Concerted Regulation of Inhibitory Activity of α₁-Antitrypsin by the Native Strain Distributed throughout the Molecule*

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The native forms of common globular proteins are in their most stable state but the native forms of plasma serpins (serine protease inhibitors) show high energy state interactions. The high energy state strain of α₁-antitrypsin, a prototype serpin, is distributed throughout the whole molecule, but the strain that regulates the function directly appears to be localized in the region where the reactive site loop is inserted during complex formation with a target protease. To examine the functional role of the strain at other regions of α₁-antitrypsin, we increased the stability of the molecule greatly via combining various stabilizing single amino acid substitutions that did not affect the activity individually. The results showed that a substantial increase of stability, over 13 kcal mol⁻¹, affected the inhibitory activity with a correlation of 11% activity loss per kcal mol⁻¹. Addition of an activity affecting single residue substitution in the loop insertion region to these very stable substitutions caused a further activity decrease. The results suggest that the native strain of α₁-antitrypsin distributed throughout the molecule regulates the inhibitory function in a concerted manner.

The native forms of common globular proteins are in their most stable state and protein folding is a spontaneous process (1). However, the native forms of some proteins are not in their most stable state: typical examples are the strained native structure of plasma serpins1 (serine protease inhibitors) (2), the spring-loaded structure of the fusion protein of some viruses (3, 4), and heat shock transcription factors (5). The high energy state of the native structure of serpins is considered to be crucial to their physiological functions, such as plasma protease inhibition (1, 6), hormone delivery (7), Alzheimer filament assembly (8, 9), and extracellular matrix remodeling (10). The inhibition process of serpins can be described as a suicide substrate mechanism (11, 12), in which serpins, upon binding with proteases, partition between cleaved serpins (substrate pathway) and stable serpin-enzyme complexes (inhibitory pathway) as described in Scheme 1.

In this scheme, I denotes the serpin; E, protease; EI, noncovalent Michaelis complex; E-I, a proposed intermediate prior to partitioning; E-I*, stable enzyme-inhibitor complex; and I*, cleaved serpin. The stoichiometry of inhibition (SI, the number of moles of inhibitors required to completely inhibit 1 mol of a target protease) is given by 1 + \( k_{\text{substrate}}/k_{\text{inhibition}} \), in which \( k_{\text{substrate}} \) and \( k_{\text{inhibition}} \) are the rate constants for the substrate and inhibitory pathways, respectively. The crystal structure of a serpin-protease complex revealed that the reactive site loop of the serpin is cleaved and inserted into the major β-sheet, sheet A, in the complex, whereas the target protease is attached to the cleaved loop of the serpin as an acyl intermediate (13). The conformational conversion during complex formation accompanies the distortion of the protease active site (13), which prevents catalytic deacylation and results in trapping the stabilized complex. Flexibility of the native serpin structure is required for conformational conversion, and the high energy state of the native conformation appears to be a driving force for this conversion.

To understand the structural basis and mechanistic consequences of the high energy state of native serpins, we have characterized stabilizing amino acid substitutions of human α₁-antitrypsin (α₁AT), a prototype serpin (14–18). In the crystal structure of the native α₁AT (19, 20), unfavorable interactions such as side chain overpacking, buried polar groups, internal cavities, and surface hydrophobic pockets occur (16, 17). These unfavorable interactions can be replaced by stabilizing substitutions (21, 22). We screened thermostable mutations over the entire α₁AT molecule and found stabilizing mutations that influence the flexibility of the native state distributed throughout the molecule (17). If the increase in the stability of the native state of α₁AT is manifested in the formation of the inhibitory complex, there should be a correlation between that increase in stability and the loss of inhibitory activity. Interestingly, however, only the stabilizing substitutions in the loop insertion region, such as Lys\(^{335}\) (23) and Gly\(^{117}\) (18) of sheet A, decreased the inhibitory activity, and the stabilizing mutations at most other sites of α₁AT did not cause the activity loss (17). It was shown recently that cavity filling substitutions designed at several sites of α₁AT increased the stability of the molecule, but activity affecting mutations among them were localized in the region that appears to be mobilized during the loop insertion (22). Thus, the high energy state in the loop insertion region appeared to be a nature’s
design for functional regulation but such a role of the strain at most other sites was not obvious. In the present study, we addressed this point by examining very stable mutations that were made through combining various single residue substitutions that did not affect the inhibitory activity individually. Characterization of the stable mutations suggests that the strain of αTAT scattered over the molecule does regulate the inhibitory activity.

