Characterization of a Human α₁-Antitrypsin Variant That Is as Stable as Ovalbumin*

Kee Nyung Lee‡, Hana Im, Sang Won Kang§, and Myeong-Hee Yu¶

From the Division of Protein Engineering, Korea Research Institute of Bioscience and Biotechnology, P. O. Box 115, Yusong, Taejon 305-600, Korea

The metastability of inhibitory serpins (serine proteinase inhibitors) is thought to play a key role in the facile conformational switch and the insertion of the reactive center loop into the central β-sheet, A-sheet, during the formation of a stable complex between a serpin and its target proteinase. We have examined the folding and inhibitory activity of a very stable variant of human α₁-antitrypsin, a prototype inhibitory serpin. A combination of seven stabilizing single amino acid substitutions of α₁-antitrypsin, designated Multi-7, increased the midpoint of the unfolding transition to almost that of ovalbumin, a non-inhibitory but more stable serpin. Compared with the wild-type α₁-antitrypsin, Multi-7 retarded the opening of A-sheet significantly, as revealed by the retarded unfolding and latency conversion of the native state. Surprisingly, Multi-7 α₁-antitrypsin could form a stable complex with a target elastase with the same kinetic parameters and the stoichiometry of inhibition as the wild type, indicating that enhanced A-sheet closure conferred by Multi-7 does not affect the complex formation. It may be that the stability increase of Multi-7 α₁-antitrypsin is not sufficient to influence the rate of loop insertion during the complex formation.

The serpin (serine proteinase inhibitor) superfamily includes inhibitors such as α₁-antitrypsin (α₁AT), antithrombin, α₁-antichymotrypsin, C1 inhibitor, and non-inhibitory members such as ovalbumin and angiotensinogen (1). Serpins share a common tertiary structure composed of three β-sheets and several α-helices (Fig. 1). One of the intriguing aspects of serpin structure is that the native conformation of the inhibitory serpins is strained (2–4). Proteolytic cleavage of the reactive center loop or the conversion into the more stable latent form (Fig. 1) accompanies a complete insertion of the reactive center loop or the conversion into the more stable latent form (12). It is possible that the native strain of inhibitory serpins is utilized for the facile loop insertion during the complex formation with a target proteinase (9, 12–14). However, precise information is needed on the mechanism underlying the complex formation is yet to be elucidated.

Previously we have identified several hydrophobic core mutations of α₁AT that increased the conformational stability, presumably by enhancing the closing of the A-sheet (12). The mutations did not affect proteinase binding, as revealed by the unchanged association rate constants with porcine elastase. It was suspected, however, that these mutations would affect the formation of the stable complex upon proteinase binding by retarding the loop insertion, resulting in substrate-like behavior. In the present study we tested this assumption by analyzing a very stable α₁AT carrying seven stabilizing mutations (Fig. 1: F51L, T59A, T68A, A70G, M374I, S381A, and K387R) identified previously (12). The mutant was designated Multi-7. The mutational effects on the inhibitory activity as well as other structural properties such as folding-unfolding transition were also examined.

MATERIALS AND METHODS

Recombinant α₁AT Proteins and Plasmids—The plasmid for α₁AT expression in Escherichia coli (15), the purification of recombinant α₁AT protein (15), and the detailed method for in vitro translation products of α₁AT (16) were described previously. The plasmid for the in vitro translation of ovalbumin was constructed by substituting α₁AT cDNA in the in vitro translation vector, p[B(LG)AT] (16), with cDNA of ovalbumin from pYOV5 (gift from Dr. H. J. Kim). Concentrations of α₁AT were determined in 6 M guanidine hydrochloride using a value of A₂₈₀ = 4.3 at 290 nm, calculated from the tyrosine and tryptophan content of the α₁AT protein (17) and based upon Mᵣ = 44,250. α₁AT activity was measured as residual porcine pancreatic elastase activity employing 1 mM N-succinyl-(Ala)₇-p-nitroanilide as a chromogenic substrate (18). Individual thermostable mutations of α₁AT were previously reported (12). Combination of the mutations of α₁AT and site-specific mutations of ovalbumin were made by oligonucleotide-directed mutagenesis (19).

