α_1-Proteinase Inhibitor Variant T345R. Influence of P14 Residue on Substrate and Inhibitory Pathways†

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ABSTRACT: To test whether the presence of charged residues at position P14 of the reactive center region of noninhibitory members of the serpin family of protein proteinase inhibitors is responsible for their lack of proteinase inhibitory properties, we expressed a variant of the α_1-proteinase inhibitor (α_1-PI) with arginine substituted for threonine at this position (T345R) and characterized its functional properties. Although the T345R variant reacted with proteinases principally as a substrate, it was still capable of forming stable complexes with the three serine proteinases examined, human neutrophil elastase (HNE), porcine pancreatic elastase (PPE), and trypsin. The fraction of T345R α_1-PI that formed a complex with proteinase was quantitated by autoradiography of SDS gels of the variant incubated with ^{125}I-labeled proteinase. The stoichiometry of inhibition (S.I.) (number of mol of α_1-PI required to completely inhibit 1 mol of proteinase), which was 1 for both plasma α_1-PI and wild-type recombinant α_1-PI interacting with each of the proteinases, was very much greater than 1 for T345R variant α_1-PI. Values of 9.5, 45, and about 70 were estimated for variant α_1-PI inhibition of trypsin, HNE, and PPE, respectively. An inverse relationship between the apparent second-order rate constant and the S.I. for inhibition of PPE by T345R α_1-PI suggested that the mutation did not affect the rate-determining step of formation of a transient intermediate complex. Following cleavage of the reactive center loop, there was a large increase in protein stability and changes in the CD spectrum, both consistent with insertion of the reactive center loop into β-sheet A. This behavior is similar to that of wild-type α_1-PI. We conclude that the presence of a charged residue at P14 does not prevent reactive center loop insertion or the functioning of α_1-PI as an inhibitor of serine proteinases but does significantly alter the relative rates of the substrate and inhibitory pathways in favor of the former, probably by reducing the rate of the latter reaction.

α_1-Proteinase inhibitor (α_1-PI)[1] is a protein proteinase inhibitor and a member of the serpin superfamily of proteins (Carrell & Travis, 1985), which are related both by primary structure homology (Hunt & Dayhoff, 1980; Huber & Carrell, 1989) and, more importantly, by close tertiary structure similarity (Stein & Chothia, 1991). Its primary target is neutrophil elastase, but it can inhibit other proteinases such as porcine pancreatic elastase and trypsin (Pannell et al., 1974). An unusual property of serpins is that the native conformation of the protein appears to be a metastable state. The difference in free energy between this state and the most stable state acts as the driving force for a conformational rearrangement that is essential for the inhibitory mechanism of the serpin (Gettins et al., 1993). This structural rearrangement is thought to involve unfolding of the α-helical reactive center region of the serpin, containing the recognition site for proteinase binding, as well as partial insertion of the unfolded, extended polypeptide into an extensive β-sheet as an additional strand (Huber & Carrell, 1989; Gettins et al., 1993). Proof that this unfolding and insertion occurs upon formation of a stable complex between serpin and target proteinase is lacking, since there are no X-ray structures of such complexes. However, it has been shown for several serpins cleaved at the P1–P1′ [2] bond that such integration into β-sheet A of the reactive center region does occur (Loebermann et al., 1984; Mourey et al., 1990; 1993; Baumann et al., 1991, 1992) and that the cleaved serpin is very much more stable than the native protein (Bruch et al., 1988; Gettins & Harten, 1988; Haris et al., 1990; Perkins et al., 1992).

Despite the family name serpin (*ser*ine *p*roteinase *in*hibitor), some members of the family are not proteinase inhibitors but act as substrates. Other serpins act as both inhibitors and substrates, with the proportions depending upon the reaction conditions and the particular proteinase–serpin pair. These observations are consistent with a branched, suicide substrate inhibition mechanism in which one or the other pathway or both are operative (Waley, 1991).

The most prominent structural difference between inhibitory and noninhibitory serpins is the presence of a noncharged amino acid at position P14 in the former and of a charged residue at the same position in the latter (Huber & Carrell, 1989; Gettins et al., 1993). Examples of this are the arginines

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[1] Abbreviations: α_1-PI, α_1-proteinase inhibitor; T345R, variant in which residue 345 has been changed from threonine to arginine; PMSF, phenylmethanesulfonyl fluoride; NPGB, *p*-nitrophenyl guanidinobenzoate; SDS, sodium dodecyl sulfate; HNE, human neutrophil elastase; PPE, porcine pancreatic elastase; dNTP, deoxynucleotide triphosphate; S.I., stoichiometry of inhibition; CD, circular dichroism.

[2] The nomenclature system of Schechter and Berger (1967), used to describe the subsites of interaction between a proteinase and its substrate, designates the residues on either side of the substrate scissile bond as P1 and P1′. Residues N-terminal to this are designated P2, P3...P*n*, and residues C-terminal are designated P2′, P3′...P*n*′.

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at the P14 positions of the reactive center loops of the noninhibitory serpins ovalbumin and human angiotensinogen and the glutamate in the equivalent position of rat angiotensinogen. Since P14 is close to the hinge region for the proposed loop insertion into the β-sheet, it has been suggested that a charged residue at this position prevents loop insertion and thus precludes action as an inhibitor (Wright et al., 1990). We therefore wanted to test the relationship between the nature of the residue at P14 and the properties of the serpin by creating an arginine-containing P14 variant (T345R) of the inhibitory serpin α_1-PI and determining whether or not it had lost its ability to inhibit its normal target proteinases. A related, but distinct, recombinant α_1-PI containing arginine substitutions at both P14 and P1 positions has been previously examined and found not to be inhibitory toward thrombin (Schulze et al., 1991).

We found that although T345R α_1-PI was no longer an efficient inhibitor of proteinases, it had not completely lost its inhibitory properties. With trypsin, the stoichiometry of inhibition (S.I.), defined as the number of moles of the serpin necessary to completely inhibit 1 mol of proteinase, was 9.5 compared with a value of 1 for the wild-type inhibitor. S.I. values of about 45 and 70 were obtained with HNE and PPE, respectively, compared to values of 1 for the wild-type inhibitor in each case. The rate of formation of the intermediate complex, measured for PPE, was not affected, however, implying that the mutation did not affect initial proteinase recognition but did affect the step committing to formation of a stable complex. Circular dichroism studies showed that the cleaved form of the variant was significantly more stable than the native protein, consistent with reactive center loop insertion into β-sheet A. We conclude that arginine at position P14 of α_1-PI slows the rate of loop insertion, but does not prevent it, and thereby reduces the efficiency of, but does not eliminate, inhibition by α_1-PI.

MATERIALS AND METHODS

Cloning, Expression, and Mutagenesis of Wild-Type and T345R Variants of α_1-Proteinase Inhibitor. Full length wild-type α_1-proteinase inhibitor cDNA was isolated from a human liver library in Uni-Zap XR bacteriophage and subcloned into pBluescript. Sequencing was carried out by the dideoxynucleotide chain termination method (Sanger et al., 1977). The resulting sequence agreed well with previously reported α_1-PI cDNA sequences (Kurachi et al., 1981; Bollen et al., 1983), with only conservative base changes in the coding region, and confirmed that the cDNA was full length.