MATERIALS AND METHODS

Chemicals—Ultrapure guanidine hydrochloride was purchased from ICN Biochemicals, Inc. (Aurora, IL). Porcine pancreatic elastase (PPE), human leucocyte elastase (HLE), N-succinyl-(Ala),-p-nitroanilide, and N-methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroanilide were purchased from Sigma. All other chemicals were reagent grade.

Recombinant αTAT Proteins—The plasmid for αTAT expression in *Escherichia coli* and the purification of recombinant proteins were described previously (14). Protein concentration was determined in 7 M guanidine hydrochloride, calculated from tyrosine and tryptophan content of the αTAT protein (24). Amino acid substitutions at specific sites were generated by oligonucleotide-directed mutagenesis and confirmed by DNA sequencing. αTAT cleaved at the reactive site loop was prepared by incubating with PPE at a molar ratio of 1:0.4 (αTAT:protease) at 37 °C for 1 h in a buffer containing 50 mM Tris-HCl, 50 mM NaCl, pH 8.0. Phenylmethylsulfonyl fluoride was added at a final concentration of 1 mM to stop the reaction. Uncleaved molecules were removed by precipitating them at 70 °C with 0.5 M guanidine hydrochloride solution of 10 mM potassium phosphate, 50 mM NaCl, 1 mM EDTA, 1 mM β-mercaptoethanol, pH 6.5, at 25 °C for 4 h. The concentration of protein was 20 μg/ml for spectrofluorometry and CD spectroscopy, respectively. Equilibrium unfolding monitored by fluorescence change was fitted to a two-state model or three-state model and changes in the unfolding stability was determined according to Pace and co-workers (25). Briefly, it was calculated with the fitted thermodynamic parameters and the equation, ΔΔG = <m> × ΔCm, where ΔCm is the difference between the values of Cm, equilibrium transition midpoint, for the wild type and mutant protein, and <m> is the average of the "m-value," a measure of the dependence of the free energy of unfolding (ΔG) on denaturant concentration. The <m> value used in the present study was 7.6 kcal mol⁻¹m⁻¹.

![FIG.1](Image 208x478 to 554x728) A stereo diagram of αTAT showing individual amino acid substitutions in combinatorial mutations. The atomic coordinates for the crystal structure of native αTAT were taken from the structure (PDB code: 1HP7) that was reported recently (26). The substitution sites are depicted by beads. The site of the activity affecting substitution, K335V, is colored red. The reactive site loop is represented in green, and the region mobilized upon insertion of the reactive site loop (s3α, s5α, hF, and the following loop) is represented in yellow. The figures were prepared with MOLSCRIPT (37).

![FIG.2](Image 365x198 to 497x460) A Guanidine hydrochloride-induced unfolding transition of the variant αTAT carrying combinatorial mutations. A, unfolding was monitored by the increase in fluorescence emission intensity at 360 nm (λex = 280 nm). Samples (10 μg/ml) were incubated in guanidine hydrochloride solution of 10 mM potassium phosphate, 50 mM NaCl, 1 mM EDTA, 1 mM β-mercaptoethanol, pH 6.5, at 25 °C for 4 h. The data were fitted to a three-state unfolding model and those of M13 and M16 were fitted to a two-state unfolding model. The dotted lines exhibit the fitted two-state unfolding monitored by fluorescence intensity.
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Molecular properties of multiple stabilizing mutations of α1AT