Chemicals—Ultrapure urea was purchased from Schwarz/Mann. [³⁵S]Methionine was purchased from NEN Life Science Products. Human plasma α₁AT, porcine pancreatic elastase, human leukocyte elastase, bovine pancreatic trypsin N-succinyl-(Ala)₇-p-nitroanilide, and N-methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroanilide were purchased from Sigma. All other chemicals were reagent grade.

Urea-induced Equilibrium Unfolding Transition—Equilibrium unfolding as a function of urea was monitored by fluorescence spectroscopy and CD spectroscopy, details of which were described previously (12, 15). The buffer used for unfolding experiments was 10 mM phosphate,
FIG. 1. **Comparison of the native, cleaved, and the latent structures of inhibitory serpins.** The **Native** structure is a ribbon diagram of the crystal structure of Multi-7 α₃AT (32). The side chain atoms for the seven stabilizing substitutions in Multi-7 are represented. The **Cleaved**
50 mM NaCl, 1 mM EDTA, 1 mM β-mercaptoethanol, pH 6.5, unless described otherwise. The native protein was incubated in the folding buffer containing various concentrations of urea at 25 °C. The protein concentration for the unfolding transition was 20 μg/ml for fluorescence spectroscopy and 50 μg/ml for CD spectroscopy. Experimental data of the fluorescence measurement were fitted to a two-state unfolding model, as described previously (12, 15).

Kinetics of Unfolding and Refolding—The kinetic study of unfolding and refolding of α1AT was performed by measuring the change of fluorescence intensity (λex = 280 nm and λem = 360 nm) of the native or unfolded proteins upon exposure to various concentrations of urea (12). The final protein concentration was 5 μg/ml, and the experimental temperature was 25 °C. The major kinetic phase was examined, and the data were fitted to a two-state (for unfolding) or three-state (for refolding) model to obtain the relaxation time: $F(t) = \sum F_i e^{-t/\tau_i} + F_{\infty}$, where $F(t)$ is the fluorescence at time t, $F_i$ is the fluorescence of phase i at zero time, $\tau_i$ is the relaxation time for phase i, and $F_{\infty}$ is the fluorescence at infinite time (Sigma Plot, Jandel Corp.).

Transverse Urea Gradient Gel Electrophoresis—Gels were prepared with a gradient of 0–8% urea perpendicular to the direction of electrophoresis with an opposing gradient of acrylamide from 15 to 11% (20). Four slab gels (100 × 80 mm) were prepared simultaneously in a multigel caster ( Hoefer) by using a gradient maker and a single channel peristaltic pump. The electrode buffer was 50 mM Tris acetate, 1 mM EDTA, pH 7.5. The native protein (20 μg/ml in 100 μl), the protein unfolded in 8% urea (10 min at room temperature), or the in vitro translation product was applied across the top of the gel. The gels were run at a constant current of 6 mA for 3 h at a controlled temperature of 25 °C. The protein bands were visualized either by Coomassie Brilliant Blue staining or by autoradiography.

Conversion into the Latent Form—Formation of the latent α1AT was examined with in vitro translation products, as described (12). Conditions for the formation of latent α1AT have been reported previously (21). The translation products labeled with [35S]methionine were incubated in assay buffer (30 mM phosphate, 160 mM NaCl, 0.1% PEG 4000, and 0.05% sodium citrate). Various concentrations of α1AT were analyzed by transverse urea gradient gel electrophoresis.

Complex Formation with Proteinases—Complex formation of Multi-7 α1AT with a proteinase was examined by monitoring the SDS-resistant α1AT-proteinase complex. Samples were incubated at 37 °C for 10 min and were analyzed by 10% SDS-polyacrylamide gel electrophoresis. The protein bands were visualized by Coomassie Brilliant Blue staining. Concentrations of α1AT were determined by A260 in 6% guanidine hydrochloride (17). The active site concentration of human leukocyte elastase was determined as described previously (22) with trypsin-titrated human plasma α1AT and a substrate, N-methoxy succinyl-Ala-Ala-Pro-Val-p-nitroanilide. The active concentration of porcine pancreatic elastase was determined by measuring the initial rates of hydrolysis of 1 mM N-succinyl-Ala-Ala-p-nitroanilide by increasing the concentration of elastase, using a previously reported standard titration curve (24). The stoichiometry of inhibition was determined by titration reactions as described (25). The reaction mixture (50 μl volume) in the assay buffer contained 100 mM porcine or human elastase. After incubation with various amounts of recombinant wild-type or Multi-7 α1AT for 10 min at 37 °C, the reaction mixture was diluted 10-fold with the assay buffer, and residual enzyme activity was determined.

