\alpha_1$-PI variants with trypsin: lanes 1–4, 2 $\mu$g (2.25 $\mu$M) of wild-type $\alpha_1$-PI, P5G-1-PI, P5Q-1-PI, P5K-1-PI; lane 5, 4.7 $\mu$g (8 $\mu$M) of HNE, lanes 6–9, 8 $\mu$g (10 $\mu$M) of wild-type $\alpha_1$-PI, P5G-1-PI, P5Q-1-PI, and P5K-1-PI incubated for 10 min at 25 °C with 1.25 $\mu$g (2.25 $\mu$M) of HNE in 0.1 M Hepes, pH 8.0, 0.5 M NaCl and 0.1% PEG 8000; lane 10, molecular mass markers (97.4, 66.4, 31 kDa). Cx stands for complex, CxC stands for cleaved complex, U stands for uncleaved $\alpha_1$-PI, and C stands for cleaved $\alpha_1$-PI. After incubation, the samples were boiled for 5 min and run on a 12% SDS-polyacrylamide gel under reducing conditions and stained with Coomassie Blue. B: reaction of $\alpha_1$-PI variants with trypsin: lanes 1–4, 2 $\mu$g (2.25 $\mu$M) of wild-type $\alpha_1$-PI, P5G-1-PI, P5Q-1-PI, and P5K-1-PI incubated for 15 min at 25 °C with 1.05 $\mu$g (2.25 $\mu$M) of trypsin in 0.1 M Hepes, pH 7.4, 0.1 M NaCl, 10 mm CaCl2, and 0.1% PEG 8000; lane 10, molecular mass markers. After incubation, the samples were boiled for 5 min and run on a 12% SDS-polyacrylamide gel under reducing conditions and stained with Coomassie Blue. Note that in both gels the amount of $\alpha_1$-PI indicated is total protein. This includes some inactive polymer as indicated under “Experimental Procedures.”

### RESULTS AND DISCUSSION

#### Activity of $\alpha_1$-Proteinase Inhibitor P2 Mutants

| Proteinase | $\alpha_1$-PI variants | $k_{\text{app}}$ | SI | $k'_{\text{app}}$ | Relative values |
|------------|------------------------|-----------------|----|----------------|----------------|
| HNE        | Wild-type              | $9.78 \times 10^8$ | 1.1 | $1.08 \times 10^7$ | 100            |
|            | P5G-$\alpha_1$-PI      | $1.18 \times 10^8$ | 3.5 | $4.47 \times 10^6$ | 41.4           |
|            | P5Q-$\alpha_1$-PI      | $1.51 \times 10^8$ | 4.2 | $6.41 \times 10^6$ | 59.3           |
|            | P5K-$\alpha_1$-PI      | $2.74 \times 10^8$ | 2.0 | $5.40 \times 10^6$ | 50             |
| Trypsin    | Wild-type              | $1.84 \times 10^9$ | 1.0 | $1.86 \times 10^6$ | 100            |
|            | P5G-$\alpha_1$-PI      | $3.43 \times 10^8$ | 2.1 | $7.24 \times 10^4$ | 38.9           |
|            | P5Q-$\alpha_1$-PI      | $3.28 \times 10^8$ | 2.7 | $8.82 \times 10^4$ | 45.5           |
|            | P5K-$\alpha_1$-PI      | $3.37 \times 10^8$ | 9.9 | ND              | ND             |

*For this mutant, the analysis of $k_{\text{app}}$ was not performed since trypsin cleaves at a site other than P1-P2 bond (i.e. preferentially at P2-P3 bond), thus it is not possible to separate the inactivation of the serpin from the inactivation by cleavage at P1-P2 bond.

#### Table I

The SI values and apparent second order rate constants ($k_{\text{app}}$ and $k'_{\text{app}}$) for inhibition of trypsin and HNE by $\alpha_1$-PI and $\alpha_1$-PI-P5 variants. Conditions of assays are described under “Experimental Procedures.”
Activity of α₁-Proteinase Inhibitor P₅ Mutants

The Role of the P₅ Residue in Proteinase Inhibition and Complex Stability—The P₅ Glu makes important structural contacts in both the intact β-strand conformation and in the cleaved form of α₁-PI (5, 17). Therefore, it was likely that mutating this residue would lead to changes in the inhibitory properties of the serpin. From the similar decrease in $k_{app}$ and $k'_{app}$ for all three variants with both proteinases it can be concluded that removal of the glutamic acid (rather than addition of positive charge in the case of the lysine variant) reduced the rate at which trypsin and HNE interacted with the serpin. With this residue changed, the interactions that stabilize the canonical conformation of the loop can no longer occur, hence the loop probably adopts a different conformation, such as a helical one, as this might now be the most stable conformation for the loop to adopt in this context (18).