| Mutation | Locations | Component substitution | ΔG° | SI^d | Relative activity^e | k_sub/k_ hid | kcal mol^-1 |
|----------|-----------|------------------------|-----|------|---------------------|---------------|-------------|
| M2a      | hA        | A34G, S36R             | 3.0 | 0.97 | 1.03                | ~0             |             |
| M2b      | s3A       | A183F, F189V           | 5.9 | 1.03 | 0.97                | ~0             |             |
| M5a      | s3A, hF, thF,e3A   | A183V, F189V, K163T, G164V, L172V | 6.0 | 0.98 | 1.02                | ~0             |             |
| M5b      | hB, hC, s4B, s5B, h6B | F51L, T59A, T68A, M174I, S381A | 7.1 | 0.95 | 1.05                | ~0             |             |
| M7       | M5b + hC, s5B | M5b + A70G, K378R | 10.1 | 1.00 | 1.00                | ~0             |             |
| M8a      | M7 + s2C    | M7 + L2638            | 10.6 | 1.01 | 0.99                | ~0             |             |
| M8b      | M7 + s3A    | M7 + F189V            | 13.2 | 1.10 | 0.91                | ~0             |             |
| M8c      | M7 + s3A    | M7 + A183V            | 14.7 | 1.32 | 0.76                | 0.3            |             |
| M9       | M7 + hA     | M7 + A34G, S36R       | 13.5 | 1.19 | 0.84                | 0.2            |             |
| M13      | M9 + hI, thI,e5A | M9 + F189V, L293M, Q305P, N314D | 15.3 | 1.43 | 0.70                | ~0             |             |
| M14a     | M9 + s2B, s3B, hF, h1C | M9 + L241I, A248V, H269Y, F275L, K368R | 15.2 | 1.47 | 0.68                | 0.5            |             |
| M14b     | M7 + s3A    | M13 + A183V           | 18.5 | 2.78 | 0.36                | ~0             |             |
| M16      | M7 + hF, thF,e3A | M13 + K163T, G164V, L172V | 20.1 | 11.11 | 0.09                | 10.1           |             |

*a The nomenclature of secondary structures is as in Huber and Carrolli (2).

b A one-letter code for amino acids was used.

ΔG is calculated using equation, ΔΔG = (m) × ΔC_m, where ΔC_m is the difference between the value of C_m, equilibrium transition midpoint, for the wild-type (0.65 M) and mutant protein, and (m) is the average of the “m-value,” 7.6 kcal mol^-1. The experimental errors are 0.2 kcal mol^-1.

d The SI values towards HLE were determined as described in Fig. 3. The experimental errors are 0.03.

e Relative activity is indicated as the inverse of the ratio of the SI toward HLE of each mutant type over that of the wild-type (1.0 ± 0.03).

f The partitioning ratios between the substrate and inhibitory pathways (k_substrate over k_inhibition) were obtained from the SI values (SI-1).

Inhibitory Function of Stable α1AT Variants—To investigate the effect of the stabilizing mutations on the inhibitory activity of α1AT, the SI value of the variants α1AT toward HLE was measured. Fig. 3 shows that M13, M14b, and M16, but not M7, increased the SI, and the degree of increase correlated with the stability increase. The SI values of other variants α1AT are summarized in Table I. Table I also shows the mutational effect on the inhibitory activity that was obtained from the SI values of the wild type and the variant α1AT (relative activity). When the SI values toward PPE were measured for several variant forms of α1AT, similar degrees of activity decrease were observed (data not shown). Table I also shows the ratios of partitioning between the substrate and inhibitory pathways (k_substrate over k_inhibition) for each variant α1AT, which were obtained from the SI values (SI-1). When the wild-type α1AT interacts with HLE, the partitioning ratio is close to 0 because most of α1AT molecules form the inhibitory complex. The stable mutation, M16, increased this ratio up to 10, which indicates that less than 10% of the mutant molecules formed the inhibitory complex and the rest were cleaved by HLE. The association rate constant (k_a) of the variants α1AT with PPE did not differ significantly from the wild type value, 5.5 × 10^7 M^-1 s^-1 (data not shown). The association rate with HLE was over 10^7 M^-1 s^-1 and could not be determined precisely.