Inhibitory Parameters of α1AT—Inhibition kinetic studies for the interaction of α1AT with porcine pancreatic elastase were performed by analyzing progress curve kinetic experiments (26). The active concentration of porcine pancreatic elastase was determined as described above. The active site titration of α1AT was measured by employing 1 mM N-succinyl-Ala-Ala-p-nitroanilide and a known activity of porcine pancreatic elastase (18). The assays were performed at 25 °C in reaction buffer containing 50 mM Tris, 50 mM NaCl, 0.1% PEG 4000, and 0.05% v/v Triton X-100, pH 8.0, and started by the addition of elastase, at the final concentration of 1.25 mM. A typical progress curve experiment consisted of 6 assays (1 zero and 5 non-zero concentrations of inhibitor) and the slow development of inhibition was determined by continuously monitoring the appearance of p-nitroaniline at 410 nm. The amount of product formed was calculated by using a molar absorption coefficient of 8,800 M cm⁻³ for p-nitroaniline at 410 nm. The inhibition of the enzyme (E) by α1AT (I) is described in Scheme I, where S is the substrate, and P is p-nitroaniline.

$$\frac{K_m}{E + S} \times \frac{h_{\text{ex}}}{E + P} + I \times \left( \frac{K_i}{h_1} \right) = \frac{K_{\text{cat}}}{E I}$$

(The data were fitted by nonlinear regression to Equation 1 describing this mechanism of slow, tight-binding mechanism as below (28).)

$$P = V_0 + \frac{(V_{-1} - V_0)(1 - d)}{d} \log \left( \frac{(1 - d \exp(-k' t))}{1 - d} \right)$$

where $P$ is the amount of product formed at time, $t$, $d$ is a function of $E$, $I$, and $k'$ is a function of these parameters and the observed second-order rate constant ($k_0$) for the interaction between the inhibitor and enzyme. The analyses yield values for apparent association rate constant ($k_0$) and the apparent dissociation constant ($K_{\text{cat}}$). The value of the Michaelis constants ($K_{\text{cat}}$) required in these calculations was 1.15 mM for Suc-Ala-Ala-p-nitroanilide (24).

RESULTS

Equilibrium Stability of Multi-7 α1AT—Equilibrium unfolding transition of Multi-7 α1AT was examined as a function of increasing urea concentration to 25 °C. When the transition was monitored by intrinsic fluorescence intensity (Fig. 2A), the midpoints of transition of the wild-type and the mutant protein were 1.8 and 4.8 M urea, respectively, yielding $\Delta \Delta G$ of 8 kcal mol⁻¹. A similar shift in the transition midpoint was observed by far UV CD signal (Fig. 2B) or by transverse urea gradient gel electrophoresis (Fig. 2C). The unfolding transition of the wild-type α1AT monitored by far UV CD signal or urea gradient gel electrophoresis exhibited at least two phases, indicative of at least one equilibrium unfolding intermediate. Because of the midpoint shift of the first unfolding phase, the equilibrium unfolding intermediate was not detected with the Multi-7 mutant protein. The unfolding transition was reversible for both the wild-type and the mutant proteins, as revealed by refolding on urea gradient gel electrophoresis (Fig. 2C, Refolding). The transition of Multi-7 was rather abrupt at the transition midpoint.

