Effects of Mutations in the Hinge Region of Serpins†

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ABSTRACT: An expression system for α_1-antitrypsin in *Escherichia coli* was developed using a T7 RNA polymerase promoter. Addition of rifampicin to inhibit the *E. coli* RNA polymerase after induction of the T7 RNA polymerase gene resulted in about 30% of newly synthesized protein being α_1-antitrypsin. This expression system was then used to examine the effect of mutations in the hinge region of α_1-antitrypsin on its activity. The mutations were based on ones in antithrombin III that had previously been shown to have adverse effects on activity. Mutation of Ala^{347} to threonine in α_1-antitrypsin did not affect the kinetic behavior of the protein with trypsin or human leukocyte elastase. In contrast, mutation of Gly^{349} to proline converted the majority of the protein into a substrate for both proteinases. The small fraction of this mutant that was active, however, had kinetic parameters that were indistinguishable from wild-type α_1-antitrypsin. Cleavage within the reactive-site loop of wild-type α_1-antitrypsin causes a conformational change in the molecules (the *S*-to-*R* transition) and results in a marked increase in heat stability. This increase in heat stability was also seen upon cleavage within the reactive-site loops of both of the α_1-antitrypsin mutants. The results are discussed in terms of a kinetic mechanism for serpin–proteinase interactions, in which after the formation of an initial complex the serpin partitions between the formation of a stable complex and a cleavage reaction. The hinge region of the reactive-site loop of serpins would appear to be important for the partitioning in favor of the stable complex.

The serpins are a family of serine proteinase inhibitors that share the same overall tertiary structure, consisting of three β-sheets surrounded by eight α-helices (Stein et al., 1990; Wright et al., 1990; Loebermann et al., 1984; Mottonen et al., 1992; Baumann et al., 1991). This shared structure is also maintained in ovalbumin, one of the several members of the family with no known inhibitory function.

The region of a serpin that binds to the active site of its target proteinase is an exposed loop that has been called the reactive-site loop. The reactive-site loops of small proteinase inhibitors, such as those of the Kunitz family, are held in a tightly constrained conformation by interactions with the body of the inhibitor. The tertiary structure of this loop is conserved between inhibitors from different families, and this structure has been called the canonical form. The conformation of the reactive-site loop of small inhibitors is essentially the same in the free and bound inhibitor, and it is thought that the stabilization of its conformation by interactions with the body of the inhibitor is important for the formation of the tight complex with the cognate proteinase (Bode & Huber, 1991). In contrast to the tightly constrained loop of the small inhibitors, the serpins have mobile reactive-site loops which have the ability to move in and out of the A β-sheet (Bode & Huber, 1992; Carrell & Evans, 1992). It has been proposed that this mobility is critical to serpin function in that it allows the insertion of the loop into the A β-sheet, which results in a smaller, more tightly constrained loop that could adopt the canonical form similar to that of the reactive-site loop of the Kunitz inhibitors (Bock, 1990; Bode & Huber, 1992; Carrell et al., 1991).

As with other inhibitors, the residue that binds at the primary specificity pocket of the target proteinase is denoted the P_1 residue (Schechter & Berger, 1967). The reactive-site loop of serpins extends from P_{15} to $P_{5'}$, and alignment of this region of serpins indicates no discernible sequence conservation for the majority of the residues except for a region between the P_9 and P_{15} residues. The P_9–P_{12} residues are usually short-chain amino acids, predominantly alanines; P_{13} is glutamic acid; P_{14} again usually has a short side chain, often serine or threonine; and P_{15} is almost always glycine. In the crystal structure of ovalbumin (Stein et al., 1990), it can be seen that this so-called hinge region (P_9–P_{15}) is where the reactive-site loop turns and joins the A β-sheet as strand 5A. Divergence from this hinge-region consensus is usually associated with a lack of inhibitory activity. It has been proposed that the larger or polar side chains present in this region of noninhibitory serpins prevent the partial insertion of the loop into the sheet, locking the reactive-site loop into the conformation seen in ovalbumin (Stein et al., 1990). Naturally occurring mutations in this region of inhibitory serpins interfere with their activity. Gross divergence from the consensus sequence, such as the P_{10} proline antithrombin III (Perry et al., 1989; Mohlo-Sabatier et al., 1989; Caso et al., 1991) or the P_{12} glutamic acid C1 inhibitor (Skriver et al., 1991), results in a complete loss of inhibitory activity and the emergence of substrate-like behavior toward the cognate proteinase. Mutations to other small amino acids, such as P_{12} threonine or P_{10} serine in antithrombin III (Devraj-Kizuk et al., 1988; Perry & Carrell, 1989; Ireland et al., 1991; Perry et al., 1991), P_{10} threonine in C1 inhibitor (Donaldson et al., 1990), and an alanine insertion in α_1-antiplasmin (Holmes et al., 1987), have a more variable effect, but they are always disruptive to normal activity. Thus, this region is seen to have a critical role in the function of the serpins as inhibitors.

The conservation of the hinge-region amino acid residues between different members of the serpin family suggests that they play the same role in all members. In order to investigate this hypothesis and to examine the role of the hinge region, two mutations that have been reported for antithrombin III (P_{12} threonine and P_{10} proline) were made in the α_1-antitrypsin, and the properties of the mutant proteins determined.

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MATERIALS AND METHODS

Materials. Restriction enzymes were obtained from New England Biolabs, and oligonucleotides were synthesized by members of the Department of Biochemistry, University of Cambridge. *Escherichia coli* BL21 (DE3) was obtained from Dr. F. W. Studier, Brookhaven National Laboratories, Upton, NY. Rifamipicin was obtained as Rifampin (Merryl Dow). [^{35}S]Methionine was obtained from Amersham (Amersham, U.K.). Trypsin, *Staphylococcus aureus* V8 protease, *p*-nitrophenyl guanidinobenzoate, *N*-methoxysuccinyl-Ala-Ala-Pro-Val *p*-nitroanilide, and polyclonal rabbit antibodies against human α_1-antitrypsin were obtained from Sigma (St. Louis, MO). D-Pro-Phe-Arg *p*-nitroanilide (S-2302) was obtained from Kabi-Pharmacia (Uppsala, Sweden). Human leukocyte elastase (HLE)[1] was a gift from Dr. P. M. George (Department of Pathology, Christchurch Hospital, New Zealand). Other reagents were of the highest grade available commercially.