A new *Hin*d III restriction site was introduced before the start of the coding region, by insertion of a synthetic oligonucleotide duplex at the *Pst* I site, to permit excission of the 1.45 kb *Hin*d III–*Xho* I fragment covering the α_1-PI cDNA and its insertion into the expression vector pMAStop to create pMA-α_1-PI. Site-directed mutagenesis was performed by subcloning the *Hin*d III–*Xba* I fragment containing the α_1-PI cDNA into M13mp19, using the method of Kunkel (Kunkel, 1985; Kunkel et al., 1985).

Mutation of threonine 345 to arginine used the 30 b oligonucleotide 5′GGCCCCAGCAGCTTCACGCCCTTTCTCGTC3′ (the triplet corresponding to the change to arginine is underlined), complementary to the coding strand. The mutation was confirmed by sequencing (Figure 1).

Expression vector pMA-α_1-PI(T345R) was created by ligating the *Hin*d III–*Xba* I fragment excised from double-stranded mutated M13mp19 into *Hin*d III–*Xba* I doubly digested pMA-α_1-PI. Baby hamster kidney cells were

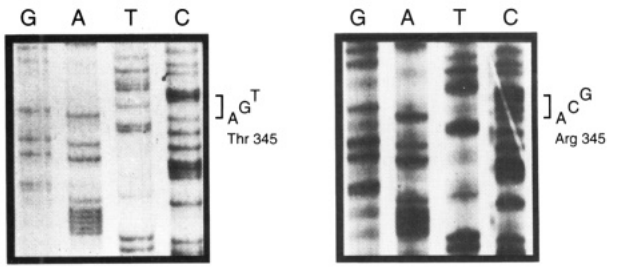


FIGURE 1: DNA sequencing gel of wild-type and T345R variant cDNA in the vicinity of residue 345. The sequence of the noncoding strand in the 5′ → 3′ direction can be read from bottom to top. The brackets show the position of the anticodons for threonine (5′AGT3′) in the wild-type cDNA and arginine (5′ACG3′) in the T345R variant cDNA.

cotransfected with plasmids pMA-α_1-PI (wild-type or T345R variant) (20 μg), pSV2dhfr (5 μg), and pRMH140 (5 μg) as described previously for production of human antithrombin (Zettlmeissl et al., 1988; Fan et al., 1993).

Production of both wild-type and T345R variant α_1-PIs was from confluent monolayers of stably transfected BHK cells in 1.5 L roller bottles. The growth medium was cycled every 24 h between medium containing both drugs (methotrexate and neomycin) and 10% fetal calf serum and medium lacking these supplements. Supernatants from the serum-free cycles were collected, made 1 mM in phenylmethanesulfonyl fluoride (PMSF) to inhibit proteolysis, and stored at 4 °C until ready for isolation of α_1-PI.

Recombinant wild-type and T345R variant α_1-PIs were isolated from pooled serum-free supernatants by hydrophobic interaction chromatography, thiol–disulfide exchange chromatography, and DEAE ion exchange chromatography as described previously (Pannell et al., 1974; Baugh & Trabis, 1976). Both proteins were dialyzed against 0.1 M Tris-HCl, pH 8.0, concentrated to 4–7 mg mL^{-1}, and stored in aliquots at −70 °C until needed. Purity was checked by SDS–PAGE in 10% slab gels according to the procedure of Laemmli (1970) and by activity measurements. Inhibitory activity of the wild-type inhibitor was determined against both human neutrophil elastase and porcine pancreatic elastase by the method of Bieth et al. (1974) and found to be comparable to that of plasma α_1-PI. Protein concentrations were determined spectrophotometrically using the same extinction coefficient as that for the plasma protein, $E^{1\%}_{280nm}$ = 5.3 (Pannell et al., 1974).

Isolation of Plasma α_1-PI. Human plasma α_1-PI was isolated from pooled outdated human plasma (Vanderbilt Blood Bank) as described previously (Pannell et al., 1974; Baugh & Travis, 1976). Protein concentrations were determined spectrophotometrically using an extinction coefficient of $E^{1\%}_{280nm}$ = 5.3 (Pannell et al., 1974) and a molecular weight of 52 000 (Pannell et al., 1974).

^{125}I-Labeling of Proteinases. Bovine trypsin, porcine pancreatic elastase, and human neutrophil elastase were radioiodinated by the iodogen method (Fraker & Speck, 1978). For each iodination, 20 μg of 1,3,4,6-tetrachloro-3α,6α-diphenylglycouril was dried onto the walls of a microfuge tube; 50 μg of proteinase and 1 equiv of Na^{125}I were added to give a total volume of 100 μL. The mixture was reacted for 15 min at 4 °C before chromatography on a GF-5 Presto desalting column to remove free Na^{125}I. Labeled proteinase fractions were pooled and stored in aliquots at −70 °C until needed.

Standardization of Proteinases and Recombinant Wild-Type α_1-PI. Bovine trypsin was active site titrated with *p*-nitrophenyl guanidinobenzoate (Chase & Shaw, 1970). This was used in turn to determine the activity of recombinant wild-type α_1-PI from the concentration of α_1-PI necessary to inhibit a known amount of active trypsin in a spectrophotometric assay of residual amidolytic activity, using *N*-benzoyl-DL-arginine-*p*-nitroanilide as substrate (Erlanger et al., 1961). A stoichiometry of inhibition of 1 was obtained with wild-type α_1-PI. Wild-type α_1-PI was then used as a secondary standard for determining the activities of HNE and PPE, by spectrophotometric assay of residual amidolytic activity using methoxysuccinyl-$(Ala)_2$-Pro-Val-*p*-nitroanilide (Nakajima et al., 1979) and *N*-succinyl-$(Ala)_3$-*p*-nitroanilide, respectively, as substrates (Bieth et al., 1974).

Quantitation of Proteinase–α_1-PI Complex Formation. ^{125}I-Labeled proteinase was incubated at pH 6.0 in 0.1 M phosphate buffer with the indicated amount of wild-type or variant α_1-PI and the reaction allowed to proceed for 30 min before electrophoresis in 10% slab gels under reducing denaturing conditions (Laemmli, 1970). The gels were dried on Whatman 3MM paper. Bands containing ^{125}I were quantitated using a Phosphor Imager (Molecular Dynamics) following exposure of the dried gels to the phosphor screen for 18 h. The program Image Quant, supplied with the Phosphor Imager, was used for quantitation.

