Distribution of the Native Strain in Human α₁-Antitrypsin and Its Association with Protease Inhibitor Function*

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Serine protease inhibitors (serpins) are metastable in their native state. This strain, which is released upon binding to target proteases, is essential for the inhibitory activity of serpins. To understand the structural basis of the native strain, we previously characterized stabilizing mutations of α₁-antitrypsin, a prototypical inhibitory serpin, in regions such as the hydrophobic core. The present study evaluates the effects of single point mutations throughout the molecule on stability and protease inhibitory activity. We identified stabilizing mutations in most secondary structures, suggesting that the native strain is distributed throughout the molecule. Examination of the substitution patterns and the structures of the mutation sites revealed surface hydrophobic pockets as a component of the native strain in α₁-antitrypsin, in addition to the previously identified unusual interactions such as side chain overpacking and cavities. Interestingly, many of the stabilizing substitutions did not affect the inhibitory activity significantly. Those that affected the activity were confined in the regions that are mobilized during the complex formation with a target enzyme. The results of our study should be useful for designing proteins with strain and for regulating the stability and functions of serpins.

Human α₁-antitrypsin (α₁AT) is a prototype of the serpin (serine protease inhibitor) superfamily that shares a common tertiary structure composed of three β-sheets and several α-helices (1). Serpins include protease inhibitors in blood plasma such as α₁AT, α₁-antichymotrypsin, antithrombin III, plasminogen activator inhibitor-I, C1 inhibitor, and α₂-antiplasmin, as well as non-inhibitory members such as ovalbumin and angiotensinogen (1, 2). One salient feature of the inhibitory serpin structure is the strain in the native conformation (3–7), which is necessary for biological functions such as protease inhibition and ligand binding (2, 1, 9, 8). The inhibition process of serpins can be described as a suicide substrate mechanism (10–12) in which serpins, upon binding proteases, partition between cleaved serpins and stable serpin-enzyme complexes. The stoichiometry of inhibition (SI; the number of moles of inhibitor required to completely inhibit 1 mol of a target protease) is designated as 1 + \( k_{\text{substrate}}/k_{\text{inhibition}} \) in which \( k_{\text{substrate}} \) is the rate constant for the substrate pathway toward the cleaved serpin and \( k_{\text{inhibition}} \) is the rate constant for the inhibitory pathway toward the complex formation. For cognate target proteases, most serpin molecules partition into the complex formation, bringing the SI values close to 1. During the complex formation, the reactive center loop (RCL) of inhibitory serpins is cleaved (13–15) and inserted into the major β-sheet, A sheet, forming a stable complex between the serpin and the protease (11, 12, 15–17). It has been suggested that the rate of loop insertion is critical for inhibitory function; retardation of the loop insertion would alter the partitioning between the inhibitory and substrate pathways in such a way that the SI values would increase (11, 12, 18, 19). Release of the native strain of serpins, upon interaction with a protease, may regulate conformational changes such as loop insertion during the complex formation.

To elucidate the structural basis and functional roles of the native strain, we have previously characterized the effects of single amino acid substitutions of α₁AT, which increased the stability of the molecule presumably by releasing the native strain at the substitution sites (5, 20). Our previous studies focused on the hydrophobic core (strands 4–6 of B-β-sheet and helix B) and the region that presumably accepts the inserting RCL during the inhibitory complex formation (strands 3 and 5 of A-sheet, helix F, and the loop connecting helix F and strand 3 of A-sheet). Various unfavorable interactions such as overpacking of side chains, buried polar groups, and cavities were suggested as the structural basis of the native strain in α₁AT (5, 6, 20).

In the present study, the mutational analyses were expanded over the entire molecule, in order to locate additional sites critical to its stability and activity. We excluded the RCL from the target region because mutations at almost every site in the RCL are likely to affect the inhibitory activity (18) without any relevance to the strain, as in the case of many genetic variations (21). Results from the current study revealed that the native strain of inhibitory serpins is distributed throughout the molecule. Interestingly, many of the stabilizing substitutions did not affect the inhibitory activity significantly. The activity-regulating strain appears to be highly localized to those regions that are presumably mobilized during the complex formation with a target enzyme.