Construction of the α_1-Antitrypsin Expression Vector and Site-Directed Mutagenesis. The α_1-antitrypsin cDNA was inserted as a *Bam*HI–*Pst*I fragment (Avron et al., 1991) into pTZ18R (Pharmacia). A synthetic *Eco*RI–*Bam*HI portable translation initiation site (Pharmacia) was then cloned in front of the α_1-antitrypsin gene to give pATH, wherein the α_1-antitrypsin gene was placed under the control of a T7 RNA polymerase promoter (Studier & Moffat, 1986). This was then further modified (Crowther, 1992) by placing a transcriptional terminator and a pSC101 par locus downstream of the α_1-antitrypsin gene to give pTermat.

Site-directed mutagenesis was performed on the α_1-antitrypsin cDNA cloned into M13mp9 by the method of Kunkel (1985). Mutant α_1-antitrypsin genes were identified by DNA sequencing using the method of Sanger et al. (1977) and the Sequenase reagents (USB, Cleveland, OH) and subcloned into pTermat as *Bst*XI–*Ava*I fragments.

In Vivo Radiolabeling of Proteins. E. coli strain BL21 (DE3) (Studier & Moffat, 1986) was transformed with pTermat. A 4-mL aliquot of exponentially growing culture in 2xTY was harvested and resuspended in 4 mL of prewarmed M9 medium (Sambrook et al., 1989). A 50-μL sample was removed at zero time, and then 40 μL of 1 M IPTG was added to the culture to induce the expression of the T7 RNA polymerase gene. This and subsequent samples of 50 μL of this culture were removed to prewarmed sterile 1.5-mL Eppendorf tubes containing 5 μL of 5 μCi/mL [^{35}S]methionine in M9 medium. The samples were incubated for 5 min at 37 °C, after which the incubation was stopped by the addition of reducing SDS-PAGE loading buffer (Laemmli, 1970) and heating to 95 °C for 5 min. After 30 min, a further 50-μL sample was removed and rifampicin dissolved dimethyl sulfoxide was added to the remainder to a final concentration of 100 μg/mL. At 30-min intervals thereafter, further samples were removed for labeling. For the pulse–chase labeling experiment, 250 μL of culture was removed after the addition of rifamipicin, 25 μL of 5 μCi/mL [^{35}S]methionine in M9 medium was added, and 5 min later the first sample was removed and treated as above, after which 12.5 μL of unlabeled 10 mM L-methionine was added. At 30-min intervals thereafter, 50-μL samples were removed and treated as above. All samples were then subjected to SDS-PAGE on a 10% acrylamide gel (Laemmli, 1970). After the samples were run; the gels were stained with Coomassie Blue R250, dried, and autoradiographed. The autoradiographs were scanned using a Microtek Greyscale Scanner.

Expression of Wild-Type and Mutant α_1-Antitrypsin. E. coli strain BL21 (DE3) transformed with pTermat was grown in a 20-L fermenter (LSL Biolafitte S.A., St-Germain-en-Laye, France) containing 10 L of medium. The medium used contained, per L, 11.25 g of K_2HPO_4, 1.25 g of KH_2PO_4, 1.25 g of sodium citrate, 2.5 g of $(NH_4)_2SO_4$, 12.5 g of casamino acids, and 6.25 g of yeast extract. This medium was autoclaved *in situ* and adjusted to pH 7.0 with NaOH. A 500-mL fresh overnight culture was used as an inoculum. During the inoculation, the following were added: 20 mL of 1 M $MgCl_2$, 20 mL of 50% glucose, and 10 mL of 0.1 g/mL ampicillin. The growth and production phases were performed at 37 °C. Growth was monitored by both OD_{600} and % saturated oxygen. When the culture has reached an OD_{600} of 4.0, the fermentation was fed with a solution of 25% glucose, 10% yeast extract, and 10% casamino acids at 2.0 mL/min. The T7 RNA polymerase gene resident in the DE3 lambda lysogen was induced when the OD_{600} had reached 10.0 by adding 1.0 g of IPTG. After 30 further min of growth, the *E. coli* RNA polymerase was selectively inhibited by the addition of 10 mL of 0.1 g/mL rifamipicin dissolved in dimethyl sulfoxide. The production phase was allowed to proceed for a further 3 h, after which the cells were harvested by repeated centrifugation "pellet-on-pellet" at 6000*g* for 20 min. The cell paste thus obtained was stored at −70 °C until purification was carried out. To confirm the presence of the desired mutation, plasmid DNA was purified from 4 g of this cell paste by alkaline lysis and CsCl density-gradient centrifugation (Sambrook et al., 1989) and sequenced.

Purification of Recombinant α_1-Antitrypsins. All purification steps were carried out at 4 °C. The purification protocol used was adapted from that of Bischoff et al. (1991). The frozen cells were thawed in an equal quantity (w/v) of 300 mM NaCl, 50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 1 mM PMSF, and 1 mM β-mercaptoethanol and disrupted by two passages through a French press (Aminco, Urbana, IL). Poly(ethylene glycol) (M_r 8000) was added to the lysate to a final concentration of 8% (w/v), and after the mixture was stirred for 1 h, the cell debris was removed by centrifugation at 10 000*g* for 1 h. Further poly(ethylene) glycol (M_r 8000) was added to the supernate to a final concentration of 28% (w/v), and after the mixture was stirred for 1 h, a second centrifugation was performed. The pellet was dissolved in 50 mL of 20 mM sodium phosphate, pH 6.8, containing 5 mM EDTA, 1 mM PMSF, and 1 mM β-mercaptoethanol. This solution was centrifuged to remove undissolved material and filtered through a 0.2-μm filter before being loaded onto a 2.6-cm × 40-cm DEAE-Sephacel column equilibrated with 20 mM sodium phosphate, pH 6.8, and 1 mM β-mercaptoethanol. The α_1-antitrypsin was eluted with a 1-L linear gradient of 0–250 mM NaCl in the equilibration buffer. The eluted fractions were analyzed by rocket immunoelectrophoresis using polyclonal anti-α_1-antitrypsin antibodies. Those fractions richest in α_1-antitrypsin were pooled and loaded onto a 1.6-cm × 15-cm column of Zn^{2+}-charged chelating Sepharose equilibrated in 20 mM Tris-HCl, pH 8.0, and 150 mM NaCl. The α_1-antitrypsin was eluted with a linear gradient of 0–75 mM glycine in the equilibration buffer. Fractions which contained α_1-antitrypsin, as determined by rocket immunoelectrophoresis, were analyzed by SDS-PAGE on a 10% acrylamide gel (Laemmli, 1970). Those fractions that were over 95% pure were pooled; poly(ethylene glycol) (M_r 6000)

[1] Abbreviations: HLE, human leukocyte elastase; IPTG, isopropyl thiogalactoside; PMSF, phenylmethanesulfonyl fluoride; P_{12}Thr, α_1-antitrypsin with a threonine substituted for Ala^{347} (position P_{12}); P_{10}Pro, α_1-antitrypsin with a proline substituted for Gly^{349} (position P_{10}).

was added to 0.1%, EDTA to 1 mM, and β-mercaptoethanol to 0.5 mM. Aliquots of 1 mL were frozen in liquid nitrogen before being stored at −70 °C.

