Single Amino Acid Substitutions of $\alpha_1$-Antitrypsin That Confer Enhancement in Thermal Stability*

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A recombinant $\alpha_1$-antitrypsin variant which increased thermal stability was obtained from random mutagenesis followed by screening. The clone was identified as having a single mutation of Phe$^{31}$ -> Cys. Heat denaturation of purified recombinant $\alpha_1$-antitrypsin produced in Escherichia coli revealed that the mutation slowed down the deactivation rate 10-fold at 57°C, increasing thermal stability of recombinant protein to almost that of natural glycosylated plasma form. The mutant protein also exhibited increased stability against denaturant. The urea-induced unfolding monitored by the changes in fluorescence intensity at 360 nm showed that the mutation shifted midpoint of the transition from 1.9 k to 2.8 k. The mutation site is particularly interesting in that some genetic variants mapped at adjacent positions were shown previously to cause aggregation of the polypeptides, while the Phe$^{31}$ -> Cys mutation decreased aggregation rate significantly during heat deactivation. The association rate constant with porcine pancreatic elastase revealed that the mutation did not affect inhibitory activity significantly. The site identified may be critical for regulating stability of $\alpha_1$-antitrypsin. Characterization of various single amino acid substitutions at position 51 suggests that volume and flexibility of hydrophobic side chain at the site are critical factors for enhancing the stability of $\alpha_1$-antitrypsin.

$\alpha_1$-Antitrypsin ($\alpha_1$-AT) is a member of the serine protease inhibitor (serpin) family, which includes antithrombin, $\alpha_1$-antichymotrypsin, C1 inhibitor, ovalbumin, angiotensinogen, and hormone-binding globulins (1). Serpins share a common tertiary structure composed of three $\beta$-sheets and several $\alpha$-helices that connect the strands in the sheets (1). Inhibitory serpins have a native strained (S) conformation in which the reactive center loop is open to proteolytic cleavage. The cleavage accompanies an irreversible transition to a very stable relaxed (R) form where the newly created N-terminal portion of the cleaved loop is completely inserted as a strand of sheet A and cleavage of the reactive center loop does not induce a conformational switch as in $\alpha_1$-AT (2). A few other conformations of serpins have been identified. The active form of plasminogen activator inhibitor-1 (PAI-1) can be spontaneously converted into a more stable latent form (6). Recent structural determination (7) showed that intact reactive center loop in latent PAI-1 is inserted into the major $\beta$-sheet (sheet A) forming a strand as in cleaved $\alpha_1$-AT. The rest of the loop stretches over the edge of the protein and joins a strand within the top $\beta$-sheet (sheet C). A stable locked conformation of antithrombin and other serpins could be induced from the native form under mild denaturing conditions, in which intact reactive center loop is presumably inserted into the sheet A in latent form of PAI-1 (8). The existence of a more stable latent structure suggests that the native structure of serpins is not the thermodynamically most stable form. In case of $\alpha_1$-AT it was also shown that oligomers are formed on prolonged incubation under mild denaturing conditions or during heat denaturation via a process called loop-sheet polymerization, in which reactive center loop of one molecule is inserted into sheet A of another (9, 10).

Structural comparisons of various forms of serpins and thermostability studies of serpins complexed with synthetic peptides carrying sequences of the reactive center loop (8, 11) support a notion that the enhancement in stability appears to be mainly due to insertion of the cleaved loop into the sheet A with concomitant increase in the number of strands in the sheet and buried surface area. However, latent PAI-1, which is more stable than the active form, is less so than the cleaved form (6), showing that insertion of the loop was not sufficient to confer maximum stability exhibited by cleaved form. In the case of peptide-annealed antithrombin, it was shown that the complex was as stable toward heat denaturation as cleaved antithrombin (8). All the stable forms of serpins so far identified, including ovalbumin, are not active as an inhibitor. The reactive center loop structure of active serpins is considered to be mobile and partially inserted into sheet A of the molecule (8, 12, 13). The presence of such a mobile loop is presumably critical for the inhibitory function of serpins. There has been an indication that the stress of native serpin is not limited to the reactive center loop but may be distributed throughout the molecule. Studies of 1H-2H exchange and Fourier transform infrared spectroscopy with the intact and cleaved form of $\alpha_1$-AT showed that stability enhancement in cleaved form is related to increase in contents of stable secondary structure in many parts of the molecule without much variation in tertiary interaction (14). In the present study we identified single amino acid substitutions of native human $\alpha_1$-AT that confer increased thermal stability but maintain inhibitory activity. The mutation also increased stability toward urea denaturation. Characterization of stable variants will help define the relationship between strain and the tertiary fold of the native $\alpha_1$-AT structure. Such mutants might be also of practical use because the thermal stability of recombinant $\alpha_1$-AT was shown to relate to the biological turnover rate of the protein (15).