Determination of Inhibition Rates of PPE by α_1-PI. The rate of inhibition of PPE by wild-type recombinant α_1-PI was determined at 25 °C under second-order conditions using 92 nM α_1-PI incubated with 46 nM PPE in 100 mM phosphate buffer, pH 6.0. At various times, the residual enzymatic activity was determined by diluting an aliquot of the reaction mixture into the assay solution, which contained 0.5 mM *N*-succinyl-$(Ala)_3$-*p*-nitroanilide substrate, and measuring the change in absorbance at 405 nm relative to that produced by known concentrations of active PPE. The large excess of substrate over α_1-PI and the dilution of the reaction mixture effectively stopped the α_1-PI–proteinase inhibition reaction. The rate constant for the reaction was obtained from a semilog plot of [residual inhibitor]/[residual proteinase] against time. For the inhibition of PPE by the T345R variant, pseudo-first-order conditions were used, with concentrations of proteinase and inhibitor of 23 nM and 8.1 μM, respectively. The pseudo-first-order rate constant was determined from a semilog plot of residual enzyme activity against time.

Preparation of Reactive Center-Cleaved Recombinant α_1-PI. Reactive center-cleaved wild-type recombinant α_1-PI was prepared by reaction of native protein with papain in a 250:1 ratio. Papain was used because it is not efficiently inhibited by α_1-PI. Papain reacts at two sites within the reactive center to give a cleaved form lacking residues P8–P1 (Mast et al., 1992). The reaction was stopped by addition of 10 mM iodoacetamide, and the products were separated by size-exclusion chromatography on G50. Reactive center-cleaved T345R variant α_1-PI was prepared by reaction of native protein with a 1:1 ratio of PPE. The reaction was stopped by addition of PMSF to 1 mM, following which the products were separated by size-exclusion chromatography on G150. In both cases, SDS–PAGE was used to characterize the reaction products and demonstrated that the reaction had gone to completion and produced reactive center-cleaved α_1-PI.

Circular Dichroism Spectroscopy. CD spectra were recorded on a JASCO 720 spectropolarimeter, which was calibrated prior to each run with a standard of 0.06% ammonium camphor sulfonate in a 1 cm cell. To follow changes in protein secondary structure as a function of temperature, the ellipticity was monitored at 0.5–1° intervals at 222 nm in a 0.5 cm temperature-controlled cell. A heating rate of 20°/h was maintained using a Neslab RTE-110 water bath. Full spectra were recorded using a 1 mm cell for the range 260–215 nm and a 0.1 mm cell for the range 215–178 nm. A spectrum of the reference blank was subtracted from the sample spectra, and the latter were smoothed. Protein solutions were about 0.6 mg mL^{-1} in 20 mM sodium phosphate buffer, pH 7.0. Samples were filtered and degassed.

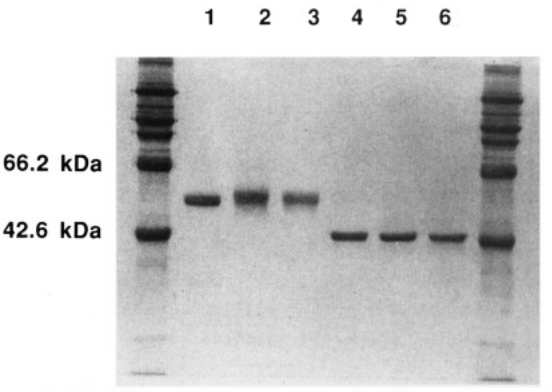


FIGURE 2: Mobility and heterogeneity of α_1-PI species on an SDS poly(acrylamide) gel, before and after deglycosylation. Lanes 1–3, normal protein; lanes 4–6, samples after deglycosylation with *N*-glycosidase F. Lanes 1 and 4, plasma α_1-PI; lanes 2 and 5, wild-type recombinant α_1-PI; lanes 3 and 6, T345R recombinant α_1-PI. Molecular weight standards are shown at the extreme left and right, with the positions and masses of bovine serum albumin and human serum albumin indicated.

Materials. Human neutrophil elastase was from Athens Research (Athens, GA); bovine trypsin was from Cooper Biomedical (Freehold, NJ). *p*-Nitrophenyl guanidinobenzoate was from Calbiochem (San Diego, CA). Methoxysuccinyl-$(Ala)_2$-Pro-Val-*p*-nitroanilide, *N*-succinyl-$(Ala)_3$-*p*-nitroanilide, *N*-benzoyl-DL-arginine-*p*-nitroanilide, porcine pancreatic elastase, (+)-amethopterin (methotrexate), ampicillin, and lysozyme were from Sigma (St. Louis). Dulbecco's modified Eagle's medium, fetal calf serum, and G418 (neomycin) were from Gibco. The human liver cDNA library in the Uni-Zap XR vector, the R408 interference-resistant helper phage, and *Escherichia coli* XL1-Blue host cells were from Stratagene (San Diego, CA). Restriction endonucleases *Eco*R I, *Xho* I, *Pst* I, *Hin*d III, *Sal* I, and *Xba* I, T4 polynucleotide kinase, T4 ligase, M13mp19, T3 and T7 promoter primers, M13 universal primer, RNase, and β-agarase were from New England Biolabs Inc. (Beverly, MA). *E. coli* strains LE392, CJ236, MC1060, and NK7085 were generous gifts from Dr. R. Stephen Lloyd (UTMB; Galveston, TX). Sequenase was from United States Biochemical (Cleveland, OH). High-purity dNTPs were from Perkin Elmer (Norwalk, CT). [^{125}I]-Sodium iodide, [α-^{32}P]- and [γ-^{32}P]ATP were from New England Nuclear (Boston, MA). Iodogen and Presto desalting columns were from Pierce (Rockford, IL). Radial immunodiffusion kits for assay of recombinant and variant α_1-PIs were from The Binding Site Ltd. (Birmingham, U.K.).

RESULTS

Characterization of Recombinant Wild-Type and T345R Variant α_1-PIs. Both wild-type and variant recombinant human α_1-PIs gave single diffuse bands on an SDS poly-(acrylamide) gel, with mobilities very similar to that of human plasma α_1-PI (Figure 2). After treatment with *N*-glycosidase F, each protein gave a sharp band with higher, identical

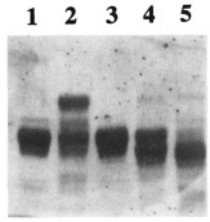


FIGURE 3: Inhibitory and substrate reactions of wild-type and T345R variant α_1-PIs with PPE. SDS poly(acrylamide) gel (7.5%) of reaction mixtures of 2 μg of α_1-PI incubated with PPE for 10 min at room temperature in 20 mM phosphate buffer, 150 mM NaCl, pH 7.4. The reactions were terminated by addition of PMSF to 1 mM. Lane 1, wild-type α_1-PI; lane 2, wild-type α_1-PI with 0.5 equiv of PPE; lane 3, T345R α_1-PI; lane 4, T345R α_1-PI with 0.05 equiv of PPE; lane 5, T345R α_1-PI with 0.25 equiv of PPE.