Combination of the Multiple Stabilizing Substitutions with Activity Affecting Single Residue Substitution—Some of the previously identified single residue substitutions caused activity decrease although they increased the stability much less than M7 did. To examine if the stable multiple substitutions constructed in the present study affect the inhibitory activity in a similar way to that of the previously identified activity affecting single residue substitutions, K335V (Fig. 1, colored red) was combined with M5b, M7, and M14a. Table II shows that the combinations increased the stability as much as expected from the sum of individual effects, and decreased the inhibitory activity more than K335V.

DISCUSSION

We examined the combinatorial effect of the stabilizing substitutions of α1AT that did not affect the activity individually. Loss of inhibitory activity was observed with the current set of the stabilizing mutations only when the stability increase was described (11). Various amounts of purified recombinant α1AT proteins were incubated with 100 nm PPE or HLE at designated molar ratios of α1AT to protease in 50 µl of assay buffer (30 mM phosphate, 160 mM NaCl, 0.1% PEG 8000, and 0.1% Triton X-100, pH 7.4). After incubation with the protease at 37 °C for 10 min, the reaction mixture was diluted 10-fold with the assay buffer and residual enzyme activity was determined. The active concentration of PPE was determined by measuring the initial rates of hydrolysis of 1 mM benzoyl-arginine-p-nitroanilide (26). The active concentration of HLE was determined as described previously (27) with trypsin-titrated human plasma α1AT and a substrate, N-succinyl-alanyl-alanyl-p-nitroanilide. The activity inhibition was extrapolated to yield the minimum molar ratio of α1AT to the protease giving 100% inhibition. The association rate constant (k_a) for the interaction of recombinant α1AT with PPE was measured under second order conditions (28) in a reaction mixture containing equimolar concentrations (8 nm) of the protease and the inhibitor.
substantial (Table I). In Fig. 4, correlation between the stability increase of $\alpha_1$AT and the decrease in the inhibition toward HLE was examined (filled circles). Any significant decrease in activity was not observed with the stability increase up to 13 kcal mol$^{-1}$, but the activity leveled off as the stability increased further with a correlation of about 11% activity loss per kcal mol$^{-1}$. This degree of activity reduction toward HLE was approximately similar to that toward PPE (data not shown). These results clearly showed that a substantial stability increase by the multiple substitutions could also affect the inhibitory function of $\alpha_1$AT, although each component substitution has inert effect on the activity. The results suggest that high energy states of $\alpha_1$AT distributed throughout the molecule regulate inhibitory function in a collective manner. Some single residue substitutions in the loop insertion region, such as those at Gly$^{117}$ or Lys$^{335}$ site, increased the stability up to 6 kcal mol$^{-1}$ with a concomitant decrease in the inhibitory activity (16–18, 22). Gly$^{117}$ and Lys$^{335}$ sites lie beneath helix F and the following loop that cover $\beta$-sheet A at the bottom half in the loop insertion region (Fig. 1), and flexibility in this region appears to be critical for the loop insertion during the complex formation. Since simultaneous stabilization at many other sites throughout the molecule is as detrimental as the stabilization in the loop insertion region (Table I), flexibility throughout the $\alpha_1$AT molecule may also be critical for the complex formation.