EXPERIMENTAL PROCEDURES

Plasmid Construction and Protein Expression—Construction of an expression plasmid, pEATS, encoding the $\alpha_1$-AT cDNA under control of
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the phage T7 promoter (16) has been described (17). The plasmid encodes an \( \alpha_1 \)-AT polypeptide in which the first residue of the authentic \( \alpha_1 \)-AT, glutamate, is substituted by methionine. A transformant of *Escherichia coli* BL21(DE3) was grown on M9 medium (per liter: 1 g of 
\[ \text{NH}_4\text{Cl}, 3 \text{ g of KH}_2\text{PO}_4, 6 \text{ g of Na}_2\text{HPO}_4, 4 \text{ g of glucose, 0.45 g of MgSO}_4, 10 \text{ mM of NaCl containing 37 °C ampicillin.}
\]
When the \( A_{260} \) reached 0.8, isopropyl-\( \beta \)-D-thiogalactopyranoside was added to a final concentration of 0.4 mM and growth was prolonged for 3 h at a designated temperature. The growth temperature affects the solubility of expressed \( \alpha_1 \)-AT encoded by pEAT8 plasmid (17). For the purpose of screening mutants, \( \alpha_1 \)-AT was expressed at 37 °C as a soluble form. However, for the purpose of purification, the protein was expressed at 40 °C as inclusion body.

**Mutagenesis**—Random mutagenesis was performed by a modified polymerase chain reaction (PCR) as described by Eckert and Kunkel (18). The 1260-base pair restriction fragment of \( \alpha_1 \)-AT cDNA was cloned into phage M13mp18. Single-stranded template DNA was isolated, and two M13 primers (Sigma) of reciprocal orientations were used for PCR. The reaction mixture was dATP-limiting condition (0.1 mM dATP, 1.0 mM each of three other dNTPs) in 10 mM MgCl\(_2\). After 25 cycles, BstI/BstXI fragment (770-base pair encoding amino acid positions 17-275) was isolated and exchanged with an equivalent fragment of pEAT8. Site-specific mutagenesis was performed by the method of Kunkel (19). Multiple amino acid substitutions were carried out by a mutagenic oligonucleotides (30-mer) containing NN/C/G (N is equimolar mixture of four dNTPs) at the target position. Nucleotide sequencing was performed with Sequenase 2.0 (U. S. Biochemical Corp.).

**Screening of Thermostable \( \alpha_1 \)-AT**—The colonies resulting from the mutagenesis were inoculated into 0.1 ml of supplemented minimal medium (per liter: 1 g of \( \text{NH}_4\text{Cl}, 3 \text{ g of KH}_2\text{PO}_4, 6 \text{ g of Na}_2\text{HPO}_4, 2 \text{ g of glucose, 0.2 g of yeast extract, 3 g of casamino acids} \) containing 1 mM isopropyl-\( \beta \)-D-thiogalactopyranoside and 50 \( \mu \)g/ml ampicillin on 96-well microtiter plates. The plates were incubated at 37 °C overnight on a shaker. The cultures were lysed by adding 25 \( \mu \)l of lysozomal buffer containing 250 mM Tris-Cl, pH 8.0, 25 mM EDTA, 0.025 % Triton X-100, 0.5 mg/ml lysozyme. The plates were incubated at room temperature for 1 h with continuous shaking. Following lysis the plates were incubated at 60 °C for 2 h to selectively release the wild type \( \alpha_1 \)-AT activity. Mutant \( \alpha_1 \)-AT that survived the above heat deactivation was identified by a chromogenic assay developed for dithiothreitol-sensitive mutants of bovine pancreatic trypsin inhibitor (20). Porcine pancreatic elastase (Sigma) was used as a protease at a 1.5 mM final concentration and N-succinyl-Ala-Ala-Ala-p-nitroanilide (Sigma) was used as a substrate at a 0.3 mM final concentration.