This conformation is no longer optimal for binding to the proteinase, hence $k'_{app}$ is reduced. The results are in agreement with the predictions made from the native structure of α₁-PI describing the loop in a canonical conformation (5). According to Fig. 1, the serpin and the enzyme first form a reversible Michaelis complex and then a reaction takes place to give the intermediate [EI]. This latter step involves the formation of optimal subsite interactions between the serpin and the enzyme, such as through the P₁’ and S₁’ subsites (19), as well as the initiation of loop insertion (20, 21). Thus, by changing the conformation of the reactive center loop by loss of the glutamic acid at P₅, both steps might be reduced. The increase in SI represents a reduction in the rate of loop insertion into β-sheet A. The basic pocket surrounding P₅ in the native molecule is critical for stabilizing the canonical structure of the loop with the hinge region ready to insert as strand 4 of β-sheet A. Part of the driving force for loop insertion might come from the disruption of this basic pocket, which occurs during the serpin conformational change (5). In the case of the variants this driving force would be lost as the interaction with the basic pocket probably does not occur. Presumably then, the rate of loop insertion would be slowed down enough to have an influence on the SI.

One of the unresolved issues for serpins is the structure of the serpin-proteinase complex. Although it is likely that full, or at least partial, loop insertion accompanies complex formation (12, 22, 23), the interactions stabilizing the complex are unknown. The active site of the proteinase in the complex is disrupted such that it cannot catalyze the deacylation reaction (24). It is also possible that in the complex deacylation might be prevented by exclusion of the water, which acts as a nucleophile during this step, from the active site of the proteinase. In addition, there are conformational changes away from the active site that make the proteinase susceptible to cleavage by free proteinase (25, 26). This cleavage probably fully inactivates the proteinase thereby preventing even residual catalyzed deacylation from taking place and preventing further complex dissociation (27). The results on the dissociation of the complexes formed with trypsin (Fig. 3) are entirely consistent with these studies. The plateau reached in the reactivation is explained by the released proteinase cleaving the proteinase in the complex to prevent further dissociation. The complexes with all three P₅ mutants showed regeneration at a faster rate, and from an earlier time, than with wild-type, consistent with our hypothesis that the P₅ residue is involved in the stabilization of the complex with trypsin.

The interactions that take place at the interface between the serpin and the proteinase are very critical to the stability of the complex. The area of contact between enzyme and inhibitor in complexes of known structure (such as trypsin-soybean trypsin inhibitor and elastase-ovomucoid) is about 700 square Å on each protein. This contact area involves about 10–15 residues in the inhibitor and about 17–29 residues in the proteinase (28). Of the amino acids in this interface region, in general 55% are nonpolar, 25% are polar, and 20% are charged. The structure of a serpin-enzyme complex is not known, but it might be expected that similar values will apply. Therefore, changing only one residue in the serpin...
is unlikely to have a profound effect on complex stability, but nevertheless if, as we predicted, the P$_5$ is an important residue any effect might be detectable. This was the case for complexes with trypsin. These results show that the P$_5$ residue makes important structural contributions to the stability of complexes.

In conclusion, the data we report here can be interpreted in terms of the structural information available for $\alpha$-PI, which describes the $\beta$-strand conformation of the reactive center loop (5). First, changing the P$_5$ residue affected both the interaction with the proteinase and subsequent loop insertion, through disruption of the canonical conformation of the loop in the native state and loss of one of the driving forces for loop insertion. Second, the reduced stability of complexes with trypsin is consistent with the proposal that the P$_5$ residue makes important intramolecular contacts that help to stabilize the loop-inserted form or intermolecular contacts with the proteinase that assist in the distortion of the catalytic triad. With no currently available structure of a serpin-proteinase complex, future studies with reactive center loop mutants will help our understanding of the mechanism of complex stability.

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