Engineering thermostability in serine protease inhibitors

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Unlike most globular proteins, the native form of serine protease inhibitors (serpins) is strained. Previous studies of human α1-antitrypsin, a prototype plasma serpin, revealed that various unfavorable interactions, such as over-packing of side chains, buried polar groups and cavities, are the structural basis of the strain. The local strain could be relieved by various stabilizing single amino acid substitutions, which appeared to remove these unfavorable interactions. To improve the stability of other clinically important serpin members, here we examined whether the rules found in α1-antitrypsin studies are applicable to other serpins. Amino acid substitutions were introduced at various positions in human α1-antichymotrypsin and human antithrombin III that were equivalent to the sites of stabilizing substitutions of α1-antitrypsin. Two-thirds of the substitutions increased thermostability in all serpins tested. Mutational analysis and structural examination suggest that serpins are suboptimally folded using common structural strategies at many sites, even though some structural details can vary in individual members. The results suggest that schemes discovered with α1-antitrypsin, an easily manipulative serpin, are a useful basis for engineering conformational characteristics of other clinically important serpins.

Keywords: α1-antichymotrypsin/antithrombin III-serpins/stability/strain

Introduction

The serine protease inhibitor (serpin) superfamily is composed of various proteins including protease inhibitors in plasma, such as α1-antitrypsin (α1AT), α1-antichymotrypsin (α1ACT), antithrombin III (ATIII), C1-inhibitor, plasminogen activator inhibitor-1 (PAI-1) and α2-rantiplasmin. Serpins play important roles not only in the control of proteolytic cascades involved in inflammation, complement activation, thrombosis and fibrinolysis (Huber and Carrell, 1989; Potempa et al., 1994), but also in such diverse functions as hormone transfer, fertilization, tumor suppression, neutrophism, heat shock, apoptosis and cell migration (Vaux et al., 1994; Stefansson and Lawrence, 1996; Silverman et al., 2001). If levels of functional plasma serpins drop below a critical threshold, the unopposed attack of target proteases is likely to cause various problems including tissue damage and bleeding disorders. Dysfunctional α1AT, ATIII and C1-inhibitor are indeed associated with clinical diseases such as emphysema, thrombosis and angioedema (Stein and Carrell, 1995).

The serpin family proteins share a common tertiary structure, composed of three β-sheets and several α-helices (Figure 1). An intriguing structural feature of inhibitory serpins is the strain of the native form (Huber and Carrell, 1989). The native forms of most proteins, having unique tertiary structures carrying biological activities, are in their most stable state (Anfinsen, 1973). However, the native forms of some proteins are in a metastable strained state and the native strain has been recognized as a mechanism of biological regulation (Wiley and Skehel, 1987; Huber and Carrell, 1989; Stein and Carrell, 1995; Carr et al., 1997; Im et al., 1999; Lee et al., 2000). The reactive site loop of inhibitory serpins is exposed in the strained native conformation, but is inserted into the major β-sheet, A β-sheet, upon formation of an inhibitory complex with the target protease (Engh et al., 1995; Wright and Scarsdale, 1995; Huntington et al., 2000). This process accompanies conversion of the metastable native structure into a more stable conformation. However, ovalbumin, a stable but non-inhibitory serpin,
does not undergo a drastic conformational change upon cleavage of the reactive site loop by a protease. Therefore, it is conceivable that the native strain of inhibitory serpins is utilized for the facile conformational change upon complex formation.

To understand the structural basis of the strain in the native structure, we have characterized stabilizing amino acid substitutions of human \( \alpha_1 \)AT, a prototype inhibitory serpin. We previously reported that various unfavorable interactions, such as overpacking of side chains, cavities and polar–non-polar interactions, are the structural basis of the native strain (Im et al., 1999; Seo et al., 2000). Our previous study also revealed that the native strain of inhibitory serpins is distributed throughout the \( \alpha_1 \)AT molecule (Seo et al., 2000). However, the activity-regulating strain appears to be highly localized to regions that are presumably mobilized during the complex formation with a target enzyme (Im et al., 1999). Stabilizing amino acid substitutions at those regions, such as A \( \beta \)-sheet (Figure 1, colored pink) and near the reactive site loop (Figure 1, colored yellow), concomitantly decreased the inhibitory activity. Interestingly, stabilizing substitutions at most other sites including the hydrophobic core did not affect the inhibitory activity despite a large increase in stability (Kwon et al., 1994; Seo et al., 2000). The results suggested that only local strain at critical sites is utilized for functional regulation of serpins and stabilizing mutations at other regions can be introduced without hampering functions. This can be explored for therapeutic applications such as developing stable recombinant protein drugs or gene therapy.