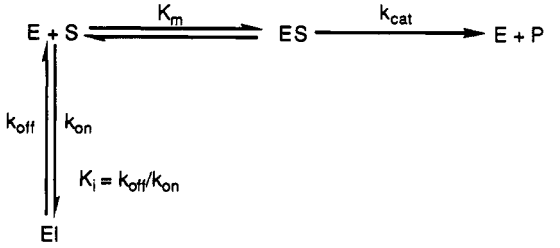
Heat Stability Assay. The heat stabilities of native and cleaved α_1-antitrypsin variants were assayed by heating the samples at different temperatures between 25 and 100 °C for 2 h in 75 mM Tris, 75 mM glycine, and 75 mM NaH_2PO_4 buffer, pH 8.0, followed by centrifugation for 20 min at 10 000*g* (Stein et al., 1989). Residual soluble protein was assayed by rocket immunoelectrophoresis. *S. aureus* V8 protease was used to cleave the mutant proteins between the P_4 and P_5 residues of the reactive-site loop (Mast et al., 1992). A change in migration of the protein in SDS-PAGE on 10% acrylamide gels confirmed the cleavage.

Assays for Cleavage of α_1-Antitrypsin Variants by Trypsin and HLE. The α_1-antitrypsins were incubated in assay buffer with HLE and trypsin at molar ratios α_1-antitrypsin:proteinase of between 0.05:1 and 3:1 for trypsin and 0.2:1 and 1:1 for HLE; inhibitor concentrations were based on the activity against trypsin. Samples were incubated at 37 °C for 10 min, and the reactions were stopped by the addition of reducing SDS-PAGE loading buffer and heating to 95 °C for 5 min. The reactions were subsequently analyzed by SDS-PAGE on a 10% acrylamide gel (Laemmli, 1970). The site of cleavage of P_{10}Pro by HLE was determined as described by Pemberton et al. (1988). Briefly, P_{10}Pro (0.29 μM; activity against trypsin) was incubated with 0.06 or 0.29 μM HLE for 10 min at 37 °C in a volume of 100 μL. In a control reaction, P_{10}Pro was incubated in the absence of HLE. An aliquot (20 μL) of the sample was analyzed by SDS-PAGE as described above, and 80 μL of 8 M guanidinium hydrochloride was added to the remaining 80 μL. This sample was fractionated by reverse-phase HPLC on a PLRP column (Polymer Laboratories, Shropshire, U.K.) using an acetonitrile gradient in 0.1% trifluoroacetic acid. This chromatographic system has previously been used to isolate the N- and C-terminal fragments of serpins cleaved at the reactive sites (Pemberton et al., 1988). In the present study, the C-terminal fragment eluted with 50% acetonitrile while the larger N-terminal fragment eluted at 64% acetonitrile. The material contained in the absorbance peaks eluting at these positions was collected and subjected to N-terminal sequence analysis (performed by Dr. R. A. Harrison, MRC Immunology, Cambridge, U.K.).

Kinetic Assays. All kinetic measurements were performed at 37 °C in 30 mM sodium phosphate buffer, pH 7.4, containing 160 mM NaCl, 0.1% (w/v) poly(ethylene glycol) (M_r 6000), and 0.1% (v/v) Triton X-100.

Determination of the Active Concentrations of Proteins. Trypsin was titrated using *p*-nitrophenyl guanidinobenzoate as described by Chase and Shaw (1970). The concentrations of active wild-type and mutant α_1-antitrypsins were determined by incubation of aliquots of serpins with 91.8 nM trypsin in assay buffer for 30 min. The residual enzyme activity was measured by adding 0.04 mM D-Pro-Phe-Arg *p*-nitroanilide and monitoring the increase in absorbance at 405 nM. The concentration of active α_1-antitrypsin was determined from linear regression analysis of the decrease in the residual activity with the increase in the amount of serpin added. The concentration of HLE was determined by titrating HLE with wild-type α_1-antitrypsin. The residual HLE activity was determined by adding 0.1 mM *N*-methoxysuccinyl-Ala-Ala-Pro-Val *p*-nitroanilide. The concentrations of mutant α_1-antitrypsins active against HLE were then determined as above.

Scheme I



Determination of Kinetic Parameters by Progress Curve Kinetics. Progress curve kinetic experiments were performed as described previously (Stone & Hofsteenge, 1986). Assays were performed in the assay buffer in the presence of substrate (38–100 μM D-Pro-Phe-Arg *p*-nitroanilide for trypsin and 98–490 μM *N*-methoxysuccinyl-Ala-Ala-Pro-Val *p*-nitroanilide for HLE). Each progress curve experiment consisted of at least seven progress curves: one in the absence of inhibitor and six others with different inhibitor concentrations. The concentrations of trypsin and HLE were respectively 8.4–230 and 89–153 pM. The assays were started by the addition of the enzyme. The increase in the concentration of *p*-nitroaniline released by cleavage of substrate was monitored by the increase in the absorbance at 400–410 nm with a Hewlett-Packard 8452A diode array spectrophotometer.