The Multi-7 α1AT Is as Stable as Ovalbumin—The stability of Multi-7 α1AT was compared with that of ovalbumin with in vitro translation products on urea gradient gel electrophoresis. As shown in Fig. 3, ovalbumin is much more stable than the wild-type α1AT, and its unfolding transition follows a two-state process. The overall transition of ovalbumin was very similar to that of Multi-7 α1AT. In vitro translation product of the wild-type ovalbumin yielded two species of the native form that exhibited a slight difference in unfolding transition on urea gradient gel electrophoresis (Fig. 3, Ovalbumin, Wt). Ovalbumin contains one disulfide bond between Cys-73 and Cys-120, which is not present in α1AT. To compare the stability of Multi-7 α1AT with that of ovalbumin without disulfide contribution, we constructed recombinant versions of ovalbumin that cannot form the disulfide bond by substituting cysteines into alanines. The mutant ovalbumin yielded only a single species of the native form on urea gradient gel with the same unfolding midpoint as Multi-7 α1AT (Fig. 3, Ovalbumin, C73A). The
amino acid substitutions themselves do not appear to contribute significantly to the stability because all three different forms of mutant ovalbumin (C73A, C120A, and C73A/C120A) showed the unfolding transition at a urea concentration similar to that of the less stable wild-type species (data not shown). The stability of Multi-7 was similar to that of the mutant ovalbumin which could not form the disulfide. The unfolding transition of both Multi-7 α1AT and ovalbumin were abrupt.

**Kinetic Analysis of Unfolding and Refolding of Multi-7 α1AT**—Kinetic unfolding and refolding of Multi-7 α1AT were performed by monitoring the intrinsic fluorescence change as a probe. Fig. 4 shows that the unfolding of α1AT occurs in an all-or-none process at all urea concentrations, and the unfolding of Multi-7 α1AT was retarded significantly. Refolding of α1AT exhibited multiple kinetic states. There were two major refolding phases in which the intensity of fluorescence decreased: a fast decrease ($\tau = 200–500$ s), and a further slow decrease ($\tau = 1,000–3,000$ s). The amplitudes of the two phases were about same at near zero urea concentration, but the amplitude of slow phase increased as a function of urea. Refolding of Multi-7 α1AT was facilitated slightly in both phases, but urea dependence of the refolding rate at lower urea concentrations disappeared in such a way that the difference in refolding rate was minimized when extrapolated to 0 M urea (the relaxation time of 339 and 106 s at 0 M urea for the fast phase of wild-type and Multi-7, respectively). In particular, the
rate of the slow refolding phase, which intersects with the unfolding kinetics at the same urea concentration (1.9 and 4.8 mM for the wild-type and Multi-7, respectively) as the equilibrium midpoint (Fig. 2A), was not significantly altered in the Multi-7 mutant (1072 s at 0 mM urea for both). The amplitude of both phases was not changed by the mutation. These results indicate that the major effect of the Multi-7 mutation in the unfolding-refolding transition is the retardation of the unfolding rate.

Conversion of Multi-7 α1AT into the Latent Form—The native structure of inhibitory serpins is considered as a kinetically trapped folding intermediate because the intact native form can convert into a more stable latent form. It was expected that the mutations that stabilize the native state of serpin will retard the conversion into the latent state. This was the case with Multi-7 α1AT, as shown in Fig. 5. Unlike the native form of α1AT that undergoes unfolding transition in urea (Fig. 2C), the latent form does not unfold even in the presence of 8 M urea. When heat denaturation was performed under conditions where the production of the latent α1AT was favored (about 50% of the wild-type α1AT converted into the latent form), the Multi-7 mutant converted into the latent form much less readily than the wild-type protein. The results showed that the Multi-7 mutation retarded the insertion of the reactive center loop into the A-sheet, although the accessibility of the loop insertion was not affected.

Inhibitory Activity of Multi-7 α1AT—To investigate the effect of the Multi-7 mutation in α1AT on the inhibitory activity, complex formation of wild-type and Multi-7 α1AT with various proteinases was examined. Both wild-type and the mutant inhibitor formed a tight SDS-resistant proteinase-inhibitor complex with porcine and human elastases (Fig. 6A). In addition, the mutation did not alter the partitioning between the inhibitory and substrate pathways, as revealed by the unchanged values of stoichiometry of inhibition in the titration of elastase by α1AT (Fig. 6B: 1.1 and 1.7 for human and porcine elastase, respectively). This was also confirmed by densitometric scanning of the SDS-resistant complex formation shown in Fig. 6A. Inhibition kinetic studies showed that the association rate constant (k<sub>a</sub>) of the wild-type and the Multi-7 mutant α1AT for porcine pancreatic elastase was 5.11 ± 0.7 × 10<sup>5</sup> M<sup>−1</sup>s<sup>−1</sup> and 5.03 ± 1.0 × 10<sup>5</sup> M<sup>−1</sup>s<sup>−1</sup>, respectively. The dissociation constant (K<sub>d</sub>) was 95 ± 24 ps and 117 ± 29 ps for the wild type and the Multi-7, respectively. The results clearly showed that the ability of α1AT to form a complex with a target proteinase was not affected by the Multi-7 mutation.