**Refolding and Purification of Recombinant \( \alpha_1 \)-AT**—Recombinant \( \alpha_1 \)-AT was purified from inclusion bodies after refolding and ion-exchange chromatography. Details of the purification method will be published elsewhere, but a brief description is as follows. The pellet of lysates acquired one clone that expressed substantially more stable \( \alpha_1 \)-AT activity than the wild type. Nucleotide sequencing was performed with Sequenase 2.0 (U. S. Biochemical Corp.) according to the manufacturer's instructions. Multiple amino acid substitutions were carried out by a mutagenic oligonucleotides (30-mer) containing NN/C/G (N is equimolar mixture of four dNTPs) at the target position. Nucleotide sequencing was performed with Sequenase 2.0 (U. S. Biochemical Corp.).

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**Identification of a Thermostable Mutation, Phe51 → Cys (F51C)**—Initial screening and heat deactivation assays with lysates acquired one clone that expressed substantially more stable \( \alpha_1 \)-AT activity than the wild type. Nucleotide sequencing identified that the 51th codon, TTC (Phe), was changed into TGC (Cys). Heat deactivation was performed at 57 °C with purified \( \alpha_1 \)-AT proteins. As shown in Fig. 1, F51C mutant recombinant protein was indeed more thermostable than the wild type recombinant protein. The stability of mutant \( \alpha_1 \)-AT was comparable with that of the glycosylated plasma \( \alpha_1 \)-AT. In order to determine the aggregation rate during heat deactivation, gel exclusion chromatography was performed with the proteins.
Gly belonged to one group with substantial inhibitory activity though polypeptides are synthesized normally. For the other folding transition experimental data to a two-state unfolding model (Fig. 1). The midpoints of transition ($C_m$) of the wild type and the mutant protein were 1.9 (± 0.1) M and 2.8 (± 0.1) M, respectively. Both transitions of the wild type and F51C appeared to be conformational stability-independent between 6 and 50 μg/ml (Fig. 3a). The transitions were fully reversible, and almost all the signals were regained in the refolding from 8 M urea, as shown in Fig. 3b. The $C_m$ values of the wild type protein were 1.9 and 2.0 M for unfolding and refolding, respectively, while the identical value of 2.7 M was obtained with the mutant protein for both unfolding and refolding. The results in Fig. 3b also showed that the native fluorescence intensity of the mutant protein was lower than that of the wild type for the same concentration (10 μg/ml as measured from the absorbance at 280 nm in 6 μM guanidine hydrochloride), while the unfolded values were similar.

Various Single Amino Acid Substitutions at Position 51—In order to identify factors at the position 51 influencing the thermal stability of α1-AT, various single amino acid substitutions were introduced. All 18 substitutions except lysine were obtained. Initial screening with lysates from cells grown at 37 °C revealed that substitutions with Pro, Trp, and charged residues (including His) did not yield any soluble activity of α1-AT although polypeptides are synthesized normally. For the other substitutions soluble activity of each lysate divided them into two groups: (i) substitutions with Leu, Val, Ile, Cys, Ala, and Gly belonged to one group with substantial inhibitory activity and (ii) substitutions with Asn, Ser, Gln, Met, Thr, and Tyr belonged to the other group with decreased activity when compared to the wild type lysate. Some of α1-AT protein carrying mutations belonging to the first group were purified, and heat stability and inhibitory activity were determined. Results are summarized in Table I. None of the mutations shown in Table I affected the function of α1-AT significantly. The heat deactivation rate indicates that amino acid residues carrying hydrophobic aliphatic side chains at position 51 increased the stability, and among them side chains with branched C3 (Val, Ile) were less effective than those with linear ones (Cys, Leu).

**DISCUSSION**

**Stability of Native Serpins**—One of the intriguing aspects of serpins is that the native conformation is not the thermody-
Table 1

| Amino acid | Association constant | Half-life | Deactivation rate |
|------------|----------------------|-----------|------------------|
| Phe (wild type) | 1.6 (± 0.3) x 10^6 | 3.1 | 0.224 |
| Cys | 1.4 (± 0.2) x 10^6 | 41.7 | 0.017 |
| Leu | 1.9 x 10^6 | 40.2 | 0.017 |
| Ile | 1.6 x 10^6 | 33.8 | 0.021 |
| Val | 1.6 x 10^6 | 33.2 | 0.021 |
| Ala | 1.6 x 10^6 | 6.9 | 0.101 |
| Human* | 1.7 (± 0.1) x 10^6 | 49.7 | 0.014 |

*Deactivation rate was calculated from fitting experimental data of remaining inhibitory activity into a single exponential decay.