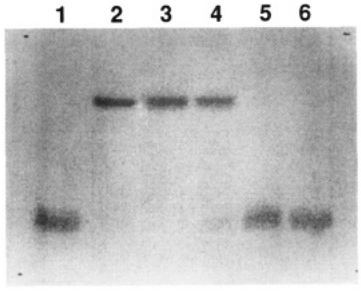


FIGURE 4: Complex formation between α_1-PI and HNE, demonstrated by the appearance of high molecular weight band on a Phosphor Imager autoradiographic scan of an SDS poly(acrylamide) gel. All lanes contain the same amount of ^{125}I-labeled HNE (2 μg). Lane 1, free HNE; lane 2, HNE incubated with 1 equiv of plasma α_1-PI; lane 3, HNE incubated with 1 equiv of wild-type recombinant α_1-PI; lane 4, HNE incubated with 45 equiv of T345R variant recombinant α_1-PI; lane 5, HNE pretreated with PMSF prior to incubation with 45 equiv of T345R variant recombinant α_1-PI; lane 6, HNE heat-denatured by incubation at 95 °C for 5 min prior to incubation with 45 equiv of T345R variant recombinant α_1-PI.

mobility (Figure 2), showing that the original diffuseness of the bands of the recombinant α_1-PIs was due to carbohydrate heterogeneity. We have observed similar diffuseness of bands which also arose from carbohydrate heterogeneity on SDS gels of human antithrombin derived from the same BHK cell expression system (Fan et al., 1993).

Wild-type recombinant α_1-PI formed a stable complex with PPE as judged by the appearance of a high molecular weight band on an SDS gel at the position expected for the complex (Figure 3, lane 2). In contrast, variant T345R α_1-PI was predominantly cleaved by PPE, resulting in the appearance of a band at a lower molecular weight than that of the starting material (Figure 3, lanes 3–5). In keeping with the catalytic nature of the reaction, substoichiometric levels of PPE were sufficient to cleave the variant. After 10 min of incubation, 0.05 equiv of PPE resulted in cleavage of about half of the α_1-PI (lane 4) and 0.25 equiv resulted in complete cleavage. We were surprised, however, to observe that the recombinant T345R variant α_1-PI also formed a stable complex, though at very low levels. Although it is difficult to discern in the figure, a trace of T345R α_1-PI–PPE complex was more clearly visible on the original gel in lanes 4 and 5.

Detection and quantitation of the T345R α_1-PI–proteinase complexes were better accomplished using autoradiography of SDS gels of ^{125}I-labeled proteinase–α_1-PI mixtures. Using this procedure, the complexes formed between HNE and both wild-type recombinant α_1-PI and plasma α_1-PI could be clearly seen (Figure 4). These occurred at the same position, though with diffuseness of the recombinant protein complex band similar to that seen with the uncomplexed protein (Figure 4,

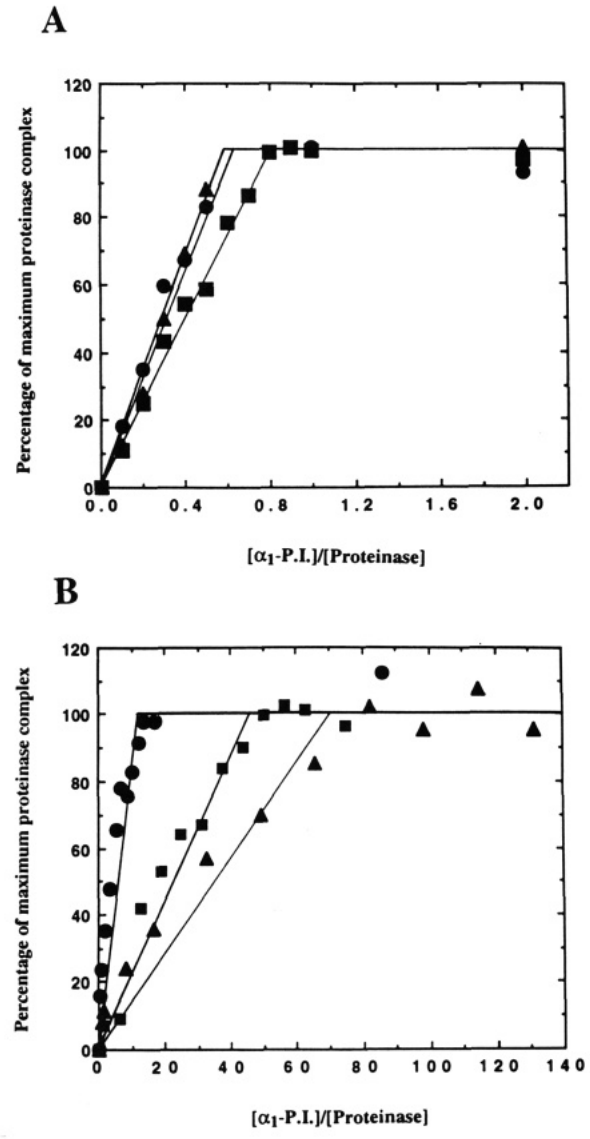


FIGURE 5: Determination of stoichiometries of inhibition of proteinase by wild-type and T345R α_1-PIs from quantitation of complex formation by autoradiography of SDS poly(acrylamide) gels. The amount of complex formed was determined by quantitating the amount of radioactivity associated with the high molecular weight complex band compared with that of the band corresponding to labeled proteinase. Conditions for the incubations are given under Materials and Methods. (A) Titration of proteinases with wild-type recombinant α_1-PI. (B) Titration of proteinases with T345R variant recombinant α_1-PI. Filled triangles, trypsin; open circles, human neutrophil elastase; open squares, porcine pancreatic elastase. In B, the active proteinase concentration determined in A was used to calculate the ratio of inhibitor to proteinase.

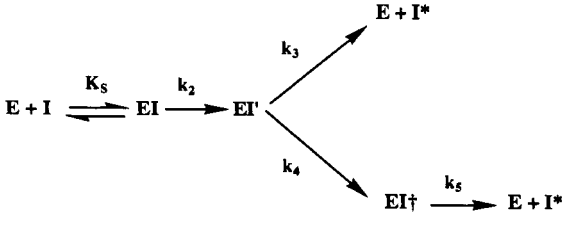
lanes 2 and 3). With the T345R variant, a much higher molar ratio of inhibitor-to-enzyme was required to completely complex all of the proteinase (Figure 4, lane 4). The appearance of the complex band for the T345R variant required both the inhibitor and the proteinase to be in active states. This was demonstrated by the finding that either inhibition of the proteinase with PMSF prior to incubation with T345R α_1-PI (Figure 4, lane 5) or heat denaturation of the T345R α_1-PI prior to incubation with the proteinase (Figure 4, lane 6) prevented complex formation.