MATERIALS AND METHODS
Recombinant α₁AT Proteins—Plasmids for α₁AT expression in Escherichia coli and purification of recombinant α₁AT protein were described previously (22). Protein concentration was determined in 6 M

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§ The abbreviations used are: α₁AT, α₁-antitrypsin; serpin, serine protease inhibitor; RCL, reactive center loop; SI, stoichiometry of inhibition; PPE, porcine pancreatic elastase; HLE, human leukocyte elastase.
guanidium hydrochloride, calculated from tyrosine and tryptophan content of the α1-AT protein (23).

Chemicals—Ultrapure urea was purchased from ICN Biochemicals. Porcine pancreatic elastase (PPE), human leucocyte elastase (HLE), human plasma α1-AT, N-succinyl-(Ala)3-p-nitroanilide, and N-methoxy-succinyl-Ala-Ala-Pro-Val-p-nitroanilide were purchased from Sigma. All other chemicals were reagent grade.

Screening of Thermostable Mutants—Random mutagenesis at target regions of α1-AT cDNA was performed as described previously, using degenerative oligonucleotides (5, 20, 22). The randomly mutated clones were expressed in E. coli, and the recombinant cells were lysed with lysozyme and Triton X-100. The lysates were heated for 90 min at 80 °C to inactivate unstable α1-AT activity. Mutant clones showing thermostable α1-AT activity were identified after PPE treatment. Representative clones obtained in the first screening were analyzed further by kinetic measurement of heat-induced activity loss of the lysates at 53 °C. The residual inhibitory activity was detected by treatment with PPE. The clones that showed increased half-life in heat-induced inactivation were selected for further analyses. The mutation sites were identified by DNA sequencing. Most mutations were single amino acid substitutions, and many such substitutions were obtained more than once, suggesting that the mutations were saturated. Several mutants carried substitutions at more than one residue. To evaluate the mutational effect at each site, these multiple substitutions were separated to investigate the effect of the mutations on the inhibitory activity, the mutations did not affect the inhibitory activity significantly.

RESULTS

Characterization of Thermostable Mutations of α1-AT—Thermostable mutations of α1-AT were obtained at 50 sites, which were distributed over most secondary structures of α1-AT (Fig. 1) except strand 6 of A sheet (s6A), strands 1 and 3 of B sheet (s1B, s3B), and strands 3 of C sheet (s3C). These mutations were selected based on the enhanced kinetic stability against heat-induced inactivation (see “Materials and Methods”). The conformational stability of each mutant α1-AT was measured through equilibrium unfolding in urea by monitoring changes in intrinsic tryptophan fluorescence intensity (Fig. 2). The midpoint of the unfolding transition, Cm, of the wild-type α1-AT in urea at 25 °C was 1.8 M. Most of the thermostable mutations shifted the Cm values to a higher urea concentration, which indicates that conformational stability was also increased. Changes in the free energy of stabilization by these substitutions are summarized in Table I. Results from the previous studies on the mutations at the hydrophobic core region and the RCL insertion site are also included. Half of the 50 substitutions documented in Table I increased the conformational stability more than 1 kcal mol−1, while 11 of them increased the conformational stability less than 0.3 kcal mol−1. To investigate the effect of the mutations on the inhibitory activity, the SI values toward PPE were measured (Fig. 3; Table I). Most of the mutations did not affect the inhibitory activity signifi-

FIG. 1. A schematic diagram of the native structure of α1-AT showing the distribution of thermostable mutations. Mutation sites are marked by beads. Denotations of the secondary structures are as in Ref. 1. The activity-affecting mutations are colored red, and the other mutation sites are colored blue. The amino acid residues are shown by one-letter codes. The RCL and the regions presumably involved in the insertion of RCL during the complex formation with a target protease are indicated in green. The diagram on the right is the rear view of the diagram on the left. The figures were prepared with Molscript software.
The experimental errors are ±0.16 kcal mol⁻¹. ΔCₐ is the difference between the transition mid point of the mutant and that of the wild-type α₁AT in urea-induced equilibrium unfolding.