The inhibitory mechanism for each of the serpins investigated is well described by Scheme I, where E, I, S, and P represent the enzyme, inhibitor, *p*-nitroanilide substrate, and product (*p*-nitroaniline), respectively. The association rate constant (k_{on}) and dissociation rate constant (k_{off}) are related to the inhibition (dissociation) constant (K_i) for the complex by the relationship given in Scheme I. Progress curve data were fitted to the equation that describes this mechanism by nonlinear regression (Stone & Hofsteenge, 1986) to yield values for the apparent association rate constant ($k_{on'}$) and apparent inhibition constant ($K_{i'}$). True values of these constants were calculated by correcting for the concentrations of substrate used in the experiments by using the following expressions (Morrison & Walsh, 1988):

$$K_i = K_{i'}/(1 + [S]K_m)$$

$$k_{on} = k_{on}(1 + [S]/K_m)$$

The values of K_m required for these calculations were determined by standard initial velocity studies (Hofsteenge et al., 1986). Estimates of 19.4 ± 1.2 and 85.5 ± 2.5 μM were obtained for the K_m values of D-Pro-Phe-Arg *p*-nitroanilide with trypsin and *N*-methoxysuccinyl-Ala-Ala-Pro-Val *p*-nitroanilide with HLE, respectively. For the interaction of wild-type and mutant α_1-antitrypsins with HLE, the value of $K_{i'}$ was too low to be determined. The estimates of $K_{i'}$ obtained were either negative or had large standard errors. Consequently, $K_{i'}$ was set to 0 in these analyses, and only an estimate of k_{on} was obtained. The reversibility of the reactions of the complexes with trypsin was demonstrated by preincubating trypsin (0.2 nM) and α_1-antitrypsin (36 nM) for 20 min in the absence of substrate. The assay was then started by the addition of substrate (100 μM). An increase in the velocity of substrate cleavage was observed, and the steady-state velocity achieved after 30 min was not significantly different from that observed when the reaction was started by the enzyme; this result is expected for a reversible, slow-binding inhibitor (Morrison & Walsh, 1988). In a similar experiment, 90 pM HLE was preincubated with 480 pM α_1-antitrypsin in the absence of substrate for 45 min. After the addition of substrate (200 μM), neglible HLE activity could be detected,

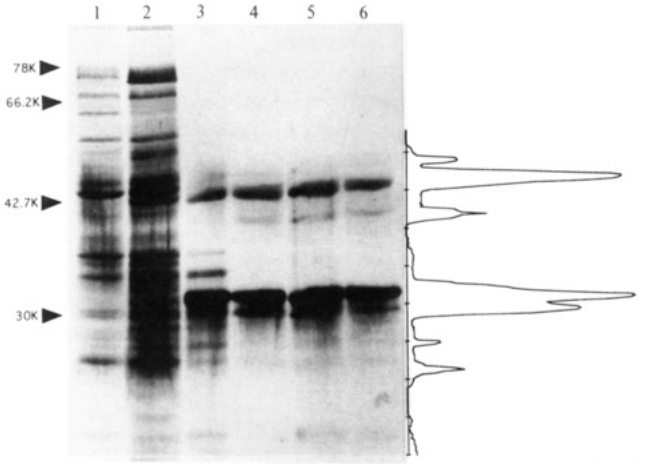


FIGURE 1: Autoradiograph and densitometry scan of *in vivo* labeled proteins. Exponentially growing BL21(DE3) pTermat (50 μL) was labeled with 25 nCi of [^{35}S]methionine for 5 min. Lane 1, prior to induction with IPTG; lane 2, 30 min after induction with IPTG; lanes 3, 4, 5, and 6, 30, 60, 90, and 120 min after addition of 100 μg/mL rifampicin, respectively. The upper of the three dominant bands in lanes 3–6 corresponds to α_1-antitrypsin.

which confirmed that the HLE–α_1-antitrypsin complex was essentially irreversible under the conditions of the assay.

RESULTS

Expression and Purification of Recombinant α_1-Antitrypsin. Variants of α_1-antitrypsin were expressed as intracellular proteins in *E. coli* using a T7 promoter-based expression system enhanced by the use of rifampicin. Without the use of rifampicin, the vector gave very low levels of α_1-antitrypsin production, typically 0.05% of soluble cell protein. Rifampicin inhibits *E. coli* RNA polymerase but not T7 RNA polymerase (Gurgo, 1980), with the result that the cellular metabolism is entirely funneled toward producing proteins encoded on the plasmid that are transcribed by the T7 RNA polymerase. The effectiveness of this strategy for the expression of recombinant α_1-antitrypsin is demonstrated in Figure 1, which shows the metabolic labeling of newly synthesized proteins after induction of the T7 RNA polymerase by IPTG and inhibition of the *E. coli* RNA polymerase by rifampicin. Around 30% of the newly synthesized protein after induction and rifampicin addition was α_1-antitrypsin, as measured by scanning densitometry. Moreover, these levels of expression were obtained without significant formation of inclusion bodies. Pulse–chase labeling experiments failed to show any significant degradation of the protein for 3 h following induction and rifampicin addition (data not shown). Induction and rifampicin addition also resulted in high levels of a protein with a molecular mass of about 30 kDa (Figure 1). This protein was assumed to be β-lactamase; a protein of similar molecular mass was also observed by Studier and Moffat (1986). The DNA encoding α_1-antitrypsin was cloned in the same direction as the β-lactamase open reading frame, such that transcription beyond the α_1-antitrypsin gene would give rise to mRNA encoding for β-lactamase. On scale-up of the procedure to a 10-L fermentation, the level of expression of α_1-antitrypsin was approximately 2% of total soluble protein.

Purification of the proteins was based on the protocol of Bischoff et al. (1991), incorporating poly(ethylene glycol) fractionation as an alternative to diafiltration, followed by ion-exchange and immobilized metal affinity chromatography. The purity of the final preparation exceeded 95%, as determined by densitometric scanning of a SDS-PAGE gel stained with Coomassie Blue R250. Overall yields were usually about 55%, giving typically 0.8 mg of purified α_1-antitrypsin per gram of wet cell paste.