DISCUSSION

It has been suggested that rapid insertion of the reactive center loop into the A-sheet during the complex formation with a target proteinase is critical for the inhibitory activity of serpins (8, 11). In the present study, we examined the effect of Multi-7 mutation on the inhibitory activity of human α1AT, which stabilized the native state of the molecule and retarded opening of the A-sheet. The mutant α1AT could form a stable complex with various target proteinases, and the stoichiometry of inhibition was not affected. These results indicate that the A-sheet closure conferred by the stability increase of Multi-7

![Image](https://example.com/image.jpg)

**FIG. 4.** Kinetics of unfolding and refolding Multi-7 α1AT. The relaxation time of urea-dependent unfolding (filled symbols) and refolding (open symbols) of the wild-type (●, ○, and □) and Multi-7 (▲, △, and ∇) α1AT monitored by the fluorescence signal was plotted. α1AT in the native or unfolded state was subjected to structural change in various concentrations of urea, and the time taken to reach a new equilibrium was analyzed by monitoring the fluorescence intensity (λ<sub>m</sub> = 280 nm and λ<sub>em</sub> = 360 nm). The final protein concentration was 5 μg/ml. For refolding, the native protein was unfolded in 8 M urea for 5 min, and refolding was initiated by the addition of the unfolded protein to urea at the designated concentrations.

**FIG. 3.** Stability of Multi-7 α1AT and ovalbumin on urea gradient gel electrophoresis. Unfolding of in vitro translation products of Multi-7 α1AT and ovalbumin analyzed on transverse urea gradient gel electrophoresis. The translation products labeled with [35S]methionine for 1 h were analyzed on urea gradient gel electrophoresis. The protein bands were visualized by autoradiography.
α1-antitrypsin does not affect the complex formation with target proteinases. The results support the notion that, in addition to intramolecular interactions, other interactions such as contact between the inhibitor and its target proteinase contribute significantly to the conformational change needed for the complex formation.

Conformational Properties of Multi-7 αAT—The equilibrium unfolding of the wild-type αAT exhibited at least two phases with one equilibrium intermediate, which is compact (Fig. 2C) and retains approximately 70% of the native CD signal (Fig. 2B) but most of the native fluorescence is de-quenched (Fig. 2A). Unfolding of the Multi-7 αAT molecule did not exhibit the equilibrium unfolding intermediate (Fig. 2, B and C). Moreover, the unfolding pattern of Multi-7 αAT on urea gradient gel electrophoresis was very similar to that of ovalbumin (Fig. 3). Ovalbumin, although sharing a common tertiary fold with inhibitory serpins, is more stable and is not active as a proteinase inhibitor. Molecular properties of ovalbumin are quite different from those of inhibitory serpins. For instance, the equilibrium unfolding transition of inhibitory serpins including α1AT (Fig. 2) is very complex (5, 27–29), whereas the unfolding transition of ovalbumin is more or less fitted to a two-state model when monitored either by far UV CD signal (5), intrinsic fluorescence (30), or by UV absorbency and intrinsic viscosity (31). The unfolding midpoint of C73A mutant ovalbumin, which could not form disulfide bonds, was very close to that of Multi-7 α1AT (Fig. 3). Previous studies on the reversible unfolding of disulfide-reduced authentic ovalbumin also yielded the same value (4.8 M) of transition midpoint (Cm) in urea as Multi-7 α1AT at 25 °C (30). The difference in Cm values of the wild-type and Multi-7 α1AT yields ΔG of 8 kcal/mol, using the m (measure of dependence of ΔG on denaturant concentration) value of 2.6 determined previously (12). It is interesting to note that neither the Multi-7 mutant α1AT nor ovalbumin exhibit a smooth unfolding transition seen in regular globular proteins (Fig. 3). It appears that the equilibrium unfolding of Multi-7 α1AT resembles that of ovalbumin.