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Theoretically most stable conformation. Strain of the active form of serpins appears to be very critical to inhibitory function and conformational switch that occurs after cleavage and it may well be that the mutation diminished loop-sheet polymerization. The effect of F51C mutation on the conformational stability was investigated by equilibrium unfolding in urea. The guanidine-induced equilibrium unfolding of α1-AT has been studied previously by various groups (4, 11, 29, 30), and a thermodynamic reversibility of unfolding has been demonstrated in the transition measured by fluorescence emission intensity (29). Our initial studies of the guanidine-induced unfolding monitored by the changes in intrinsic tryptophan fluorescence did not show an obvious effect of the mutation, which was probably due to the fact that both transitions occurred at very low guanidine concentration (data not shown). In order to observe the effect of the mutation on unfolding more clearly, urea-induced unfolding was performed. The results in Fig. 3 showed that the mutation shifted the transition midpoint from 1.9 to 2.8 M urea, increasing the free energy of stabilization (ΔGf) by 3 kcal/mol. Concentration independence and reversibility, shown in Fig. 3, indicated that the aggregation under the experimental conditions is negligible. It was reported that the recombinant α1-AT tended to aggregate during unfolding (29), but a much higher concentration (0.1–0.5 mg/ml) of α1-AT was used in that experiment. In our refolding experiment, recombinant α1-AT was found to recover 50% activity at 0.2 mg/ml but over 90% activity below 30 μg/ml for both wild type and the mutant proteins. The unfolding transitions of α1-AT monitored by fluorescence intensity could be fitted to a two-state model very well. However, the transitions monitored by circular dichroism spectroscopy (3, 29) or transverse urea gradient gel electrophoresis (13, 29) suggested that the unfolding of α1-AT is multi-phasic and continues at higher concentrations of denaturant. Therefore, the transitions shown in Fig. 3 reflect only the step of unfolding to which the fluorescence signal is sensitive, and the calculated energy difference is not that of the native and the unfolded states. There are two different ways of increasing the free energy of stabilization: first, lowering the energy level of the native folded state, and second, increasing the energy level of unfolded (partially unfolded in our case) state. These possibilities are to be distinguished to understand the mechanism of F51C mutation in detail.

The transition shown and changed by the mutation in urea denaturation may be the same as the transition from the native to the open conformation described above. Trp234, the major contributor of the signal among two tryptophans in α1-AT (29, 30), is likely to be a sensitive probe for sheet opening because it is located at the top of s5A and is hydrogen-bonded to the side chain of Asp41 (top of s4A) in the cleaved form (2) and also to the backbone of Lys49 in model native α1-AT (31). The mutational effect of F51C on aggregation during heat deactivation is likely to be a secondary effect resulted from the change in conformational stability shown in urea denaturation. The site identified in the present study may be critical for regulating stability of α1-AT.

Side-chain Contribution at Position 51—In the crystal structure of the cleaved form of α1-AT, the side chain of Phe31 is involved in hydrophobic interaction with several residues including Phe35 and Phe36 (2). This part of the structure is quite similar in the cleaved form of α1-AT and the crystal structure of
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ovalbumin (5), i.e. an intact serpin, and may not be different in the intact form of $\alpha_1$AT. Results of various amino acid substitutions showed that charged and polar residues are not tolerable at the site, consistent with the hydrophobic environment of position 51. The fact that smaller aliphatic hydrophobic side chains at 51 are more effective than the wild type residue, Phe, in enhancement of thermal stability of $\alpha_1$AT and among them linear chains at the $\beta$ carbon are more efficient than the branched ones (Table I) suggests that volume and flexibility of hydrophobic side chains at 51 are critical factors for the stability of $\alpha_1$AT. Phe$^{51}$ is moderately conserved among various serpins (1). Most of the amino acid substitutions that are shown to increase stability of $\alpha_1$AT against heat deactivation (Ala, Val, Ile, and Leu) are the ones found in sequences of other serpins. The fact that smaller aliphatic hydrophobic side chains at position 51 might have improved an overall tertiary packing. Structural studies on these mutant proteins will shed light on understanding the mutational effects and eventually the stability of serpins in general.