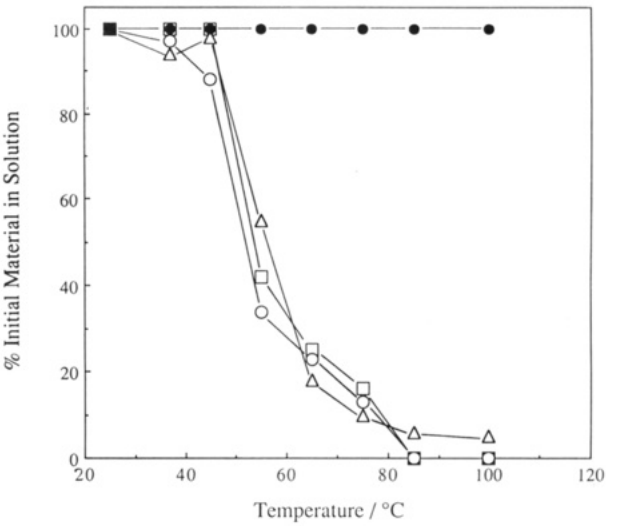


FIGURE 2: *S*-to-*R* transition assay of recombinant proteins. The proteins, either uncleaved or cleaved with *S. aureus* V8 protease, were heated at a range of temperatures for 2 h and assayed by measuring residual soluble protein by rocket immunoelectrophoresis after centrifugation. All cleaved proteins gave the same profile, represented by ●. Uncleaved proteins are presented by the following symbols: wild-type, □, P_{10}Pro, △; P_{12}Thr, ○.

Characterization of the Purified Proteins. Using the above expression system, wild-type recombinant α_1-antitrypsin and the mutants Ala^{347}→Thr and Gly^{349}→Pro were produced. Ala^{347} and Gly^{349} are found in the P_{12} and P_{10} positions, respectively, and thus the mutants are referred to as P_{12}Thr and P_{10}Pro. Double-stranded DNA sequencing of the DNA extracted from the same culture as that from which the proteins were purified confirmed the existence of the desired mutation in each case. Amino acid sequencing of α_1-antitrypsin yielded the sequence Xaa-Asp-Pro-Gln-Gly, which is the expected N-terminus of the recombinant protein. The initial yield was, however, lower than expected (ca. 15%), which suggests that the N-terminal formyl group had not been efficiently removed. Amino acid analysis of the purified proteins gave the expected composition. The concentration of protein determined by amino acid analysis was used to standardize rocket heights in immunoelectrophoresis, and this permitted rapid and accurate determination of α_1-antitrypsin concentrations during the purification process. Isoelectric focusing (results not shown) revealed the same microheterogeneity as that reported by Bischoff et al. (1991). It is interesting to note that microheterogeneity is also noted in α_1-antitrypsin purified from plasma, where it has been primarily attributed to glycosylation variations (Crawford, 1973; Jeppsson et al., 1985; Nowiki & Freier, 1990).

S-to-R Transition. Upon cleavage within the reactive-site loop of inhibitory serpins, the proximal portion of this loop inserts into the A β-sheet to form a 6-stranded antiparallel β-sheet, and a dramatic increase in heat stability is observed. This conversion to a more heat stable form upon cleavage has been termed the *S*-to-*R* transition (Carrell & Owen, 1985); absence of this change is only seen amongst the noninhibitory serpins (Stein et al., 1989). As expected, recombinant wild-type α_1-antitrypsin was found to undergo this transition (Figure 2). In addition, both α_1-antitrypsin mutants (P_{10}Pro and P_{12}-Thr) demonstrated the ability to undergo the *S*-to-*R* transition (Figure 2), indicating that the overll structural transformation was not prevented by either mutation in α_1-antitrypsin.

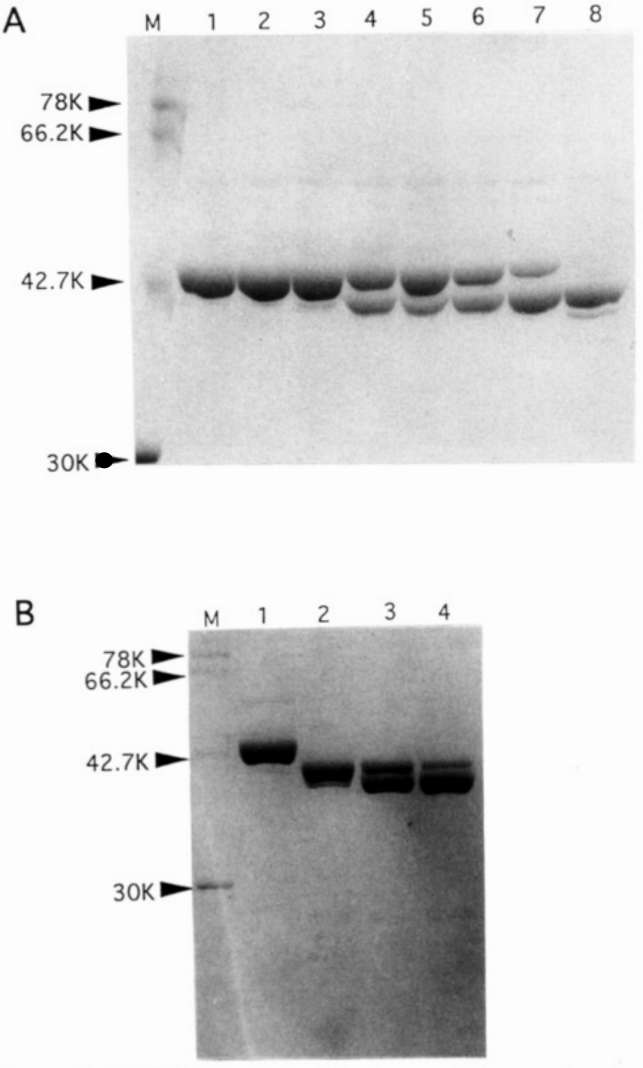


FIGURE 3: SDS-PAGE analysis of the cleavage of the α_1-antitrypsin mutant P_{10}Pro by trypsin and human leukocyte elastase. (A) P_{10}Pro (0.54 μM in 100 μL of kinetics buffer) was incubated at 37 °C for 10 min with increasing concentrations of trypsin. The concentrations of trypsin were 0.0, 0.03, 0.14, 0.29, 0.57, 0.86, 1.15, and 1.72 μM for lanes 1–8, respectively. SDS-PAGE was performed as described in Materials and Methods. P_{10}Pro concentrations are based on its activity against trypsin. (B) P_{10}Pro (0.29 μM in 50 μL of kinetics buffer) was incubated at 37 °C for 10 min with increasing concentrations of HLE. The concentrations of HLE were 0.0, 0.06, 0.17, and 0.29 μM for lanes 1–4, respectively. P_{10}Pro concentrations are based on its activity against trypsin.