Retarded A-sheet Opening of Multi-7 α1AT—The x-ray crystal structure of Multi-7 α1AT (Fig. 1) was determined with 2.7-Å resolution recently (32). The uncleaved wild-type structure has yet to be determined. Mutation sites of Multi-7 α1AT appear to be well packed as in ovalbumin (33) and the presumed native state of the wild-type around the mutation sites would increase steric hindrance. The reactive center loop of Multi-7 α1AT is not inserted into the A-sheet at all, as in the crystal structure of intact α1-antichymotrypsin (34). A peculiar feature of Multi-7 α1AT is that strands 3 and 5 of the A-sheet are hydrogen-bonded all the way up to the top of the sheet, which is not the case in other native structures of inhibitory serpins (34–36) and ovalbumin (33).

The following experimental results support that the A-sheet of the wild-type α1AT is more open than that of Multi-7, although the structure of the intact wild-type α1AT is not known. First, kinetic analysis revealed that the unfolding of the Multi-7 mutant α1AT was retarded significantly, and refolding was facilitated only slightly (Fig. 4). The refolding rates of Multi-7 in both phases were urea-independent at lower urea concentrations and approach the refolding rates of the wild type. One interpretation of such urea independence is that the observed change in the refolding rates is due to the stabilization of a kinetic folding intermediate, as with the case of ubiquitin mutants (37, 38). If this is the case with Multi-7 α1AT, the intrinsic refolding rates are not altered significantly by the Multi-7 mutation. The results are consistent that the stability increase by the Multi-7 mutation is mainly due to the stabilization of the native state as opposed to the destabilization of the unfolded state. Another line of experimental evidence is that Multi-7 α1AT is not as readily converted into the latent form as the wild type (Fig. 5). The native form of α1AT can be converted into the latent form upon heat treatment in the presence of 0.7 M citrate (21), which did not show unfolding transition in urea gradient gel electrophoresis (Fig. 5). It was shown previously that the reactive center loop of the latent form produced under this condition was not accessible for the proteolytic attack by V8 proteinase (12). Since the conversion into the latent form requires the insertion of the reactive center loop into the A-sheet (3), it is very likely that the A-sheet is more closed in Multi-7 than in the wild-type α1AT.

Inhibitory Activity of Multi-7 α1AT—Various biochemical and structural studies with the mutant forms of inhibitory serpins suggest that the loop insertion is necessary for the formation of a stable complex although not sufficient to confer inhibitory activity (8–10). Alteration of inhibitory function by oversized or charged amino acid substitutions at the P-even positions (P14, P12, and P10) in the reactive center loop region was attributed to a retarded loop insertion (39–43). It was
expected therefore that mutations that interfere with the loop insertion, like Multi-7 in this study, would affect the formation of the stable complex by retarding the loop insertion, thus inducing substrate-like behavior, even if binding proteinase was not affected. The Multi-7 α₁AT did not affect the inhibitory activity measured by inhibition kinetic experiments, nor did the Multi-7 mutation significantly influence the partitioning between the inhibitory pathway and the substrate pathway (Fig. 6). In addition, the stability of the complexes was not influenced by the Multi-7 mutation up to 72 h examined (data not shown). It was reported that the partitioning of C1 inhibitor between the inhibitory and substrate pathways are temperature-dependent; the substrate pathway is more favorable at low temperatures (44). This suggests that a kinetic step is involved in complex formation. We examined the complex formation at a low temperature (20 °C) but could not detect any mutational effects, although more of the cleaved forms are produced at 20 than at 37 °C in all cases (data not shown). These results suggest that enhanced A-sheet closure conferred by the stability increase of the Multi-7 molecule is not sufficient to influence the complex formation with target proteinases.