Regulation of Inhibitory Activity by the Conformational Properties of $\alpha_1$AT—Various studies suggested that rapid loop insertion in a serpin (12, 29–32) and active site distortion of the protease (13, 33) confer inhibitory activity on the serpins. Experimental data showed a direct correlation between retardation of the loop insertion and the activity decrease (15, 34). The stability increase in the current set of mutant proteins may also influence the complex formation by retarding the loop insertion. Why then is the activity affected only when the stability increase is over 13 kcal mol$^{-1}$? Unfolding of the wild type $\alpha_1$AT is biphasic, as probed by CD signal, although the change in fluorescence intensity monitors only the first transition (Fig. 2). Very stable variants that decreased the inhibitory activity such as M13 and M16 showed a single superimposable unfolding transition, whereas M7, which did not affect the activity, showed biphasic transition (Fig. 2B). Biphasic transition of equilibrium unfolding indicates that the molecule is made of two folding domains. The intrinsic fluorescence change monitors the environmental change near Trp$^{194}$ (35), the unique buried tryptophan residue at the top of strand 3 of sheet A. It is very likely that equilibrium fluorescence change reflects initial opening of sheet A, and unfolding of $\alpha_1$AT can be considered as opening of sheet A followed by further unfolding of the remaining secondary structures. For those mutations that show a single transition, opening of sheet A appear to be coupled with complete unfolding. It may be that such conformation property of $\alpha_1$AT as domain folding is important for proper complex formation with a target protease. There is another possibility that the energy difference between the strained native state and a more stable state in the complex becomes too small to trap the protease-inhibitor complex as an acyl-enzyme inhibitor. It was suggested that the energy difference is utilized for trapping the protease-inhibitor complex (32, 36), presumably by distorting the active site (13). If the difference becomes too small, the proposed distortion and trapping may become inefficient. Further studies will elucidate

**Table II**

| Combinations | $\Delta\Delta G$ | Relative activity of HLE | Relative activity of PPE |
|--------------|-----------------|-------------------------|------------------------|
| K335V        | 1.51            | 6.5                     | 0.50                   |
| K335V + M5b  | 2.49            | 14.0                    | 0.28                   |
| M5b          | 1.59            | 7.1                     | 1.05                   |
| K335V + M7   | 2.88            | 17.0                    | 0.30                   |
| M7           | 1.98            | 10.1                    | 1.00                   |
| K335V + M14a | 3.51            | 21.7                    | 0.14                   |
| M14a         | 2.65            | 15.2                    | 0.68                   |

$^a$ Change in the conformational stability by the mutation ($\Delta\Delta G$) was calculated as in Table I.

$^b$ Relative activity is indicated as the inverse of the ratio of the SI toward HLE or PPE of each mutant type over that of the wild-type (for HLE: 1.0 $\pm$ 0.03, for PPE: 1.6 $\pm$ 0.06).
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detailed mechanisms how the stability of a serpin molecule regulates its inhibitory activity.

Concerted Regulation of the Inhibitory Function by the Native Strain—We examined how the stable multiple substitutions constructed in the current study might influence the activity affecting single residue substitutions. The activity of K335V decreased further down when a multiple substitution, such as M5, M7, or M14, was combined (Table II; Fig. 4, open symbols). The results suggest that at least two distinct steps are involved in the regulation of the complex formation. The first step appears to be the destabilization of the interactions between helix F and sheet A, which is affected by the activity affecting single residue substitutions in this region (e.g. K335V) but not by such multiple stable substitutions as M5 or M7. The step is likely to be independent of the changes in fluorescence property and secondary structure contents. However, the activity affecting single residue substitutions also shift the unfolding midpoints monitored by fluorescence and CD signals with a good correlation with the activity increase (16, 18). Therefore, the step is likely to be prerequisite to opening of sheet A detected by fluorescence and CD.

The second step is opening of sheet A for the insertion and locking of the reactive site loop. This step appears to require flexibility of the whole molecule, because the inhibitory activity is affected when the step is coupled with global stability and with the very stable multiple substitutions. Whereas the interactions at specific location are important in the first step, collective molecular properties may be as important in the second step. Our results clearly suggest that high energy states of α1AT regulate concertedly various steps of the complex formation.

In summary, we probed the functional role of the native strain of α1AT that is distributed throughout the molecule. Although stability of the wild type α1AT is optimized and is designed in such a way that the activity is not sensitive to a minor change in the stability, a substantial stability increase of α1AT affected the inhibitory activity of the molecule. The opening of sheet A, a critical step for the loop insertion, appears to be regulated by such conformational properties as domain folding and global stability as well as local high-energy states in the loop insertion region.

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