In the present study, we searched for common themes generally applicable to inhibitory serpins to increase stability. There are some residues conserved among inhibitory members of serpins such as \( \alpha_1 \)ACT, ATIII and PAl-I and hormone-binding globulins, but not in non-inhibitory members such as ovalbumin and angiotensinogen (Huber and Carrell, 1989). Such residues might be critical for either functionality or the native strain of the inhibitory serpins. Substitutions, which increased conformational stability in \( \alpha_1 \)AT, were also introduced at the equivalent positions in \( \alpha_1 \)ACT and ATIII and mutational effects were investigated. We measured thermostability of the serpins. There is a correlation between the conformational stability of \( \alpha_1 \)AT and its thermostability (Kwon et al., 1994). Thermostable variants of serpins may be of practical use because heat-induced deactivation of serpins was shown to be a concentration-dependent aggregation process (Lomas et al., 1993; Kwon et al., 1994). In addition, thermostability of recombinant \( \alpha_1 \)AT was shown to relate to the biological turnover rate of the protein (Travis et al., 1985). The results obtained in this study provide valuable information for engineering clinically important inhibitory serpins.

**Materials and methods**

**Chemicals**

Porcine pancreatic elastase, bovine \( \alpha \)-chymotrypsin, human plasma thrombin, porcine mucosa heparin, N-succinyl-(Ala)\(_3\)-p-nitroanilide, N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide and a rabbit anti-human ATIII antibody were purchased from Sigma (St Louis, MO). Chromozym-TH was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). Bac-N-Blue transfection kit and High 5 cells were purchased from Invitrogen (Leek, The Netherlands) and *Spodoptera frugiperda* (Sf9) cells from Clontech Laboratories (Palo Alto, CA). Econo-Pac heparin cartridge was purchased from Bio-Rad Laboratories (Hercules, CA). All other chemicals were of reagent grade.

**Recombinant \( \alpha_1 \)AT and recombinant \( \alpha_1 \)ACT proteins**

Plasmids for the wild-type human \( \alpha_1 \)AT and human \( \alpha_1 \)ACT expression in *Escherichia coli* have been described previously (Im and Yu, 2000). Substitution mutations of \( \alpha_1 \)AT and \( \alpha_1 \)ACT were generated by oligonucleotide-directed mutagenesis (Kunkel et al., 1987). Expression of recombinant \( \alpha_1 \)AT proteins and recombinant \( \alpha_1 \)ACT proteins was induced in M9ZB media (1% tryptone, 0.5% NaCl, 0.1% NH\(_4\)Cl, 0.3% KH\(_2\)PO\(_4\), 0.6% Na\(_2\)HPO\(_4\), 0.4% glucose, 1 mM MgSO\(_4\)) by addition of 0.1 mM IPTG as described previously (Kwon et al., 1994).

**Recombinant ATIII proteins**

Human ATIII cDNA was previously obtained by polymerase chain reaction (Im and Yu, 2000). A pair of complementary oligonucleotides coding for baculovirus gp64 signal sequence were introduced in front of ATIII sequences to direct the synthesized protein to extracellular culture media. Substitutions of ATIII sequences were introduced by oligonucleotide-directed mutagenesis. After deoxyxynucleotide sequencing, the DNA fragment containing ATIII gene was transferred to pBlueBac plasmid (Invitrogen) to co-transfect insect cells with a linearized wild-type baculovirus DNA. Recombinant blue plaques formed on Sf9 cell lawns were purified and stored at 4°C. Wild-type baculovirus, recombinant baculovirus harboring wild-type ATIII gene and recombinant baculovirus harboring mutant ATIII gene were used in parallel to infect High 5 cells. Serum-free media (Invitrogen) were used in this study to avoid any contaminating antithrombin activity from serum. Conditioned media were harvested 4–5 days after infection with recombinant baculovirus and clarified by centrifugation at 9000 g for 20 min. Samples were buffer-exchanged to ATIII assay buffer (0.1 M Tris–Cl, pH 8.0, 0.12 M NaCl, 0.01% sodium azide and 0.1% bovine serum albumin). ATIII was partially purified on a heparin-affinity column by elution with a NaCl gradient. Expression of ATIII was confirmed by inhibitory activity assays and western blot analyses using a rabbit anti-human ATIII antibody.