Stoichiometries of Inhibition for Wild-Type and T345R Variant α_1-PIs. S.I. values for the inhibition of trypsin, HNE, and PPE were determined for both wild-type and T345R variant α_1-PIs by quantitation of autoradiograms of SDS poly(acrylamide) gels of incubations of each α_1-PI with each ^{125}I-

labeled proteinase. The S.I. value was taken as the intercept between the best fit lines to the portions of the graph below and above complete inhibition of proteinase. S.I. values for wild-type α_1-PI of 0.58, 0.61, and 0.80 were determined for inhibition of trypsin, HNE, and PPE, respectively (Figure 5A). These values are less than the maximum theoretical value of 1.0 expected for a 1:1 complex and probably result from less than fully active radiolabeled proteinase. Taking these proteinase activities into account, the S.I. values for inhibition by T345R α_1-PI were found to be very much greater than those found for inhibition by wild-type α_1-PI (Figure 5B), indicating that the variant could inhibit each of the proteinases but that the substrate pathway was much more efficient than the inhibitory pathway. S.I. values of 9.5, 45, and 70 were estimated for inhibition of trypsin, HNE, and PPE, respectively. The fit of the data for HNE and PPE is poorer than that for trypsin, probably resulting from the longer time needed to reach complete proteinase inhibition for large S.I. values. Importantly, however, the S.I. values are very different for each proteinase.

A concern in ascribing unexpected inhibitory properties to the variant α_1-PI was that the protein might, for unknown reasons, have contained a small percentage of functional wild-type α_1-PI. If this had been the case, the observed S.I. values for the variant α_1-PI preparation would have been a weighted S.I. based on the percentage of each type of protein and the S.I. for each form of the inhibitor. Taking the extreme case of variant α_1-PI being incapable of functioning as an inhibitor (S.I. = ∞), the apparent S.I. for T345R would have been constant for each of the three proteinases, since the S.I. for wild-type α_1-PI with each proteinase is 1. The result that we obtained, that T345R α_1-PI gave a different S.I. for each proteinase, ranging from 9.5 for trypsin inhibition to 70 for PPE inhibition is incompatile with a contamination by wild-type α_1-PI but, rather, indicated that the observed S.I.s reflect inhibition of proteinases by T345R variant α_1-PI with different efficiencies. The results of the temperature dependence of the CD signals of native and cleaved forms of the T345R variant sample are in accord with this (see below), since they must report the properties of the dominant species, which in this case must be the T345R variant.

Rate of Inhibition of PPE by α_1-PI Species. Serpins are thought to interact with proteinases as suicide substrate inhibitors which can form stable 1:1 complexes but which can also be cleaved as substrates according to the reaction scheme outlined below (Patston et al., 1991). In this reaction scheme, the inhibitory pathway proceeds with a rate constant, k_4, to form the stable complex EI†. This complex typically has very



long term stability on the order of hours to days, so that k_5 is very slow (Matheson et al., 1991; Cooperman et al., 1993). Under conditions where $k_5 \ll k_4$ and $[I]_0 \ll K_S$, the observed pseudo-first-order rate constant for inhibition is given by eq 1.

$$k_{obs} = \frac{k_2[I]_0}{K_S}\frac{k_4}{k_4 + k_3} \quad (1)$$

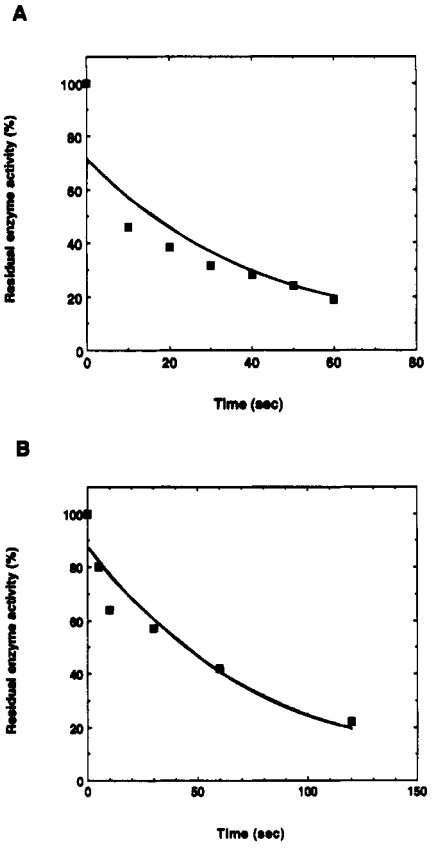


FIGURE 6: Time course of inhibition of PPE by α_1-PI. Residual enzymatic activity, measured by discontinuous assay, is shown as a function of reaction time. (A) wild-type α_1-PI (92 nM) reacted with 46 nM PPE (second-order reaction conditions). (B) T345R α_1-PI (8.1 μM) reacted with 23 nM PPE (pseudo-first-order reaction conditions). The solid lines represent the best fits to the data generated with the parameters in Table 1.

The apparent second-order rate constant (k_{app}) can be obtained from this in two ways. The first is to divide k_{obs} by the total α_1-PI concentration ($[I]_0$). This results in the value for k_{app} given in eq 2.

$$k_{app} = \frac{k_2}{K_S}\frac{k_4}{k_4 + k_3} \quad (2)$$

The second way to calculate k_{app} is to divide k_{obs} by the "effective" α_1-PI concentration, which is the fractional concentration that forms a stable complex and is given by $[I]_0$ divided by the S.I. Since the S.I. can also be represented as $(k_4 + k_3)/k_4$ for this mechanism, this results in a simplification of k_{app} to the form given in eq 3.

$$k_{app} = \frac{k_2}{K_S} \quad (3)$$

For wild-type α_1-PI, these values are identical, since the S.I. is indistinguishable from 1.0.[3] For T345R, the values differ by the magnitude of the S.I. The time courses of inhibition of PPE are shown in Figure 6 for both wild-type and the T345R variant. When these data were used to calculate $k_2/K_S \times k_4/(k_4 + k_3)$ for the inhibition of PPE by each α_1-PI species, very different values were obtained, 2.8×10^5 M^{-1} s^{-1} for wild-type and 1.6×10^3 M^{-1} s^{-1} for T345R variant α_1-PI (Table 1). However, when these were converted to k_2/K_S,

[3] As long as $k_4 \gg k_3$, the S.I. is 1.0 within experimental error even for significant changes in either k_3 or k_4.

Table 1: Kinetic Constants for the Reaction of PPE with Recombinant Wild-Type and Reactive Center Loop Variant α_1-PI Species

α_1-PI	k_2/K_S[a] (M^{-1} s^{-1})	$k_2/K_S \times k_4/(k_4 + k_3)$[b] ($M^{-1}$ s^{-1})	S.I.[c]
wild-type	2.8×10^5	2.8×10^5	1.0
T345R	1.1×10^5	1.6×10^3	70

[a] Calculated using the total concentration of α_1-PI. [b] Calculated using the fractional concentration of α_1-PI that forms a complex with PPE, derived from S.I. and k_2/K_S. [c] S.I. values were calculated from the relative proportions of α_1-PI that were cleaved as substrate and formed complexes with PPE, determined from Figure 4.

the values were much more similar (Table 1), indicating that the steps up to the branch point of the pathway that leads to substrate and inhibition reactions proceeded at similar rates for both wild-type and variant α_1-PI species. Similar behavior was observed in a study by Hopkins et al. (1993) for P10 glycine → proline and P12 alanine → threonine variants of α_1-PI. Although only values for k_2/K_S were reported, we estimated S.I. values from the enzymatic activities they reported. Taking the S.I. of their wild-type α_1-PI for trypsin as 1.0, we estimated S.I. values for inhibition of trypsin by the A347T variant of 1.3 and by the G349P variant of 5.5. Interestingly, the reported inhibitory activity of the G349P variant for HNE implied an S.I. of about 160. This shows the same order of S.I.s toward different enzymes as we found here for the T345R variant.