Characterization of the Activity-affecting Mutation Sites—Among the newly identified mutations that affected the inhibitory activity more than 10% (Table I, bold numbers), G117V increased the conformational stability substantially. This substitution mapped to the region that presumably is mobilized during the complex formation (Fig. 1). However, the other four mutations, R196S, Q212E, L245I, and P362L, increased the stability marginally. To understand the relationship between the stability increase and the activity loss at these sites, various other substitutions were introduced at each site. The effects of individual substitutions on the stability and the inhibitory activity toward both PPE and HLE are summarized in Table II. Except for the substitutions at Gln-212, which did not affect the inhibitory activity toward HLE, most of the substitutions caused the loss of activity toward both elastases. The activity losses by the mutations at Leu-245 and Pro-362 were substantial, but were not correlated with the stability increase at all. In order to examine if any of these substitutions affect protease binding, the second order association rate constant...
The experimental errors are 6.

Errors are against PPE or HLE were 1.67 and 1.03, respectively. The experimental induced equilibrium unfolding.

a constant (was determined. As shown in Table II, the association rate with HLE was over 10^7 M^-1 s^-1 in all cases.

was determined. As shown in Table II, the association rate constant (k_a) of the variant α_AT with PPE did not differ significantly from the wild-type value, 5.5 × 10^5 M^-1 s^-1. The association rate with HLE was over 10^-7 M^-1 s^-1 in all cases.

**TABLE II**

| Mutations | ΔG | Relative SI PPE | Relative SI HLE | k_a (PPE) | k_a (HLE) |
|------------|----|----------------|----------------|-----------|-----------|
| R196S      | 1.4 | 0.79           | 0.87           | 3.2 × 10^6 | 3.2 × 10^6 |
| R196A      | 0.7 | 0.75           | 1.01           | 4.8 × 10^6 | 4.8 × 10^6 |
| R196K      | 0.4 | 0.87           | 0.86           | 4.8 × 10^6 | 4.8 × 10^6 |
| Q212E      | 0.8 | 0.83           | 1.01           | 2.9 × 10^6 | 2.9 × 10^6 |
| Q212A      | 0.6 | 1.00           | 1.00           | 6.8 × 10^6 | 6.8 × 10^6 |
| Q212D      | 0.2 | 0.87           | 1.06           | 7.1 × 10^6 | 7.1 × 10^6 |
| Q212K      | -0.1| 0.90           | 1.00           | 3.2 × 10^6 | 3.2 × 10^6 |
| L245V      | 0.6 | 0.96           | 1.01           | 6.1 × 10^6 | 6.1 × 10^6 |
| L245I      | 0.3 | 0.75           | 0.75           | 3.7 × 10^6 | 3.7 × 10^6 |
| L245Q      | 0.0 | 0.40           | 0.58           | 5.2 × 10^6 | 5.2 × 10^6 |
| L245F      | -0.4| ND             | ND             | ND        | ND        |
| L245A      | Unstable | ND             | ND             | ND        | ND        |
| P362F      | 1.3 | 0.89           | 0.94           | 3.7 × 10^5 | 3.7 × 10^5 |
| P362G      | 1.3 | 0.84           | 0.88           | 4.7 × 10^5 | 4.7 × 10^5 |
| P362L      | 1.1 | 0.73           | 0.69           | 3.8 × 10^4 | 3.8 × 10^4 |
| P362V      | 0.9 | 0.67           | 0.94           | 2.8 × 10^4 | 2.8 × 10^4 |
| P362A      | 0.5 | 0.75           | 0.99           | 5.5 × 10^4 | 5.5 × 10^4 |
| P362N      | 0.0 | 0.46           | 0.93           | 3.8 × 10^4 | 3.8 × 10^4 |
| P362G      | -0.7| ND             | ND             | ND        | ND        |
| K168I      | 1.6 | 0.85           | 0.91           | 7.1 × 10^5 | 7.1 × 10^5 |

**DISCUSSION**

The present mutational studies addressed several specific questions about native strain in α_AT. What parts of the molecule are strained? What is the structural basis of the native strain in α_AT? Is the native strain at each identified site related to the functional regulation? Results from the current studies revealed that the native form of α_AT is suboptimally folded and the strain is distributed throughout the molecule. However, the strain directly regulating the protease inhibitory activity appears to be highly localized.