**Measurement of inhibitory activity**

The concentration of porcine pancreatic elastase was determined by measuring the initial rates of hydrolysis of 1 mM N-succinyl-(Ala)\(_3\)-p-nitroanilide (Travis and Johnson, 1981). After recombinant wild-type or mutant \( \alpha_1 \)AT proteins had been incubated in 50 \( \mu \)l of elastase assay buffer (30 mM phosphate, 160 mM NaCl, 0.1% PEG 6000 and 0.1% Triton X-100, pH 7.4) with 20 mM porcine pancreatic elastase at 37°C for 10 min, the reaction mixture was diluted 10-fold with the assay buffer and the residual proteolytic activity was determined using 1 mM N-succinyl-(Ala)\(_3\)-p-nitroanilide as a substrate for porcine pancreatic elastase.

The concentration of bovine \( \alpha \)-chymotrypsin was determined by measuring the initial rates of hydrolysis of 0.1 mM N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide. After recombinant wild-type or mutant \( \alpha_1 \)ACT proteins had been incubated in 50 \( \mu \)l of chymotrypsin assay buffer (100 mM Tris–Cl, 10 mM CaCl\(_2\) and 0.005% TritonX-100, pH 7.8) with 20 nM bovine \( \alpha \)-chymotrypsin at 37°C for 10 min, the reaction mixture was...
diluted 10-fold with the assay buffer and the residual proteolytic activity was determined using 0.1 mM N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide as a substrate.

Thrombin activity was measured using 0.1 mM Chromozym-TH as a substrate. Partially purified recombinant ATIII was mixed with 25 ng of heparin and incubated with 20 nM human plasma thrombin at 37°C for 10 min at ATIII assay buffer (0.1 M Tris–Cl, 0.12 M NaCl, 0.01% sodium azide and 0.1% bovine serum albumin, pH 8.0). The reaction was diluted 10-fold with the assay buffer and the remaining proteolytic activity was measured using 0.1 mM Chromozym-TH as a substrate.

**Thermal deactivation of recombinant serpins**

α1AT and α1ACT proteins were expressed in M9ZB medium at 37°C as a soluble form. Cells corresponding to A_{660} = 1 were harvested by centrifugation and resuspended in 500 µl of buffer (50 mM Tris–Cl, 50 mM NaCl, pH 8.0). Cells were disrupted by sonication using a W-380 sonicator (Heat Systems, New York) and insoluble materials were removed by centrifugation. Soluble fractions containing recombinant α1AT or α1ACT proteins were assayed for the inhibitory activity against porcine pancreatic elastase or bovine α-chymotrypsin, respectively. Amounts of α1AT or α1ACT proteins, which barely inhibit 90% of the target protease used, were included in each assay, so that subtle changes in serpin activity could be easily monitored. It was previously shown that, at least up to 50 µg/ml α1AT, the concentration dependence of polymerization was negligible in urea-dependent equilibrium unfolding and refolding experiments (Kwon et al., 1994). Since all mutant α1AT proteins retained comparable inhibitory activity and concentration ranges of active inhibitors (~0.8 µg/ml) used in this study were far below 50 µg/ml, it is not likely that some concentration difference between mutant proteins would seriously modify the kinetics of thermal inactivation. The thermal stability of recombinant α1AT and α1ACT proteins was measured by following the kinetics of inactivation at 53 and 52°C, respectively. Samples were taken at various time points during thermal deactivation and the remaining inhibitory activity was determined as described above.

Thermal stability of the partially purified recombinant ATIII proteins, in the absence and presence of added heparin, was measured by following the kinetics of inactivation at 59 and 64.5°C, respectively. Aliquots of samples were taken along the time course of inactivation and the remaining ATIII activity was determined as described above.

**Structural examination of native serpins**

The crystal structure of α1AT was from the Protein Data Bank with a coordinate number 2PSI (Elliott et al., 1998) and 1ATU (Ryu et al., 1996). The native structure of α1ACT was kindly provided by D. Christianson (University of Pennsylvania). The active ATIII structure in heterodimeric form was from the Protein Data Bank with a coordinate number 1ANT (Carrell et al., 1994). Structures were examined using the Insight II program (MSI).