Inhibitory Properties of the α_1-Antitrypsin Variants. The specific activity of wild-type and mutant proteins were determined by titration against trypsin and HLE; the total concentrations of the proteins were evaluated by amino acid analysis. Titration of the recombinant wild-type α_1-antitrypsin against trypsin indicated that 85% of the inhibitor was active; a similar specific activity (75%) was found for α_1-antitrypsin purified from plasma. The mutant P_{12}Thr displayed a comparable specific activity of 65%. In contrast, the mutant P_{10}Pro was found to be only 15% active. The low specific activity of P_{10}Pro was due to cleavage of the inhibitor by trypsin. Figure 3 shows that incubation of P_{10}Pro with trypsin (or HLE, see below) resulted in the appearance of two new bands. At lower concentrations of trypsin (Figure 3A, lanes 4 and 5), the apparent molecular mass of P_{10}Pro decreased by about 3 kDa, which is consistent with cleavage of the inhibitor at the reactive center (Figure 3A); the decrease was about the same as that observed when the reactive-site loop of the inhibitor was cleaved between P_4 and P_5 by *S. aureus* V8 protease (data not shown). The material shown in lanes 4 and 5 of Figure 3A underwent the *S*-to-*R* transition as determined by its heat stability (data not shown), which confirmed that the cleavage had occurred in the reactive-site loop. At higher concentrations of trypsin, a second cleavage occurred that led to a further reduction of about 3 kDa in the molecular mass of P_{10}Pro (Figure 3A).

Titration with wild-type α_1-antitrypsin was used to estimate the concentration of HLE, and the mutants were then titrated against this proteinase. The mutant P_{12}Thr displayed a specific activity with HLE that was indistinguishable from that of wild-type α_1-antitrypsin. In contrast, titration of HLE with P_{10}Pro indicated that only 4% of the molecules that inhibited trypsin were able to inhibit HLE. Thus, the specificity activity of this mutant with HLE was less than 1%. Analysis of the interaction of P_{10}Pro with HLE by SDS-PAGE indicated that the lower activity of this mutant with HLE was again due to cleavage of the inhibitor (Figure 3B). As was observed with P_{10}Pro and trypsin, further degradation of the inhibitor occurred at higher concentrations of HLE (Figure 3B). The sites of cleavage of P_{10}Pro by HLE were determined by fractionation of the cleaved material with reverse-phase HPLC, followed by N-terminal sequence analysis of the resultant peaks. In the absence of HLE, only a single peak was obtained. After treatment of with HLE, two peaks were obtained. At the low concentrations of HLE (0.06 μM HLE:0.29 μM P_{10}-Pro (activity against trypsin), Figure 3B, lane 2), the first peak yielded the sequence Ser-Ile-Pro-Pro-Glu, which is the sequence from P'_1 to P'_5 of α_1-antitrypsin, and this confirmed that cleavage of P_{10}Pro by HLE occurred at the reactive-site bond. The sequence of the material in the second peak corresponded to the N-terminus of α_1-antitrypsin (Xaa-Asp-Pro-Gln-Gly). With a higher concentration of HLE (0.29 μM HLE:0.29 μM P_{10}Pro, Figure 3B, lane 4), the sequence of the first peak again coincided with that of P'_1–P'_5 of α_1-antitrypsin. However, two major sequences were obtained from the second peak; the first sequence was that of the N-terminus of α_1-antitrypsin and the second was Asp-Thr-Ser-His-His. The second sequence was that of residues 12–16 of α_1-antitrypsin and, thus it seems likely that cleavage of P_{10}Pro between Thr^{11} and Asp^{12} was the secondary cleavage observed by SDS-PAGE (Figure 3B).

The kinetic parameters for the inhibition of both HLE and trypsin by the recombinant α_1-antitrypsins were determined by progress curve kinetics; representative data sets are shown in Figures 4 and 5. The kinetic parameters for the inhibition of both trypsin and HLE by recombinant wild-type α_1-antitrypsin were similar to those previously observed for both natural and recombinant proteins (Avron et al., 1991; Rosenberg et al., 1984; Beatty et al., 1980). The association rate constant (k_{on}) for wild-type α_1-antitrypsin with trypsin was 2.2×10^5 M^{-1} s^{-1}, and the dissociation constant (K_i) of the α_1-antitrypsin–trypsin complex was 0.63 nM (Table I). The value of k_{on} for wild-type α_1-antitrypsin with HLE was 1.2×10^7 M^{-1} s^{-1} (Table I), but the K_i for the α_1-antitrypsin–HLE complex was too low to be reliably determined; considering the concentrations of inhibitor used (20–200 pM), an upper limit of 1 pM can be assigned to K_i. The data obtained for the inhibition of HLE by the mutant P_{12}Thr are shown in Figure 4; the kinetic constants for the inhibition of HLE remained essentially unchanged from those with the wild-type α_1-antitrypsin: k_{on} was 1×10^7 M^{-1} s^{-1} and K_i was again less than 1 pM (Table I). The values of kinetic constants for the inhibition of trypsin by this mutant were also similar to those obtained with wild-type α_1-antitrypsin: estimates of

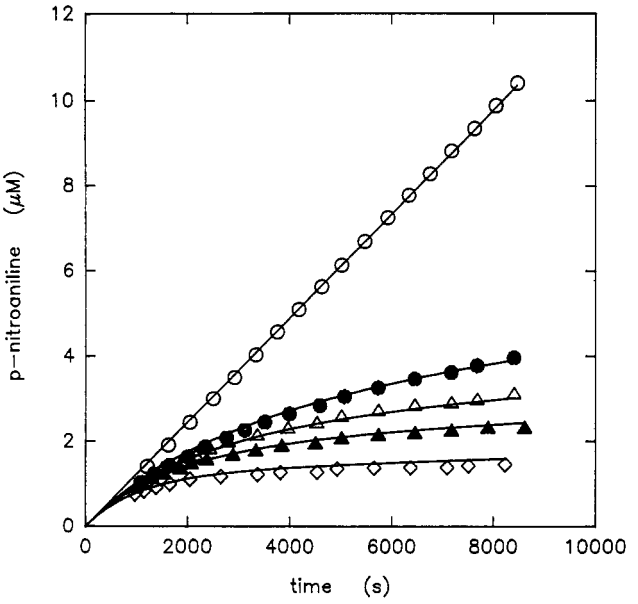


FIGURE 4: Progress curves for inhibition of HLE by the α_1-antitrypsin mutant P_{12}Thr. Assays were performed as described in Materials and Methods with a concentration of 490 μM *N*-methyoxysuccinyl-Ala-Ala-Pro-Val *p*-nitroanilide. The concentration of HLE was 89 pM. The assays contained the following concentrations of P_{12}Thr: 0, ○; 192, ●; 256, △; 320, ▲; and 480 pM, ◇. Nonlinear regression was used to fit the data to the equation describing the mechanism presented in Scheme I, and the lines drawn show the fit of the data to this equation. Data points at times less than 1000 s are not shown, and only every other point thereafter is displayed. Progress curves obtained with 160 and 224 pM P_{12}Thr that were used in the fitting of the data are not shown.