Implication on the Mechanism of the Complex Formation—It is very likely that the metastability of inhibitory serpins is utilized for the facile loop insertion during the complex formation with a target proteinase (9, 12–14). It was suggested that the drive toward a more stable state due to metastability results in trapping the proteinase-inhibitor complex as an acyl-enzyme inhibitor (13, 14), possibly accompanying conformational change in proteinases also (45, 46), including a distortion of the active site (47). We have constructed a variant α₁AT that is as stable as ovalbumin (ΔΔG > 8 kcal/mol), but the mutational effect on the inhibitory activity is not comparable with the dramatic shift in stability. How can one reconcile such a paradox? It is possible that the drive stemmed from the hydrophobic core of the serpin molecule can indeed influence the conformational shift during the complex formation, but such a drive is only slightly diminished for Multi-7 α₁AT. The increase in stability by Multi-7 may be only a small fraction of much greater binding energy of the complex between a serpin inhibitor and a target proteinase, and consequently the stability increase of Multi-7 is not sufficient to influence the rate of loop insertion during the complex formation. Since the stoichiometry of inhibition is defined by (1 + k_{substrate}/k_{inhibitory}), the value is not sensitive to the changes of partitioning when one of the pathways is dominant (e.g., either when the value is close to 1 as with the interactions of many inhibitory serpins and their cognate target proteinases or when the value is very large as with ovalbumin). For instance, increasing the rate of the inhibitory pathway of ovalbumin significantly by hinge region mutations does not lead to detectable complex formation (48). There is, however, another class of mutations that particularly affects the stoichiometry of inhibition significantly. Many of the mutations in the reactive loop region such as G349P (22) or T345R (40) of α₁AT induce the substrate pathway more effectively, even if they may not increase the conformational stability as much as Multi-7. They may exert the mutational effect by interfering with the loop insertion more directly (e.g., blocking accessibility). None of the seven mutations in Multi-7 is located in the region directly involved in the loop insertion or when the value is large as with ovalbumin). For instance, increasing the rate of the inhibitory pathway of ovalbumin significantly by hinge region mutations does not lead to detectable complex formation (48).

Finally, our results suggest that target enzymes contribute to the complex formation with serpins. The mutational effect of Multi-7 on the inhibitory function was not manifested with the

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**Fig. 6.** Mutational effects on the formation of proteinase-α₁AT complex. A, complex formation of α₁AT at increasing concentrations of elastase. Wild-type (lanes 2–5) or Multi-7 (lanes 6–9) α₁AT was incubated with increasing amounts of human leukocyte elastase (HLE, top panel) and porcine pancreatic elastase (PPE, bottom panel). The molar ratios of elastase over α₁AT were 0 (lanes 2 and 6), 0.1 (lanes 3 and 7), 0.2 (lanes 4 and 8), and 0.4 (lanes 5 and 9). Conditions for the incubations are given under “Materials and Methods.” The formation of the SDS-resistant α₁AT-elastase complex was analyzed on 10% SDS-PAGE. The protein bands were visualized by Coomassie Brilliant Blue staining. Lane 1 represents molecular mass standards (from the top, 200 kDa, 97.4 kDa, 68 kDa, 43 kDa, 29 kDa, and 18.4 kDa). The C and I indicate the migration position of the complex and α₁AT, respectively. B, stoichiometries of inhibition of proteinases by α₁AT. Human leukocyte elastase (filled symbols) or porcine pancreatic elastase (open symbols) was incubated with increasing amounts of wild-type (● and ○) and Multi-7 (■ and □) α₁AT at 37 °C for 10 min in the assay buffer (50 mM phosphate, 160 mM NaCl, 0.1% PEG6000, 0.1% Triton X-100, pH 7.4). The residual proteinase activity was measured with N-methoxy succinyl-Ala-Ala-Pro-Val-p-nitroanilide as a substrate for human leukocyte elastase and with N-succinyl-Ala-Ala-Ala-p-nitroanilide for porcine pancreatic elastase.
same target proteinase, but the same α1AT exhibited different values of the stoichiometry of inhibition on binding human and porcine elastases (Fig. 6B: 1.1 versus 1.7). It was suggested that enzymes also play an important role in inducing conformational changes in inhibitory serpins (49). Many experimental results support that contact between serpins and proteinases is critical. For instance, the stoichiometry of inhibition of T345R ([P14 mutant] α1AT) was quite different for human neutrophil elastase, porcine pancreatic elastase, and trypsin (40). Also G349P ([P10 mutant]) α1AT conferred different effects on human leukocyte elastase and trypsin (22). The S380W variant antithrombin showed a similar result upon binding factor Xa, thrombin, and trypsin (50). These and our results clearly show that the same serpin molecule can confer a different effect on the same target proteinase, but the same α1AT may be that contact between a serpin and its target proteinase contributes significantly to the conformational change needed for the loop insertion.

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Kee Nyung Lee, Hana Im, Sang Won Kang and Myeong-Hee Yu

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