**Modes of Destabilization in the Native Form of α_AT**

**Surface Pockets**—Examination of the wild-type α_AT crystal structure (29) revealed that many of the newly identified substitutions map at surface hydrophobic pockets. For instance, the side chain of Ser-36 on helix A is close to the surface pocket formed by Leu-306 of helix I and Asn-81 of the loop connecting helix C and helix D (Fig. 4). Substitution to bulky arginine at the 36 site (S36R) may provide better packing interactions in the pocket. Another mutation site in helix A, Asn-29, is very close to Phe-82 of the loop connecting helix C and helix D (Fig. 4A). The substitution to tyrosine (N29Y) may provide good aromatic-aromatic interactions with Phe-82. Several other substitutions appear to permit better packing interactions at surface pockets, which are indicated in Table I (denoted as SP). A surface hydrophobic pocket near T114F formed between helix D and strand 2 of A sheet was also identified previously by structural examination (29). The T114F substitution increased the conformational stability by 2 kcal mol^-1, apparently due to filling of the pocket by the mutation. Our results suggest strongly that the surface pockets are part of the structural design for the metastable native structure of α_AT.

**Overpacking and Cavities**—Previous sets of thermostable mutations of α_AT (marked in Table I) showed that size reduction or cavity filling (denoted as S and C in Table I, respectively) inside the molecule increased the conformational stability (5, 20). This was surprising because size reduction inside the proteins usually makes the protein unstable (30, 31), and cavities other than active-site pockets are rarely observed (32). The substitution patterns observed in the new set also showed size reduction at several sites (Table I). Size reduction at the buried sites (for example T27S, A34G, L245I, etc.) may provide better packing by allowing rearrangement of the interlocked side chains. The mutants carrying the K163T, K174I, E199D substitutions also show the size reduction. These sites are highly exposed, and many of the substitutions show a marginal stabilizing effect. The stabilizing mechanism at these sites is not clear, and these substitutions are accordingly denoted as “S” in Table I. Several substitutions in the new set, such as A31V, G117V, A183V, A142V, A248V, and V364I, showed size increase rather than size reduction (Table I). Examination of these sites in the crystal structure (29) of α_AT revealed the existence of cavities nearby. Cavities are a likely source of energetic cost in conformational stability (33), and the stabilizing substitutions appear to provide better packing by filling the cavities.

**Other Destabilizing Mechanisms**—Previous studies of the hydrophobic core mutations (5, 6) suggested that certain buried polar groups provide another structural feature for the native strain in α_AT (designated as Po in Table I) because they do not participate in hydrogen bonding. It has been shown that unpaired polar groups cause energetic cost (34). Creation of a polar network may be another structural mechanism of the native strain in α_AT, as suggested by the R196S mutation. Arg-196, located beneath the RCL, forms a positively charged network with Arg-223 and Arg-281 (7, 29). The R196S substitution might relieve the charge repulsion at the site. Several substitutions appear to create salt bridge interactions (Table I, denoted as I). These surface substitutions marginally increased the conformational stability (<0.9 kcal mol^-1). It is not clear,
however, if any of these sites in the wild-type protein contribute to the native strain.