An alternative linear reaction scheme in which two different proteinase-reactive conformations of T345R variant α_1-PI exist, one inhibitory and one substrate-like, fails to account for the present observations, since the proportion of α_1-PI reacting by substrate or inhibitory pathways is not constant but depends on the proteinase used. If two conformers, one substrate-like and one inhibitory, were present in fixed proportions, the amounts reacting by each type of linear pathway should be constant.

Stability of α_1-PI Species and Evidence for Loop-Sheet Insertion. As a means of following changes in secondary and tertiary structure of α_1-PI species as a function of temperature, we monitored the ellipticity of samples of native and cleaved wild-type and T345R variant α_1-PIs at 222 nm, which is a wavelength that is particularly sensitive to α-helix content.

Native wild-type α_1-PI gave a CD temperature profile with an abrupt decrease in negative ellipticity centered at 58 °C (Figure 7A). From differential scanning calorimetric measurements, we know that 58 °C is the temperature of unfolding of native wild-type α_1-PI (unpublshed results). In contrast, cleaved α_1-PI, which is known from an X-ray structure determination to have the cleaved reactive center loop inserted into β-sheet A (Loebermann et al., 1984), gave a very gradually increasing ellipticity over the whole temperature range, with no sharp transition indicative of unfolding. This implies that the temperature of unfolding (T_m) has increased by at least 31 °C. Differential scanning calorimetry of the cleaved T345R variant α_1-PI also showed no unfolding transition below 100 °C (data not shown).

T345R variant α_1-PI behaved in the same way as wild-type α_1-PI over the temperature range studied (Figure 7B). Thus, the native form gave an abrupt decrease in negative ellipticity centered at 58 °C, whereas the cleaved form of the variant gave a smoothly increasing ellipticity over the whole temperature range that paralleled the behavior of the wild-type protein, which again indicated a large increase in stability as a result of the cleavage and insertion of the reactive center loop into β-sheet A.

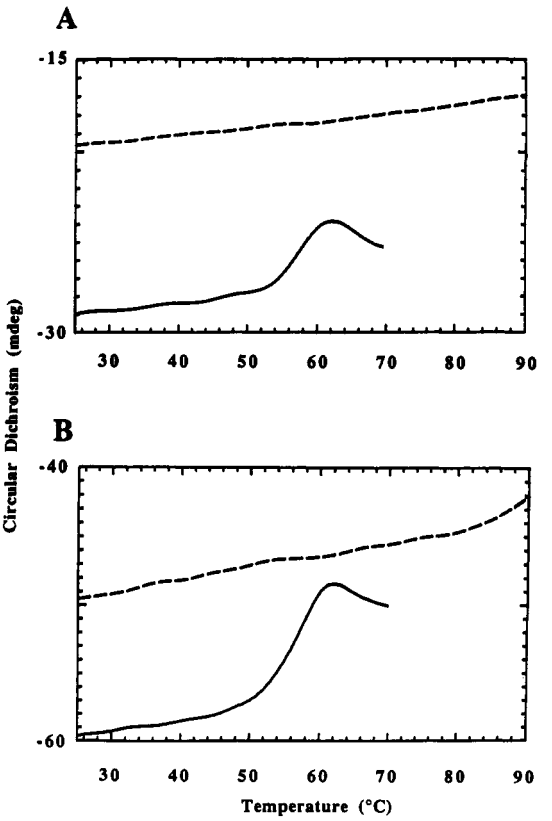


FIGURE 7: Temperature dependence of intensity of CD ellipticity at 222 nm for wild-type and variant α_1-PI species. (A) Wild-type α_1-PI. (B) T345R variant α_1-PI. Solid lines represent native protein; dashed lines represent reactive center-cleaved protein.

Similarity of CD Spectra of Wild-Type and T345R Variant α_1-PIs. CD spectra of both native and cleaved forms of both wild-type and T345R variant α_1-PIs were recorded in the UV region from 178 to 260 nm (Figure 8), which is a region dominated by the amide $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ electronic transitions and so is sensitive to the proportions of the different types of secondary structure present in the protein. The CD spectra of both native proteins are very similar, consistent with no major perturbation in secondary structure arising from the threonine → arginine replacement. The effect of reactive center cleavage of wild-type α_1-PI on the CD spectrum was a reduction in ellipticity at both extrema (220 and 194 nm), consistent with a reduction in α-helix and an increase in β-sheet. If the reactive center loop of native α_1-PI is α-helical, as is the case in ovalbumin, these are the structural changes expected from proteolytic cleavage. Variant α_1-PI showed very similar behavior, suggesting similar structural transformations as a result of cleavage in the reactive center loop.

DISCUSSION

In the present study, we have examined the ability of the T345R variant of α_1-PI to function as an inhibitor of serine proteinases despite the substitution of a charged arginine residue for the neutral threonine at the P14 position of the reactive center loop. Surprisingly, we found that for each of the three proteinases examined, trypsin, human neutrophil elastase, and porcine pancreatic elastase, T345R α_1-PI was still able to act as an inhibitor, forming SDS-stable complexes, though with a stoichiometry of inhibition in each case that was very much greater than that for the wild-type inhibitor.

A second important conclusion from the present studies is that the presence of arginine at the P14 position of the T345R

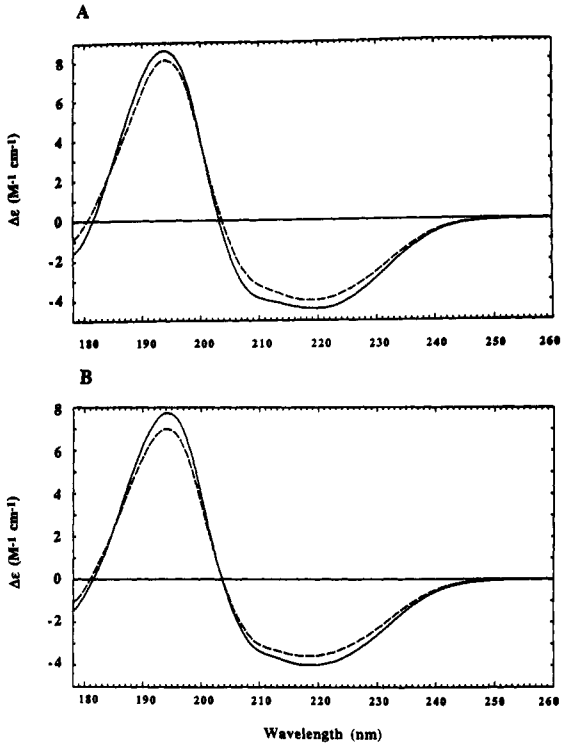


FIGURE 8: CD spectra of wild-type and T345R variant forms of α_1-PI in the UV region between 178 and 260 nm. (A) Native (27.2 μM) (solid line) and cleaved (15.9 μM) (dashed line) wild-type α_1-PI. (B) native (10.0 μM) (solid line) and cleaved (10.7 μM) (dashed line) T345R α_1-PI.

