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Characterization and suppression of dysfunctional human α_1-antitrypsin variants ☆

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Abstract

Human α_1-antitrypsin-deficient variants may aggregate in the liver, with subsequent deficiency in the plasma, which can lead to emphysema. The structural and functional characteristics of 10 dysfunctional α_1-antitrypsin variants (R39C, S53F, V55P, I92N, G115S, N158K, E264V, A336T, P369S, and P369L) were analyzed in detail. Most of them were unstable, as compared to the wild-type molecule, and many of the variants folded into an intermediate form. When five thermostable mutations (T68A, A70G, M374I, S381A, and K387R) were introduced into dysfunctional α_1-antitrypsin variants, the stabilities and inhibitory activities of most of the variants were restored to levels comparable to those of the wild-type molecule. However, the extremely unstable S53F variant was not stabilized sufficiently by these mutations so as to exhibit function. N158K variant, which carries a mutation in the region critical for the reactive site loop insertion into β-sheet A, exhibited a reduced level of inhibitory activity, despite conformational stabilization. These results show that aberrant folding caused by conformational destabilization due to mutations can be compensated for by increasing the overall stability of the α_1-antitrypsin molecule, with exception of a mutation in the highly localized region critical for functional execution.

Keywords: α_1-Antitrypsin; Conformational stability; Dysfunctional variants; Inhibitory activity; Pathogenic mutations; Serine protease inhibitors

Human α_1-antitrypsin (α_1AT) belongs to the serine protease inhibitor (serpin) superfamily, which shares a common tertiary structure that consists of three β-sheets and several α-helices [1]. In the active, native forms of inhibitory serpins [2], the reactive site loop (RSL) at one end of the molecule is exposed for protease binding (Fig. 1). Upon binding and cleavage of the RSL by the target protease, the RSL attached to the protease is inserted into β-sheet A [3,4]. The protease is then translocated over a distance of 70 Å along the side of the serpin molecule to the opposite pole [5,6], and the catalytic triad of the target protease is distorted [7]. These events markedly increase the stability of the serpin molecule [8]. Unlike other globular proteins, in which the native state is the thermodynamically most stable form, inhibitory serpins are unique in that the native form is a metastable form [4]. This metastability is thought to be important in facilitating conformational conversion into a more stable form during functional execution [9–13]. We have previously shown that unfavorable interactions occur throughout the serpin molecule, possibly to induce native strain [14]. However, incorporation of these structural aspects into the folding process exposes serpin molecules to various folding defects. Indeed, over 100 genetic variants of serpins have been reported [10].

The α_1AT is synthesized in the liver and secreted into the plasma, to protect tissues against indiscriminate proteolytic attack by neutrophil elastase [15]. Deficient α_1AT molecules in the plasma provide insufficient protection against proteolytic activity, which leads to clinical symptoms, such as pulmonary emphysema. Over 75 genetic variants of α_1AT have been reported [16]. The α_1AT-null alleles carry point mutations that introduce premature stop codons, or have a mutation in an exon–intron junction that interferes

☆ *Abbreviations:* α_1AT, α_1-antitrypsin; RSL, reactive site loop; serpin, serine protease inhibitor; TUG, transverse urea gradient.

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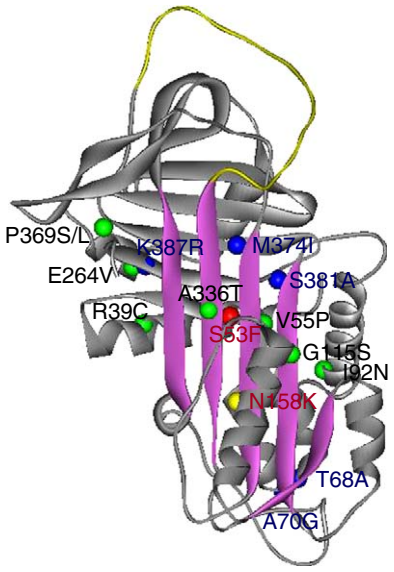