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REFERENCES

1. Huber, R., and Carré, R. W. (1989) Biochemistry 28, 8951–8966
2. Loebmann, H., Tokuoka, R., Deisenhofer, J., and Huber, R. (1984) J. Mol. Biol. 177, 531–556.
3. Bruch, M., Weiss, V., and Engel, J. (1988) J. Biol. Chem. 263, 16626–16630
4. Schulze, A. J., Huber, R., Degryse, E., Speck, D., and Bischoff, R. (1991) Eur. J. Biochem. 202, 1147–1155
5. Stein, P. E., Leslie, A. G. W., Finch, J. T., Turrell, W. G., McLaughlin, P. J., and Carré, R. W. (1989) Nature 347, 99–102
6. Katagiri, K., Okada, K., Hatton, H., and Yano, M. (1988) J. Biol. Chem. 176, 81–87
7. Mottonen, J., Strand, A., Symersky, J., Sweet, R. M., Danley, D. E., Geoghegan, K. F., Gadz, R. D., and Goldsmith, E. J. (1992) Nature 355, 270–272
8. Carré, R. W., Evans, D. L., and Stein, P. E. (1991) Nature 353, 576–578
9. Lomas, D. A., Evans, D. L., Finch, J. T., and Carré, R. W. (1992) Nature 357, 655–657
10. Lomas, D. A., Evans, D. L., Stone, R. C., Chang, W.-S. W., and Carré, R. W. (1993) Biochemistry 32, 500–508
11. Schulze, A. J., Baumann, U., Kroft, S., Jaeger, E., Huber, R., Laurell, C.-B. (1990) Eur. J. Biochem. 194, 51–56
12. Schulze, A. J., Prochnert, P. W., Engh, R. A., and Huber, R. (1992) Biochemistry 31, 7585–7585
13. Mast, A. E., Engblom, J. J., and Salvesen, G. (1992) Biochemistry 31, 2720–2728
14. Perkins, S. J., Smith, K. F., Nealis, A. S., Harris, P. L., Chapman, D., Bauer, C. J., and Harrison, R. A. (1992) J. Mol. Biol. 225, 1285–1294
15. Travis, J., Owen, M., George, P., Carré, R., Rosenherg, S., Hallewell, R., and Barr, P. (1985) J. Biol. Chem. 260, 4304–4309
16. Studier, F. W., Rosenberg, A. H., Dunn, J. J., and Dubendorff, J. W. (1990) Methods Enzymol. 185, 69–89
17. Lee, K. N., Shin, H. S., Kwon, K.-S., Park, S. D., and Yu, M.-H. (1993) Mol. Cells 3, 71–74
18. Eckert, K. A., and Kunkel, T. A. (1991) in PCR: A Practical Approach (McPherson, M. J., Quirke, P., and Taylor, G. R., eds) pp. 225–244, IRL Press, Oxford
19. Kunkel, T. A., Roberts, J. D., and Zakour, R. A. (1987) Methods Enzymol. 154, 367–382
20. Coplen, L. J., Frieden, R. W., and Goldenberg, D. P. (1990) Proteins Struct. Funct. Genet. 7, 16–31
21. Travis, J., and Johnson, D. (1981) Methods Enzymol. 80, 754–765
22. Beatty, K., Bischoff, L., and Travis, J. (1980) J. Biol. Chem. 255, 3931–3934
23. Edelhoch, H. (1987) Biochemistry 6, 1944–1954
24. Pace, C. N. (1986) Methods Enzymol. 115, 359–380
25. Curiel, D. T., Holmes, M. D., Okasaya, H., Brustow, M. L., Vogelweider, C., Travis, W. D., Stier, I. E., Perks, W. H., and Crystal, R. G. (1989) J. Biol. Chem. 264, 13938–13945
26. Suyama, K., Nukiwa, T., Takabashi, K., Miyake, K., and Kira, S. (1991) J. Biol. Chem. 266, 12627–12632
27. Lomas, D. A., Finch, J. T., Suyama, K., Nukiwa, T., and Carré, R. W. (1993) J. Biol. Chem. 268, 15335–15335
28. Stein, P., and Chothia, C. (1991) J. Mol. Biol. 221, 615–621
29. Powell, L. M., and Pain, R. H. (1992) J. Mol. Biol. 224, 241–252
30. Herve, M., and Guelin, C. (1990) Eur. J. Biochem. 181, 653–658
31. Engh, R. A., Wright, H. T., and Huber, R. (1990) Protein Eng. 3, 469–477
32. Lifson, S., and Sander, C. (1979) Nature 282, 109–111