**Results and discussion**

**Thermostability of mutant serpins**

To examine whether common themes are applicable to relieve the native strain of inhibitory serpins, single amino acid substitutions that increased the conformational stability of α1AT were introduced at equivalent positions of α1ACT and ATIII. Especially, substitutions that stabilize α1AT by >1 kcal/mol were chosen. Mutant serpins were expressed and their thermostability was measured through heat deactivation. Figure 2 shows typical heat deactivation results for stable serpin variants. All mutant α1AT proteins, which increased conformational stability presumably by compensating various
Table I. Stabilizing substitutions having common themes in all three inhibitory serpins

<table>
<thead>
<tr>
<th>Residue No. (location)</th>
<th>$\alpha_1$AT $T_{1/2}$</th>
<th>$\alpha_1$ACT $T_{1/2}$</th>
<th>ATIII $T_{1/2}$ (+ hep)</th>
<th>Stabilization mode</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mutant $^b$</td>
<td>Mutant $^b$</td>
<td>Mutant $^b$</td>
<td></td>
</tr>
<tr>
<td>51 (s6B)</td>
<td>F51L 13.0 ± 0.2</td>
<td>I49L 2.6 ± 0.1</td>
<td>F77L 1.8 ± 0.1 (8.1 ± 0.3)</td>
<td>S</td>
</tr>
<tr>
<td>59 (hB–hC)</td>
<td>T59S 2.5 ± 0.4</td>
<td>T57S 1.7 ± 0.2</td>
<td>T85S 1.0 ± 0.1 (10.0 ± 0.1)</td>
<td>S</td>
</tr>
<tr>
<td>335 (s5A)</td>
<td>K335A 44.3 ± 0.1</td>
<td>K335A 22.9 ± 2.1</td>
<td>K370A$^e$ 5.2 ± 1.1 (6.5 ± 0.7)</td>
<td>S, Po</td>
</tr>
<tr>
<td>381 (s5B)</td>
<td>S381A 3.6 ± 0.1</td>
<td>N385A 2.6 ± 0.1</td>
<td>T419A 1.8 ± 0.2 (1.3 ± 0.1)</td>
<td>S, Po</td>
</tr>
<tr>
<td>68 (hB–hC)</td>
<td>T68A 5.0 ± 0.2</td>
<td>–</td>
<td>NA$^A$ (A66) –</td>
<td></td>
</tr>
<tr>
<td>70 (hB–hC)</td>
<td>A70G 8.8 ± 0.7</td>
<td>N68G 2.7 ± 0.1</td>
<td>N96G 1.6 ± 0.1 (1.2 ± 0.1)</td>
<td>Back-bone freedom</td>
</tr>
<tr>
<td>114 (s2A)</td>
<td>T114I 17.5 ± 0.8</td>
<td>M112I 4.7 ± 0.2</td>
<td>S142I 1.1 ± 0.0 (1.0 ± 0.03)</td>
<td>SP</td>
</tr>
<tr>
<td>102 (hD)</td>
<td>T102R 1.9 ± 0.1</td>
<td>T100R 1.4 ± 0.1</td>
<td>NA$^A$ (R129) –</td>
<td>SP, I</td>
</tr>
<tr>
<td>142 (s1A)</td>
<td>A142V 26.4 ± 0.6</td>
<td>A140V 28.2 ± 0.4</td>
<td>NA$^A$ (L170) –</td>
<td>C</td>
</tr>
<tr>
<td>117 (s2A)</td>
<td>G117V 7.6 ± 0.5</td>
<td>A115V 1.1 ± 0.1</td>
<td>NA$^A$ (R145) –</td>
<td>C</td>
</tr>
<tr>
<td>183 (s3A)</td>
<td>A183V 21.6 ± 0.3</td>
<td>NA$^F$ (V181) –</td>
<td>NA$^A$ (V214) –</td>
<td>C</td>
</tr>
<tr>
<td>164 (hF)</td>
<td>G164V 4.1 ± 0.2</td>
<td>G162V 15.8 ± 0.3</td>
<td>NA$^A$ (K193) –</td>
<td>SP</td>
</tr>
</tbody>
</table>

$^a$Residue numbers of $\alpha_1$AT are used. Locations of the mutated sites on the secondary structures are shown. Denotations of the secondary structures follow those in Huber and Carrell (1989).

$^b$One-letter codes for amino acids are used. Residues in front of numbers are the wild-type residues and those following the numbers are the mutant residues.