variant α_1-PI did not prevent insertion of the reactive center loop into β-sheet A of the inhibitor upon cleavage of the P1–P1′ peptide bond. This was concluded from the similarity of the temperature dependence of the circular dichroic ellipticity of native and cleaved forms of the wild-type and variant α_1-PIs and of the changes in the CD spectra resulting from cleavage in the reactive center loop of each protein. For the wild-type inhibitor, it is known from differential scanning calorimetric data that the native protein unfolds around 58 °C and from X-ray crystallographic data that the reactive center loop is inserted into β-sheet A in the cleaved protein (Loebermann et al., 1984), which results in much greater thermal stability (Bruch et al., 1988; Haris et al., 1990; Perkins et al., 1992). The temperature dependence of the circular dichroic ellipticity of the two forms of wild-type α_1-PI reflects this structural change, showing a transition at 58 °C for the native protein but no abrupt change for the more stable cleaved form up to the highest temperature (90 °C) examined. Critically, this allows interpretation of an increase in thermal stability upon cleavage in terms of a known structural change involving loop insertion into β-sheet A. This contrasts with the behavior of native and cleaved forms of ovalbumin, which also possesses arginine at P14 but which is known from X-ray structure determination not to undergo loop insertion upon cleavage under physiological conditions (Stein et al., 1990; Wright et al., 1990). Instead of the large increase in thermal stability observed here for T345R following cleavage, there is a 4° decrease in unfolding temperature following cleavage for ovalbumin (Shitamori & Nakamura, 1983). The results obtained for the temperature dependence of the circular dichroic ellipticity of native and cleaved T345R variant α_1-PIs were very similar to those of wild-type α_1-PIs and imply that the same structural transition of reactive center loop insertion that is known from the X-ray structure to occur upon cleavage of the P1–P1′ bond in the wild-type protein also occurs in the T345R variant. The similarities in the CD spectra of the two proteins, both in the change upon cleavage and the simple comparison of a given form, imply that the known loop insertion of the wild-type protein also occurs in the T345R variant. Although this conclusion is contrary to our initial expectation based upon ovalbumin, it is what would be expected if T345R α_1-PI acts as an inhibitor, since the current understanding of the mechanism of inhibition of proteinases by serpins is that partial reactive center loop insertion is an absolute requirement for inhibition.

A third conclusion is that the threonine → arginine mutation appears not to greatly affect either the initial equilibrium leading to the Michaelis-like encounter complex (K_S) or the rate of formation of the intermediate (EI′) located at the branch point leading to the inhibitory and substrate pathways (k_2). However, the mutation does greatly alter the stoichiometry of inhibition (S.I. = $(k_4 + k_3)/k_4$), indicating a change in one or both of the rate constants k_4 and k_3. Although the kinetic data presented are limited to one enzyme and do not enable us to determine the changes in the individual rate constants, the most conservative interpretation of the altered S.I.s is that the mutation has not affected the rate of the substrate cleavage pathway (k_3), since this reaction is probably most strongly influenced by residues near the reactive peptide bond at P1–P1′. This implies that the large changes in S.I. between wild-type α_1-PI and the T345R variant result from large reductions in the rate of the inhibitory pathway step, k_4. This is qualitatively what would be expected from an increase in the activation energy for loop insertion as a consequence of the threonine to arginine change at P14. The magnitudes of the reductions can only be roughly estimated using the information that for wild-type α_1-PI $k_4 \gg k_3$ and for the variant α_1-PI $k_4 < k_3$, with the ratio of k_3/k_4 varying from 8.5 for trypsin to about 70 for porcine pancreatic elastase. Although the magnitude of the substrate reaction could be measured for the T345R variant, this is not so readily done for wild-type α_1-PI, for which the substrate cleavage reaction is insignificant. More extensive rate constant measurements of both inhibitory and substrate pathways for several proteinases will be necessary to resolve this question.

The present findings of an alteration in S.I. of α_1-PI upon changing the P14 residue to arginine without an alteration in the underlying mechanism of interaction with proteinase are in agreement with a number of recent studies on related serpins, which have been interpreted in terms of a common branched pathway, suicide substrate inhibition mechanism, with perturbation of k_4 and/or k_3 resulting in sometimes large changes in S.I. (i) Rubin and colleagues have shown that mutation of residues within the reactive center loop of α_1-antichymotrypsin altered the stoichiometry of inhibition toward different proteinases as a result of alteration in k_4 and/or k_3 (Rubin et al., 1990; Schechter et al., 1993). (ii) A P10 hinge region mutant of α_1-PI, in which glycine was substituted by proline, reacted with both trypsin and HNE predominantly as a substrate to give a more stable loop-inserted form (Hopkins et al., 1993) but continued to function as an inhibitor, albeit an inefficient one. In this case, it was also concluded from measurements of k_{app} that the mutation greatly altered the stoichiometries of inhibition toward trypsin and HNE without affecting the rates of formation of the transient intermediate, EI′. This study parallels the present one in that the most likely cause of the increased S.I. values is a decrease in the rate of the inhibitory pathway as a result of a higher activation energy for loop insertion. The only difference is that the position of the residue involved (P10 vs P14) is four

residues removed. (iii) It has been demonstrated that heparin can significantly alter the S.I. for the heparin-catalyzed reaction of thrombin with antithrombin in an ionic strength-dependent manner (Björk & Fish, 1982; Olson, 1985). This probably results from an increase in the activation energy for loop insertion as a consequence of heparin binding more tightly to the non-loop-inserted form of antithrombin. (iv) In the reaction between C1 inhibitor and kallikrein, the proportions of substrate and inhibition reactions were found to be temperature dependent, favoring the substrate reaction at low temperature and the inhibitory reaction at higher temperature (Patston et al., 1991). This could be understood in terms of the relative magnitudes of the activation energies for the substrate and inhibitory pathways, since a given temperature change will have a proportionately larger effect on the pathway with the larger activation energy.