Fig. 1. A schematic diagram of the native structure of α_1AT (2psi.pdb) [2] showing mutation sites carried by deficient α_1AT variants. The β-sheet A is shown as purple strands and the RSL is colored in yellow. Mutation sites that can be suppressed by addition of thermostable mutations (shown as blue beads) are indicated with green beads. The N158K mutation, that showed reduced inhibitory activity, is indicated with a yellow bead. The S53F mutation, which could not be stabilized enough, is shown as a red bead. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

with efficient splicing of the α_1AT mRNA [17]. Most pathogenic α_1AT alleles have single substitutions that result in the production of dysfunctional α_1AT molecules. In the clinically most significant Z-type (Glu342 → Lys) variant, severely retarded protein folding has been implicated in the accumulation of an intermediate with a high tendency to aggregate [18]. Recently, two other α_1AT variants, D256V and L41P, have also been reported to fold very slowly [19]. Although the native form of the M_{malton} (Phe52 deletion) variant is as stable and active as the wild-type protein, this variant molecule readily undergoes conformational change into a more stable, inactive, latent form under physiological conditions [20]. In the latent form, the RSL of the serpin molecule is inserted into β-sheet A without being cleaved [21]. The $\alpha_1AT_{Pittsburgh}$ (Met358 → Arg) variant, which bears an amino acid substitution at site P1 (the 'bait' residues in front of the scissile peptide bond on the RSL), exhibits a shift in target specificity from neutrophil elastase to thrombin, which results in a bleeding disorder [22]. Most variants are prone to intermolecular loop-sheet polymerization [10,23,24], although the mechanisms by which these variants form aggregates remain mostly unknown. To elucidate the basis for α_1AT deficiency, we first examined the conformational stability of 10 dysfunctional α_1AT variants with single substitutions. The ability of each variant molecule to form an inhibitory complex with the target protease was also monitored. In the case of severely destabilized variants, it was difficult to judge whether the substituted residues that caused the α_1AT destabilization also affected the inhibitory activities. Therefore, five thermostable mutations (T68A, A70G, M374I, S381A, and K387R) were introduced into each of the 10 dysfunctional α_1AT variant molecules, to suppress conformational instability and to study the detailed basis for α_1AT deficiency.

Materials and methods

Chemicals. Ultrapure urea was purchased from ICN Biochemicals (Coast Mesa, CA). Porcine pancreatic elastase and *N*-succinyl-$(Ala)_3$-*p*-nitroanilide were purchased from Sigma (St. Louis, MO). All other chemicals were of reagent grade.

Mutagenesis, expression, and purification of recombinant α_1AT. Substitution mutations were introduced on pFEAT30, the plasmid for α_1AT expression in *Escherichia coli* [25], by oligonucleotide-directed mutagenesis [26]. Recombinant α_1AT was expressed as inclusion bodies in *E. coli* and refolded as described previously [27]. Recombinant α_1AT proteins were purified on a Q Sepharose™ Fast Flow column (Amersham Bioscience Ltd.) in a buffer (10 mM phosphate, 1 mM β-mercaptoethanol, and 1 mM EDTA, pH 6.5). The protein was further purified by ion exchange chromatography on Resource Q column (Amersham Bioscience Ltd.) in the same buffer by FPLC. Concentrations of α_1AT were determined in 6 M guanidine hydrochloride using a value of $A^{1\%}_{1cm} = 4.3$ at 280 nm, calculated from the tyrosine and tryptophan content of the α_1AT protein [28] and based upon $M_r = 44{,}250$.

Conformational analysis by gel electrophoresis. Conformation of α_1AT proteins was analyzed by transverse urea gradient (TUG) gel electrophoresis [29]. TUG gels were prepared with a gradient of 0–8 M urea perpendicular to the direction of electrophoresis with an opposing gradient of acrylamide from 15% to 11%. Four slab gels (100 × 80 mm) were prepared simultaneously in a multigel caster (Hoefer Scientific Instruments) by using a gradient maker and a single-channel peristaltic pump. The α_1AT protein (20 μg in 100 μl) was applied across the top of the gel. The electrode buffer was 50 mM Tris–acetate and 1 mM EDTA (pH 7.5). The gels were run at a constant current of 6 mA for 3 h at a controlled temperature of 20 °C. The protein bands were visualized by staining with Coomassie brilliant blue.