$^c$Fold increase in the half-life of mutant protein over that of the wild-type protein during heat treatment is shown. The results are mean of three independent measurements. The half-life of the wild-type $\alpha_1$AT was 17.1 min at 53°C and that of the wild-type $\alpha_1$ACT was 24.7 min at 52°C. The wild-type ATIII has a half-life of 11.3 min at 59°C in the absence of heparin, but it retained half of the activity for 27.6 min at 64.5°C in the presence of heparin. Fold increase in the half-life in the presence of heparin is shown in parentheses.

$^d$Stabilization modes: SP, filling a surface pocket; S, residue size-reduction at a buried site; C, filling a cavity; Po, removal of a buried polar group; I, introduction of an ionic interaction.

$^e$From a previous study (Im and Yu, 2000).

$^f$NA: not applicable. The wild-type residue is shown in parentheses.

Table II. Substitutions showing different effects on stability in individual serpins

<table>
<thead>
<tr>
<th>Residue No. (location)</th>
<th>$\alpha_1$AT $T_{1/2}$</th>
<th>$\alpha_1$ACT $T_{1/2}$</th>
<th>ATIII $T_{1/2}$ (+ hep)</th>
<th>Stabilization mode</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mutant $^b$</td>
<td>Mutant $^b$</td>
<td>Mutant $^b$</td>
<td></td>
</tr>
<tr>
<td>113 (s2A)</td>
<td>T113I 8.4 ± 0.4</td>
<td>S111I 3.8 ± 0.2</td>
<td>V141I 0.3 ± 0.1 (0.6 ± 0.2)</td>
<td>SP</td>
</tr>
<tr>
<td>189 (s3A)</td>
<td>F189V 6.2 ± 0.6</td>
<td>F187V 3.6 ± 0.1</td>
<td>Y220V 0.6 ± 0.1 (0.2 ± 0.05)</td>
<td>S</td>
</tr>
<tr>
<td>172 (hF–s3A)</td>
<td>L172A 12.3 ± 0.7</td>
<td>L170A 0.72 ± 0.04</td>
<td>V201A 0.8 ± 0.1 (1.0 ± 0.1)</td>
<td>S</td>
</tr>
<tr>
<td>286 (s2C)</td>
<td>L286V 4.9 ± 0.2</td>
<td>L288V 0.1 ± 0.04</td>
<td>NA$^A$ (V318) –</td>
<td>S</td>
</tr>
<tr>
<td>248 (s2B)</td>
<td>A248V 6.1 ± 1.4</td>
<td>A247V 0.9 ± 0.1</td>
<td>NA$^A$ (I279) –</td>
<td>C</td>
</tr>
<tr>
<td>387 (s5B)</td>
<td>K387R 15.7 ± 1.1</td>
<td>K391R 0.4 ± 0.2</td>
<td>NA$^A$ (R425) –</td>
<td>SP</td>
</tr>
</tbody>
</table>

$^a$Residue numbers of $\alpha_1$AT are used. Locations of the mutated sites on the secondary structures are shown. Denotations of the secondary structures follow those in Huber and Carrell (1989).

$^b$One-letter codes for amino acids are used. Residues in front of numbers are the wild-type residues and those following the numbers are the mutant residues.

$^c$Fold increase in the half-life of mutant protein over that of the wild-type protein during heat treatment is shown as in Table I.

$^d$Stabilization modes in $\alpha_1$AT: SP, filling a surface pocket; S, residue size-reduction at a buried site; C, filling a cavity.

$^e$NA: not applicable. The wild-type residue is shown in parentheses.

structural defects of $\alpha_1$AT (Seo et al., 2000), greatly increased the half-life in heat deactivation at 53°C (Tables I and II; Figure 2A). Especially a cavity-filling mutation at Ala142 and a size-decreasing substitution at Lys335 showed profound effects on thermostability, shifting the half-life upon heat deactivation from 17 min (wild-type $\alpha_1$AT) to >400 min at this temperature. These substitutions increased the conformational stability of $\alpha_1$AT by 2.4 and 6.5 kcal/mol, respectively (Im et al., 1999; Seo et al., 2000). Other substitutions also increased the thermal stability of $\alpha_1$AT significantly (2–22-fold). The thermostability of mutant $\alpha_1$ACT, in which the same kinds of substitutions were introduced at the equivalent positions of $\alpha_1$ACT, was measured by heat treatment at 52°C (Figure 2B). Most mutant proteins showed increased thermostability compared with the wild-type $\alpha_1$ACT (Table I; Figure 2B). For example, cavity-filling substitution at Ala140, in addition to the size-decreasing mutation at Lys335, showed a dramatic increase in thermostability, as in the case of $\alpha_1$AT.