**Localization of the Strain That Regulates Inhibitory Function**

Most of the activity-affecting mutations reside either near the RCL or at the loop insertion site on α-sheet (Fig. 1, colored green). If the strain is relevant to inhibitory function, increasing the stability by releasing the strain should decrease the serpin’s inhibitory activity. This appears to be the case for the previously identified mutations at Lys-335 and Lys-331, which showed increased conformational stability with a concomitant decrease in the inhibitory activity (20). In the crystal structure of the native α1-AT (Fig. 1), these residues are located in strand 5 of A sheet (s5A) and interact with the residues in strand 3 of A sheet (s3A), helix F (hF), and the adjacent loop connecting strand 3 of A sheet and helix F. Among the newly identified sites where stabilizing substitutions reduced the activity, Gly-117 maps in this region. Gly-117 interacts with Tyr-160 of hF and Val-185 in strand 3 of A sheet. It is very likely that the residue interactions in this region must be mobilized to accept the RCL during the complex formation with a target protease. Another site where the native strain may promote the inhibitory activity is Arg-196. Arg-196 has been suggested to form a positively charged pocket near the RCL (7, 29). Such an unfavorable interaction can drive the conformational change during the complex formation with a target protease. Indeed, there is a modest correlation between activity loss and stability increase among the substitutions at Arg-196 (Table II). The strain at the identified sites may facilitate the conformational change.

None of the stabilizing mutations in the other regions of α1-AT appeared to decrease the inhibitory activity (Table I). Filling the surface hydrophobic pocket between strand 2 of A sheet and helix D was predicted to inhibit the transition to a more stable conformation (29). However, T114I, which increased the stability substantially (2 kcal mol⁻¹), presumably by filling the predicted pocket, failed to decrease the inhibitory activity significantly (Table I). These results suggest that only the strain in critical regions of α1-AT, such as the regions that are directly involved in the complex formation with a target enzyme, regulates the serpin’s inhibitory function.

**Mutations Affecting Inhibitory Activity without Stability Increase**

Thermostable mutations at some sites profoundly affected the inhibitory activity without any associated stability increase. Two such sites (Lys-168 and Ile-169) were previously identified (20), and three sites (Gln-212, Leu-245, and Pro-362) were identified in the present study. Various other substitutions at the three sites, Gln-212, Leu-245, and Pro-362, also reduced the inhibitory activity without any associated stability increase (Table II). The mutations do not seem to affect the binding of a target protease, because they have only marginal effects on the rate constant for association (kₐ) with PPE (Table II). It was not surprising that the substitutions at Pro-362 affected the inhibitory activity, because Pro-362 is located at the end of RCL (Fig. 1; P4' position, 4 residues away from the active site residue, Met-358), at the site where RCL kinks and joins C β-sheet (Fig. 1). This proline may help maintain the rigidity of the RCL needed to maximize the strain loaded on the RCL, which may be directly utilized for the distortion of the protease active site (35). Leu-245 is exposed at the end of strand 2 of B sheet (Fig. 1, s2B), and appears to fix the proximal hinge region of RCL near Thr-345 (6, 29). The importance of this hinge region of the RCL has been implied by many genetic mutations in this region of inhibitory serpins (21). Gln-212 is exposed at the end of strand 4 of C sheet and is far away from the RCL. It is not clear how the substitutions at Gln-212 affect the activity. Further studies must define the precise roles of these residues in the inhibitory mechanism.

**Conclusion**

Our examination of thermostable mutations of α1-AT showed that the native strain is scattered throughout the molecule. The structural instabilities in the native form may well be mobilized during the complex formation with a target protease. Interestingly, however, the destabilization component for regulating the inhibitory function of α1-AT is highly localized (Fig. 1 and Table I). Our approach has some potential limitations. Since the mutagenesis method adopted in this study is highly likely to yield only the residue substitutions induced by single nucleotide change (36), we might have missed some important sites where critical substitutions require more than a single nucleotide change. Despite these limitations, the current mutational analyses clearly provide us perspectives on the stabilizing-function relationship of α1-AT by revealing the distribution and structural mechanism of the native strain in α1-AT. Information obtained from this study will be a valuable guide in regulating the stability and function of serpins, including developing stable therapeutic serpins without activity compensation.

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