Denaturant-induced unfolding transition. To measure the stability of α_1AT variants quantitatively, equilibrium unfolding of the native α_1AT as a function of urea (ICN Biomedicals, Inc.) was monitored by changes in intrinsic fluorescence of α_1AT. The protein concentration was 10 μg/ml in a buffer (10 mM phosphate, 50 mM NaCl, 1 mM EDTA, and 1 mM β-mercaptoethanol, pH 6.5). The native protein was incubated in the buffer containing various concentrations of urea at 25 °C for 2 h. Equilibrium unfolding was monitored by fluorescence spectroscopy ($\lambda_{ex} = 280$ nm and $\lambda_{em} = 360$ nm, excitation and emission slit width = 5 nm for both), using Shimadzu RF-5301PC spectrophotometer as described previously [27]. Experimental data of the fluorescence measurement at 360 nm were fitted to a two-state unfolding model to measure stability of the native form.

Thermostability. To measure thermostability of α_1AT variants, purified α_1AT proteins was incubated for 30 min at various temperatures ranging from 40 to 65 °C. The protein concentration was 0.15 mg/ml in a buffer (10 mM phosphate, 50 mM NaCl, 1 mM EDTA, and 1 mM β-mercaptoethanol, pH 6.5). Disappearance of monomers and formation of oligomers of α_1AT molecules were monitored on 10% non-denaturing gels containing Tris–glycine buffer system.

Complex formation with the target protease. Inhibitory activity of α_1AT variants was followed by monitoring the ability to form SDS-stable complexes with porcine pancreatic elastase. Active concentration of porcine pancreatic elastase, a target protease, was determined by measuring the initial rates of hydrolysis of 1 mM *N*-succinyl-Ala-Ala-Ala-*p*-nitroanilide [30]. Four micrograms of α_1AT proteins was incubated with the protease at various molar ratios of α_1AT to protease at 37 °C for 10 min. For P369L variant, three times more protein was used to visualize the small amount of inhibitory complex. The buffer was

30 mM phosphate, pH 7.4, 160 mM NaCl, 0.1% PEG6000, and 0.1% Triton X-100. Inhibitory complex formation of α_1AT proteins with target proteases was examined by 10% SDS–polyacrylamide gel electrophoresis.

Determination of the stoichiometry of inhibition. The stoichiometry of inhibition was determined by titration reactions as described [11]. The active concentration of porcine pancreatic elastase was determined as described above. Various amounts of purified recombinant wild-type or mutant α_1AT proteins were incubated in 50 μl assay buffer (30 mM phosphate, 160 mM NaCl, 0.1% PEG 6000, and 0.1% Triton X-100, pH 7.4) with 100 nM porcine pancreatic elastase at designated molar ratios of α_1AT to protease. After incubation with the protease at 37 °C for 10 min, the reaction mixture was diluted 20-fold with the assay buffer and the residual enzyme activity was determined using 1 mM *N*-succinyl-$(Ala)_3$-*p*-nitroanilide as a substrate.