To test whether the same kinds of substitutions can relieve the native strain of ATIII, similar substitutions were introduced to ATIII. Due to the inherent increased stability of ATIII, the heat deactivation temperature has to be raised to 59°C. Several substitutions of ATIII, such as F77L, N96G, K370A and T419A, increased the half-life significantly under these conditions, compared with 11.3 min for the wild-type ATIII (Table I; Figure 2C). In the presence of heparin, ATIII undergoes conformational change, which greatly enhanced the inhibitory activity of ATIII (Gettins et al., 1993; Huntington et al., 1996). The resistance to heat-induced deactivation was also increased in the presence of heparin, and the heat deactivation temperature therefore had to be raised to 64.5°C. Those mutations shifted the half-time for thermal deactivation in the

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absence of heparin and also increased thermal stability in the presence of heparin (Table I). The wild-type ATIII, at several sites, already has the kinds of residues that showed stabilizing effects on \( \alpha_1 \)AT and \( \alpha_1 \)ACT proteins. Stabilizing substitutions were not applicable at these sites (denoted NA in Table I). This explains, at least partially, the inherent higher thermal stability of ATIII compared with \( \alpha_1 \)AT and \( \alpha_1 \)ACT.

**Inducing native strain in ATIII by reverse engineering**

A great stabilizing effect was obtained by substitutions at the residue 142 site (residue number of \( \alpha_1 \)AT): size increases by \( \alpha_{1AT} 142 \) of ATIII increased the half-life on thermal inactivation by 1.4- and 4.1-fold in the absence and presence of added heparin, respectively, presumably by allowing better packing at this site (Figure 3). However, substitution of a smaller residue, alanine, which is the wild-type residue in \( \alpha_{1ACT} \), decreased the thermal stability of ATIII: L170A mutation reduced the thermal half-life to 0.2- and 0.1-fold of the wild-type ATIII in the absence and presence of added heparin, respectively (Figure 3). The results confirmed that cavity-filling substitutions decreased the thermostability of ATIII. The results also suggest that the wild-type residue, Leu, of ATIII at this site appears to be suboptimally suitable for the site because size reduction to valine appears to allow better packing in the region. It appears that the existence of a cavity at this location is a structural design inducing the native strain of inhibitory serpins.

**Structural examination of stabilizing substitution patterns common among inhibitory serpins**

Table I summarizes substitutions that showed increased thermostability in all serpins when the theme found with the \( \alpha_1 \)AT study was tested. In the crystal structure of the native \( \alpha_1 \)AT, the stabilizing mutation sites are composed of many unusual interactions, which appear to be the basis of the native strain (Im et al., 1999; Seo et al., 2000). From the current mutational studies, the following structural schemes were confirmed as strategies to increase the stability of the native serpin molecule.

**Relief of side-chain overpacking.** Substitutions at positions 51, 381 and 335 (residue numbers of \( \alpha_1 \)AT) with smaller residues increased the thermostability in all three serpins examined (denoted S in Table I), possibly by releasing the native strain caused by overpacked side chains.

**Cavity filling.** Residues at positions 142 and 117 (residue numbers of \( \alpha_1 \)AT) constitute internal cavities of \( \alpha_1 \)AT and \( \alpha_1 \)ACT (denoted C in Table I) and filling the cavities by substitutions at these sites increased the thermostability. ATIII, which showed inherent higher thermostability, already has larger side chains at the equivalent sites, Leu170 and Arg145. Likewise, Thr114, Gly164 and Thr102 constitute surface pockets with surrounding residues (denoted SP in Table I). Substitutions by larger residues at these sites increased the thermostability of \( \alpha_1 \)AT and \( \alpha_1 \)ACT. Substitution of Ser142 of ATIII (equivalent to Thr114 of \( \alpha_1 \)AT) with isoleucine also increased stability. The other two surface pockets near Gly164 and Thr102, observed in \( \alpha_1 \)AT and \( \alpha_1 \)ACT, are already filled with Lys193 and Arg129 at the equivalent sites of ATIII.