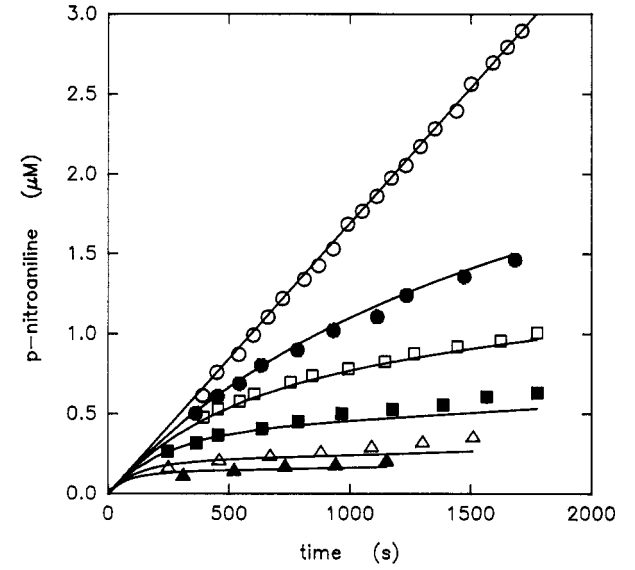


FIGURE 5: Progress curves for inhibition of trypsin by the α_1-antitrypsin mutant P_{10}Pro. Assays were performed as described in Materials and Methods with 40 μM D-Pro-Phe-Arg *p*-nitroanilide, 0.23 nM trypsin, and the following concentrations of P_{10}Pro: 0, ○; 5.85, ●; 11.7, □; 23.4, ■; 46.8, △; and 70.2 nM, ▲. Nonlinear regression was used to fit the data to the equation describing the mechanism presented in Scheme I, and the lines drawn show the fit of the data to this equation. Data points at times less than 300 s are not shown, and only every other point thereafter is displayed. The progress curve obtained with 35.1 nM P_{10}Pro that was used in the fitting of the data is not shown. The concentrations of P_{10}Pro were determined by titration against trypsin.

6.5×10^5 M^{-1} s^{-1} and 0.31 nM were obtained for k_{on} and K_i (Table I). The data for the inhibition of trypsin by P_{10}Pro are shown in Figure 5; analysis of these data yielded estimates for k_{on} and K_i similar to those obtained with wild-type α_1-antitrypsin and P_{12}Thr (5.4×10^5 M^{-1} s^{-1} and 0.44 nM for k_{on} and K_i, respectively, Table I). It should be noted, however, that the estimates of k_{on} and K_i are based on the concentrations of inhibitor active against the enzyme tested. Similarly, for the fraction of P_{10}Pro that inhibited HLE, the value of k_{on} was indistinguishable from that of the wild-type protein (Table I).

Table I: Kinetic Constants for the Reaction of α_1-Antitrypsin Variants with HLE and Trypsin[a]

	k_{on} (M^{-1} s^{-1})	10^4 k_{off} (s^{-1})	K_i (nM)
	HLE		
wild-type	$(1.20 \pm 0.05) \times 10^7$	*b*	*b*
P_{12}Thr	$(0.96 \pm 0.02) \times 10^7$	*b*	*b*
P_{10}Pro	$(0.99 \pm 0.01) \times 10^7$	*b*	*b*
	Trypsin		
wild-type	$(2.21 \pm 0.06) \times 10^5$	1.40 ± 0.09	0.63 ± 0.03
P_{12}Thr	$(6.45 \pm 0.13) \times 10^5$	2.01 ± 0.18	0.31 ± 0.02
P_{10}Pro	$(5.44 \pm 0.15) \times 10^5$	2.41 ± 0.32	0.44 ± 0.05

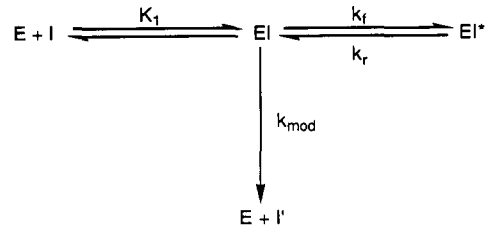
[a] Assays were performed and the data analyzed as described in the Materials and Methods section. The values for the kinetic constants were determined on the basis of the concentration of active inhibitor in the preparation and represent the weighted means of at least two determinations. [b] It was not possible to determine accurate K_i values with HLE; an upper limit of 1 pM could be estimated for the dissociation constants.

DISCUSSION

In the current study, we have used an expression system employing a T7 RNA polymerase promoter to express α_1-antitrypsin in *E. coli*. Initially, low levels of expression were obtained (about 0.05% of soluble cell protein), but the addition of rifampicin, which inhibits *E. coli* RNA polymerase but not T7 RNA polymerase (Chamberlin et al., 1970; Hesselbach & Nakada, 1977), increased expression levels 40-fold (2% of total soluble protein). In addition, these levels of expression were achieved without significant formation of inclusion bodies. Various strategies have been previously used in order to achieve high levels of expression of α_1-antitrypsin in *E. coli*. The protein has been expressed as a fusion protein (Courtney et al., 1984; Courtney et al., 1985; Jallat et al., 1986; Straus et al., 1985), with the 5′ coding and noncoding sequences modified to increase ribosome binding kinetics (Tessier et al., 1986) or with the first five amino-terminal amino acids deleted (Bischoff et al., 1991). When high levels (>1.0% of total cell protein) of expression were achieved, most of the α_1-antitrypsin was found in inclusion bodies (Courtney et al., 1984; Tessier et al., 1986). Thus, the use of rifampicin and the T7 promoter appears to have certain adantages in that high levels of expression of soluble protein were obtained without extensive engineering of the serpin.