The conclusions of a previous study on a different serpin (plasminogen activator inhibitor 1), in which distinct conformational states were shown to exist and to behave either as an inhibitor or as a substrate of tissue-type plasminogen activator (Declerck et al., 1992), are not at variance with either the present findings or the proposed branched pathway mechanism given above. The results of Declerck et al. (1992) can be interpreted in terms of the branched pathway mechanism if their "inhibitory conformer" interacted with plasminogen activator to give a stable complex under conditions where $k_4 \gg k_3$ (i.e., S.I. = 1). No detectable substrate cleavage would then be detectable. If the "substrate conformer" represented a normal serpin conformation that is generally present in fully active material, reaction would have proceeded by the branched pathway, with $k_4 \ll k_3$. If the conformation was not a normal conformation (e.g., a partially loop-inserted form), entry onto the branched pathway may not have been possible, leaving only a direct substrate-like cleavage.

The only work that appears to be in conflict with the present results is a study that examined the inhibitory properties of a variant α_1-PI that contained both a methionine → arginine mutation at P1 and the same threonine → arginine substitution at P14 as examined here (Schulze et al., 1991). The methionine → arginine mutation at P1 had been identified as a naturally occurring variant (Pittsburgh) in a boy with a bleeding disorder (Owen et al., 1983) and resulted in a specificity change from elastase to thrombin. No detectable α_1-PI–thrombin complex was formed with the double mutant despite the use of a 100-fold excess of α_1-PI over proteinase. However, different target proteinases were used in the two studies. Since the magnitude of S.I., and thus whether or not significant complex formation is detectable, depends on the *relative* values of k_4 and k_3, which is in turn proteinase dependent, different outcomes could have resulted from the use of thrombin in the earlier study and elastase and trypsin in the present study, without any inconsistency between the two results.

In light of the present surprising findings, some aspects of the structural and functional properties of serpins need to be re-examined. X-ray structures of cleaved inhibitory serpins show that the reactive center loop from residues P14 to P1 can integrate into β-sheet A in a nearly perfect antiparallel manner. In contrast, the cleaved reactive center loop of the noninhibitory serpin ovalbumin does not insert under normal conditions. It has been commonly held that the absence of loop insertion in ovalbumin is a direct consequence of the P14 arginine and that the absence of insertion in turn accounts for the lack of inhibitory properties of ovalbumin. The present findings appear to contradict this in two ways: firstly, in that a P14 arginine in α_1-PI does not prevent loop insertion and, secondly, in that the P14 arginine does not eliminate inhibitory properties. The two sets of observations can, however, be reconciled to a single mechanism of action if very different activation energies for loop insertion apply to each system, with values increasing in the order wild-type α_1-PI < T345R variant α_1-PI < ovalbumin. Thus, upon substitution of the threonine at P14 of α_1-PI by arginine, the activation energy for loop insertion probably increases significantly and results in a slower inhibitory pathway and consequently in predominant reaction as a substrate. In the case of ovalbumin, we propose that the activation energy for loop insertion is even higher, resulting in no detectable loop insertion upon reaction with proteinase and thus apparent reaction exclusively as a substrate.

Although we can thus reconcile the present findings with a common mechanism of proteinase inhibition by serpins, by recognizing that a P14 arginine in α_1-PI slows down but does not stop loop insertion, we have no satisfactory explanation of *how* loop insertion can take place with a charged residue at P14. In the structures of cleaved inhibitory serpins, the side chain of the P14 residue is buried in the protein interior. This would be clearly unfavorable for the large, charged arginine side chain. However, loop insertion is unlikely to involve the P14 residue alone as evidenced by the effects of mutations at the P12 and P10 positions in this and other serpins (Perry et al., 1989; Ireland et al., 1991; Skriver et al., 1991; Hopkins et al., 1993; Aulak et al., 1993). Thus, in determining whether loop insertion is a thermodynamically favorable process, one must consider the net changes in ΔG for insertion of all residues involved. A favorable ΔG for inserting the other reactive center loop residues may offset the energy required either to bury the arginine side chain or to distort the polypeptide in the vicinity of P14 such that it does not insert into the A sheet. An X-ray structure determination of cleaved T345R α_1-PI is required to resolve this.

The common mechanism proposed above for explaining the properties of wild-type and variant α_1-PIs and ovalbumin leads to the following predictions of ovalbumin. First, if the ovalbumin–proteinase reaction could be conducted at high enough temperature (without adversely affecting the two reactants), some inhibition of the proteinase would be observed. Second, ovalbumin might be able to undergo loop insertion to give a *thermodynamically* more stable form but is *kinetically* prevented from doing so by a very high activation energy for loop insertion. Again, raising the temperature should increase the rate of formation of such a species. In this regard, it is intriguing that a second form of ovalbumin exists, designated S-form (Smith & Back, 1968), which is only very slowly formed from native ovalbumin and which has properties consistent with it being a more stable loop-inserted form (Kint & Tomimatsu, 1979). The rate of its formation from normal ovalbumin can also be enhanced by increasing the temperature (Donovan & Mapes, 1976).

Our thermal stability data on the two forms of native α_1-PI (T345R and wild-type) also provide a test of the proposal of Carrell and co-workers that the active conformation of a serpin represents a partially loop-inserted canonical form that is in equilibrium with an inactive non-loop-inserted form (Carrell et al., 1991). This hypothesis has been used by these authors to explain the enhancing effect that heparin has on the rate of inhibition of proteinases such as factor Xa by antithrombin, by proposing a heparin-induced shift in the equilibrium toward the more active loop-inserted conformation. If such a mechanism applies generally to serpins, the activity of a given

serpin should be a measure of the position of the equilibrium between active and inactive forms. Since native α_1-PI is a very efficient HNE inhibitor, in contrast to the slow inhibition of factor Xa by antithrombin in the absence of heparin, the equilibrium in the native state should be strongly shifted toward the partially loop-inserted form. If that were the case, the thermal stability of wild-type and T345R variant α_1-PIs should be significantly different, reflecting the adverse influence of the threonine → arginine change at the position of initial loop insertion on the thermodynamic stability of such a putative loop-inserted canonical form. Instead, the stabilities of native and T345R variant α_1-PIs were found to be the same. This argues strongly against any loop insertion in the active state of the serpin.

In conclusion, the present results show that the main effect of variation in the P14 residue of α_1-PI is to alter the rate of the inhibitory and/or substrate reactions, without affecting the basic mechanism of interaction with proteinase, the rate of formation of the initial encounter complex, or the ability of the reactive center loop to insert into β-sheet A, even with a substitution as nonconservative as threonine → arginine. These findings are in accord with a common branched pathway suicide substrate inhibition mechanism for the interaction of all serpins with target proteinases, including apparently noninhibitory serpins such as ovalbumin, but contradict the belief that a charged residue at the P14 position of serpins is sufficient to preclude inhibition by the serpin. The outcome of a given serpin–proteinase interaction depends on the relative values of k_4 and k_3. This can produce a range of results, from complete inhibition at one extreme to complete substrate reaction at the other extreme, even though a common mechanism is in operation in all cases. For a given serpin–proteinase interaction, the outcome will depend on both the serpin and the proteinase, since a given mutation may have different relative effects on k_4/k_3 in different serpins and different proteinases may affect k_4/k_3 differently in the same serpin.

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