**Others.** In the case of S381A and K335A, removal of the unpaired polar groups in hydrophobic environments may contribute to the increased thermostability in all serpins tested (denoted Po in Table I). In the case of Thr68 of \( \alpha_1 \)AT, substitution with alanine increased the thermostability. \( \alpha_1 \)AT and ATIII already have alanine at the equivalent site. Buried polar groups have been recognized as a means of inducing metastable native structures in other proteins such as influenza A virus, arrestin and the spike glycoprotein of influenza C virus (Chen et al., 1998; Rosenthal et al., 1998; Hirsch et al., 1999). Increased ‘backbone freedom’ is another theme of stabilization mode in inhibitory serpins. Ala70 of \( \alpha_1 \)AT (equivalent to Asn68 of \( \alpha_1 \)ACT and Asn96 of ATIII) is located at the turn between helix B (hB) and helix C (hC) and substitution with a flexible small residue, glycine, greatly increases the thermostability of the serpin. The existence of flexible glycine at the turn seems to allow better packing of nearby residues, including those in the following helices. Introduction of favorable ionic interactions (denoted I in Table I) may be another means of stabilization. Substitution with arginine at the Thr102 site of \( \alpha_1 \)AT and Thr100 of \( \alpha_1 \)ACT might provide positive charges to neighboring Glu376 and Glu98 of \( \alpha_1 \)AT and Asp384 of \( \alpha_1 \)ACT. ATIII already has arginine at the equivalent site.

**Substitutions showing distinct effects among inhibitory serpins**

Some substitutions that increased the thermostability of \( \alpha_1 \)AT did not show stabilizing effects when introduced at the equivalent positions of \( \alpha_1 \)ACT or ATIII. Table II summarizes such substitutions. Structural examination shows distinct side-chain interactions at these sites among inhibitory serpins.
Common strain in α1AT and α1ACT but not in ATIII. Some structural strains exist common in α1AT and α1ACT but not in ATIII. Thr113 of α1AT (Ser111 of α1ACT) is located in a surface hydrophobic pocket and substitution with isoleucine at this site stabilized α1AT possibly by filling the surface cavity. However, ATIII has valine at the equivalent site, which is well packed without a surface pocket. Introduction of a larger residue at this position of ATIII destabilized the molecule. Phe189 of α1AT (Phe187 of α1ACT), located on s3A (the third strand of A β-sheet), is unfavorably overpacked underneath hF and substitutions with smaller, aliphatic side chains increased the thermal stability. However, Tyr220 of ATIII is well packed and there is no unfavorable polar–non-polar interaction with surrounding residues. Substitution to a smaller residue, valine, at this position of ATIII destabilized the molecule. Phe189 of α1AT (Phe187 of α1ACT), located on s3A (the third strand of A β-sheet), is unfavorably overpacked underneath hF and substitutions with smaller, aliphatic side chains increased the thermal stability. However, Tyr220 of ATIII is well packed and there is no unfavorable polar–non-polar interaction with surrounding residues. Substitution to a smaller residue, valine, at this position of ATIII destabilized the molecule. Phe189 of α1AT (Phe187 of α1ACT), located on s3A (the third strand of A β-sheet), is unfavorably overpacked underneath hF and substitutions with smaller, aliphatic side chains increased the thermal stability. However, Tyr220 of ATIII is well packed and there is no unfavorable polar–non-polar interaction with surrounding residues. Substitution to a smaller residue, valine, at this position of ATIII destabilized the molecule. Phe189 of α1AT (Phe187 of α1ACT), located on s3A (the third strand of A β-sheet), is unfavorably overpacked underneath hF and substitutions with smaller, aliphatic side chains increased the thermal stability. However, Tyr220 of ATIII is well packed and there is no unfavorable polar–non-polar interaction with surrounding residues. Substitution to a smaller residue, valine, at this position of ATIII destabilized the molecule. Phe189 of α1AT (Phe187 of α1ACT), located on s3A (the third strand of A β-sheet), is unfavorably overpacked underneath hF and substitutions with smaller, aliphatic side chains increased the thermal stability. However, Tyr220 of ATIII is well packed and there is no unfavorable polar–non-polar interaction with surrounding residues. Substitution to a smaller residue, valine, at this position of ATIII destabilized the molecule. Phe189 of α1AT (Phe187 of α1ACT), located on s3A (the third strand of A β-sheet), is unfavorably overpacked underneath hF and substitutions with smaller, aliphatic side chains increased the thermal stability. However, Tyr220 of ATIII is well packed and there is no unfavorable polar–non-polar interaction with surrounding residues. Substitution to a smaller residue, valine, at this position of ATIII destabilized the molecule. Phe189 of α1AT (Phe187 of α1ACT), located on s3A (the third strand of A β-sheet), is unfavorably overpacked underneath hF and substitutions with smaller, aliphatic side chains increased the thermal stability. However, Tyr220 of ATIII is well packed and there is no unfavorable polar–non-polar interaction with surrounding residues. Substitution to a smaller residue, valine, at this position of ATIII destabilized the molecule. Phe189 of α1AT (Phe187 of α1ACT), located on s3A (the third strand of A β-sheet), is unfavorably overpacke