The conservation of amino acids with small side chains in the hinge region of all inhibitory serpins strongly suggests a common structure–function relationship for this region among serpins. In this paper, the effects of mutations in α_1-antitrypsin which had already been characterized in antithrombin III, a P_{10}Pro, and a P_{12}Thr have been examined. Both of these mutations have deleterious effects on the activity of antithrombin III. The P_{10}Pro mutation converts antithrombin III from an inhibitor of α-thrombin into a substrate (Caso et al., 1991) and also modifies the *S*-to-*R* transition (unpublished results). The P_{12}Thr mutation also appears to transform antithrombin III into a substrate (Ireland et al., 1991). The effect of these two mutations in α_1-antitrypsin differed from those observed with antithrombin III. Neither of these mutations prevented the *S*-to-*R* transition (Figure 2). The

Scheme II



inhibitory properties of the α_1-antitrypsin with the P_{10}Pro mutation were similar to those of its antithrombin III homologue; it became a substrate for HLE and trypsin. In contrast, replacement of the P_{12} alanine by threonine in α_1-antitrypsin did not affect the inhibitory kinetics of the protein. The differential effect of the P_{12}Thr mutations in antithrombin III and α_1-antitrypsin indicates that while the hinge regions are well conserved between inhibitory members of the serpin family, individual serpins vary in their ability to accommodate mutations in this region.

The effects of the mutations in α_1-antitrypsin on the inhibitory properties of the protein can be understood in terms of Scheme II, which has been proposed for the interactions of C1 inhibitor with kallikrein and of antithrombin III with thrombin (Olson, 1985; Patston et al., 1991). In Scheme II, the serpin (I) functions as a suicide substrate for the target proteinase (E). After the formation of an initial (Michaelis) complex (EI), the inhibitor partitions between an inhibited complex (EI*) and a modification reaction that leads to the formation of an inactive inhibitor (I'). The relative amounts of the inhibited complex and modified inhibitor will depend on the relative rates of the two processes (k_f and k_{mod}). In previous studies on C1 inhibitor and antithrombin III, it has been shown that the relative rates of these two processes can be influenced by the reaction conditions, such as salt strength and temperature, and, in the case of antithrombin III, by the presence of heparin (Olson, 1985; Patston et al., 1991).

For the interaction of wild-type α_1-antitrypsin with HLE, the inhibitor partitioned almost exclusively into the inhibited complex, i.e., the ratio of k_f to k_{mod} is large. In addition, the k_r is exceedingly small, such that the formation of the final inhibited complex (EI*) is essentially irreversible. The mutation of the alanine at P_{12} to threonine did not markedly affect the partitioning of the inhibitor between the inhibited complex and the modified form; the inhibited complex was still the predominant product. In contrast, the P_{10}Pro mutation decreased the k_f to k_{mod} ratio such that the pathway for the formation of the modified inhibitor was the preferred pathway. The interaction of the α_1-antitrypsins with trypsin follows the same mechanism as that observed with HLE; both the wild-type and the P_{12}Thr mutant partitioned predominantly to the inhibited complex. For trypsin, however, the final complexes were not as tight as those observed with HLE. The K_i value for α_1-antitrypsin with trypsin is at least 100-fold higher than those observed with HLE (Table I). With P_{10}Pro and trypsin, however, a significant portion of the initial complex again partitioned toward the cleavage reaction; the ratio of k_{mod} to k_f was lower with trypsin than with HLE, and a smaller fraction of the inhibitor was cleaved.

The effects observed with the P_{10}Pro mutation suggest that the hinge region of serpins is important for controlling the partitioning of the inhibitor between the inhibited complex and the modified inhibitor. For other inhibitors of serine proteinases such as Kunitz inhibitors, the reactive-site loop is stabilized by either disulfide bonds or interactions with the body of the protein (Bode & Huber, 1991). The stabilized conformation of the loop is complementary to the active site of the proteinase, and a tight complex is formed. For serpins, it has been proposed that a portion of the reactive-site loop between P_{15} and P_8 inserts into the A β-sheet and that this insertion maintains the reactive-site loop in a taut conformation similar to that of the reactive-site loop of Kunitz inhibitors and leads to a stable complex between the proteinase and the serpin (Carrell et al., 1991; Skriver et al., 1991). Thus, modification of this insertion by mutation of residues in the hinge region would be expected to affect the stability of the inhibitory form of the reactive-site loop. The effect of the P_{10}Pro mutation on the interaction of α_1-antitrypsin with HLE and trypsin suggests that this mutation has destabilization the reactive-site loop such that proteolytic cleavage of the loop is favored. It is interesting to note that with both enzymes, the mutation did not affect the rate of formation of the inhibited complex; the association rate constants with both enzymes were comparable to those observed with the wild-type protein. These results indicate that the hinge region is not of crucial importance for the rate at which the initial complex forms but rather determines the proportion of inhibitor which is able to inhibit the enzyme. A similar conclusion can be drawn from the work of Björk et al. (1992a,b). A peptide corresponding to P_1–P_{14} of antithrombin III binds to the inhibitor and converts it into a substrate presumably by blocking the partial insertion of the reactive-site loop into the A β-sheet. Binding of the P_1–P_{14} peptide does not, however, affect the rate of interaction of antithrombin III with thrombin.

All previous studies have indicated a direct correlation of the ability of a serpin to undergo the *S*-to-*R* transition and its ability to function as an inhibitor [reviewed in Carrell and Evans (1992)]. The data presented here for P_{10}Pro provides an exception to this rule. This mutant was able to undergo the *S*-to-*R* transition but was a substrate for HLE and trypsin. Insertion of the reactive-site loop into the A β-sheet creates a more compact molecule with a tighter packing of residues and a corresponding resistance to denaturing conditions (Bruch et al., 1988; Haris et al., 1990; Perkins et al., 1992). The increased heat stability of P_{10}Pro cleaved with *S. aureus* V8 protease suggests that the reactive-site loop of P_{10}Pro was able to insert into the A β-sheet. This insertion would involve 11 residues from P_5 to P_{15}, and a proline at position P_{10} appears not to destabilize the binding of these residues. In contrast, the maintenance of a taut, inhibitory conformation of the reactive-site loop appears only to involve the insertion of at most three residues (P_{14}–P_{12}: Mast et al., 1992; Schulze et al., 1992), in which case the P_{10} glycine would be located in a turn near the beginning of the exposed reactive-site loop. Consequently, the substitution of a proline for glycine in the P_{10} position may destabilize the taut conformation of the reactive-site loop by disrupting the turn out of the A β-sheet or by adversely influencing the insertion of residues P_{15}–P_{12}.

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