Strains unique in α1AT. Some unfavorable interactions exist only in α1AT. Leu172 of α1AT is pointing towards A β-sheet and Lys335 is unfavorably overpacked underneath Leu172 (Figure 4). A size decrease at Leu172 greatly increased the thermostability of α1AT (Table II). Unlike the side chain of Leu172 of α1AT, the side chains of Leu170 of α1ACT and Val201 of ATIII are positioned toward helix F (hF) and interact with Lys160 (3.52 Å) and Val190 (3.73 Å) of hF, respectively (Figure 4). Hence substitution with a residue with a smaller side chain at Leu170 of α1ACT and Val201 of ATIII may reduce favorable interactions with hF and not bring hF and the following turn closer to A β-sheet. Likewise, microenvironments near Leu286, Ala248 and Lys387 of α1AT are distinct from those of α1ACT and of ATIII. For instance, ATIII has a large residue, isoleucine, at the 279 residue site (equivalent to Ala248 of α1AT). It also has Arg425 (at the equivalent site to Lys387 of α1AT), which suitably fits the site and exposes a positive charge to solvent.

Improving thermostability of inhibitory serpins

Two-thirds of the substitutions (12 out of 18) increased thermostability in all serpins tested (Figure 1; Tables I and II). Substitutions at two sites (residues sites 113 and 189 of α1AT) among the other six sites increased thermostability in α1AT and α1ACT but not in ATIII (Table II). Only the substitutions at four sites did not show a thermostability increase in other serpins when the theme found in the α1AT study was applied (Table II). These results suggest that inhibitory serpins are suboptimally folded using common structural strategies at many sites, even though some structural details could vary in individual serpins. The overall packing density (0.67) of the metastable α1AT structure is similar to those of common globular proteins (Rashin et al., 1986), but local structures of α1AT are made of a mosaic of many over- and under-packed regions (Lee et al., 2001). The existence of cavities and over-packed regions seems to be a unique structural feature in strained serpin molecules including α1AT. The results of this study suggest that rules found for α1AT can be applied to increase the thermostability of inhibitory serpins. Activity-affecting mutations are highly localized in the regions that are mobilized during the complex formation and stabilizing mutations at

![Fig. 4. Distinct side-chain interactions among inhibitory serpins in the hF region. The major β-sheet, A sheet, is shown on the left and hF is shown on the right side in ribbon diagrams. The size decreases at the Leu172 site of α1AT, α1ACT and ATIII show different effects on stability. The representative side chains surrounding the mutation site, Leu172, are shown as green sticks. Atoms with a positive and a negative polarity are indicated in blue and red, respectively. One-letter codes for amino acids are used in front of the residue numbers. Distances between representative atoms described in the text are shown in Å. The designations of secondary structures are described in the text. Crystal structure of α1AT (1atu.pdb), α1ACT (act_p_v.pdb) and ATIII (1ant.pdb) were used. The figures were prepared using the InsightII program (Molecular Simulations, San Diego, CA).](https://example.com/fig4.png)
other sites did not affect activity. Increased thermostability based on enhanced kinetic stability against heat-induced aggregation has practical implications. The formation of inactive oligomers of inhibitory serpins is frequently found in many pathological, denaturing conditions or upon storage (Stein and Carrell, 1995). Information obtained in this study, including understanding of the packing modes and stability of serpins, will be a valuable guide in designing and modulating conformational characteristics of the molecules. Especially suppression of heat-induced polymerization of clinically important serpins would contribute to the development of therapeutic serpins suited to long-term storage and administration.

Acknowledgements

We thank M.R.Kim for her excellent technical assistance. We thank Dr C.Lee and Dr E.J.Seo for the preparation of figures. This study was supported by grant number FPR02B1-01-113 of 21C Frontier Functional Proteomics Program from the Korean Ministry of Science and Technology.

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Received December 31, 2003; revised April 13, 2004;
accepted April 13, 2004

Edited by Andreas Matouschk