Results

Most of the deficient α_1AT variants are conformationally unstable

To elucidate the structural bases for the α_1AT deficiencies of the 10 variants (R39C, S53F, V55P, I92N, G115S, N158K, E264V, A336T, P369S, and P369L), the mutations carried by these variants were introduced separately into recombinant α_1AT using oligonucleotide-directed mutagenesis. After expression and purification of the variant α_1AT proteins, the conformations of the mutant serpins were analyzed by transverse urea gradient (TUG) gel electrophoresis (Fig. 2). As the urea concentration increased gradually in this gel system, the wild-type α_1AT exhibited three unfolding state transitions. Three (R39C, G115S, and P369S) of the 10 α_1AT variants tested showed moderate decreases in conformational stability as shown by unfolding of their native forms at lower urea concentrations than that required to unfold the wild-type α_1AT. However, the remaining α_1AT variant polypeptides (S53F, V55P, I92N, N158K, E264V, A336T, and P369L) exhibited drastic decreases in conformational stability, and generally folded into a molten globule-like intermediate with a lower electrophoretic mobility than the native form (Fig. 2). Low levels of a tightly folded form, which migrated to the same position as the native form in the gels, were also observed for E264V, A336T, and P369S variants. The conformational stabilities of the α_1AT variants were quantified by equilibrium unfolding in the presence of urea. The unfolding transition midpoint was 1.8 M urea for the wild-type protein, and the transition midpoints for the R39C, G115S, and P369S variants were 1.6, 1.4, and 1.1 M urea, respectively, corresponding to free energy changes ($\Delta\Delta G$) of 0.6, 1.3, and 2.2 kcal/mol, respectively. The unfolding transition values for the remaining variants could not be determined, since they folded poorly into the native form, as described above.

The thermostability of each α_1AT variant was determined by heat treatment at various temperatures for 30 min and analyzed by non-denaturing gel electrophoresis. The thermostability results for the α_1AT variants (Fig. 3) were consistent with the results of the TUG gel electrophoresis (Fig. 2). For the wild-type protein, low amounts of the polymers were first detected upon heat treatment at 50 °C. On the other hand, significant amounts of the polymers were formed at this

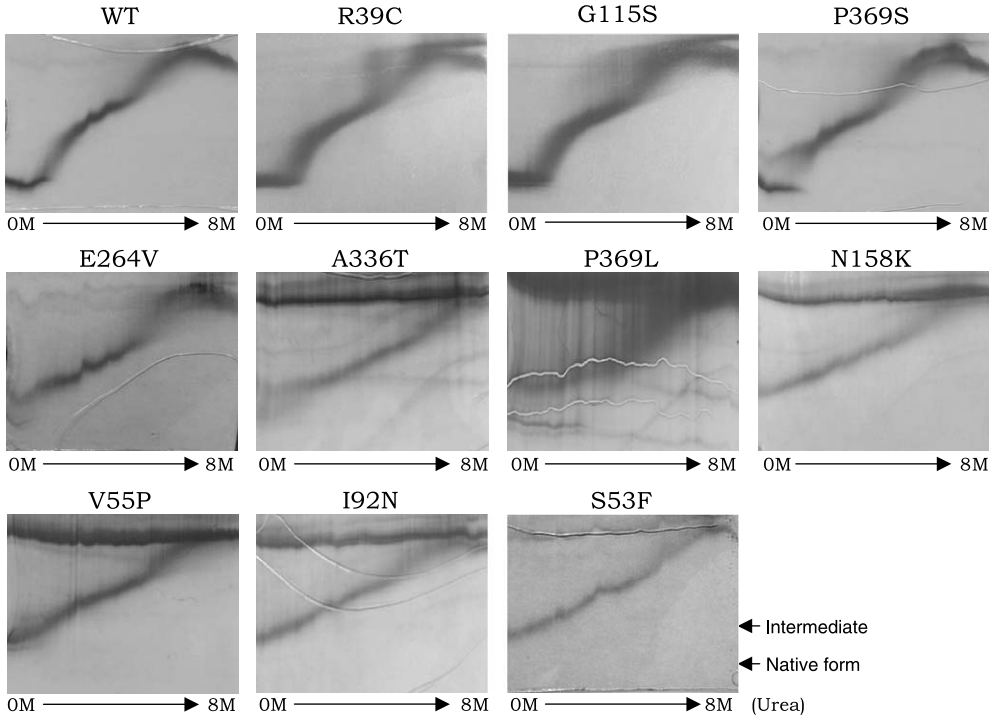

Fig. 2. TUG gel electrophoresis of α_1AT variants. The wild-type (WT) and variant α_1AT proteins were analyzed by TUG gel electrophoresis [29]. The transverse urea gradient gels contained a gradient of 0–8 M urea perpendicular to the direction of electrophoresis. Positions of the native and intermediate conformers are indicated.

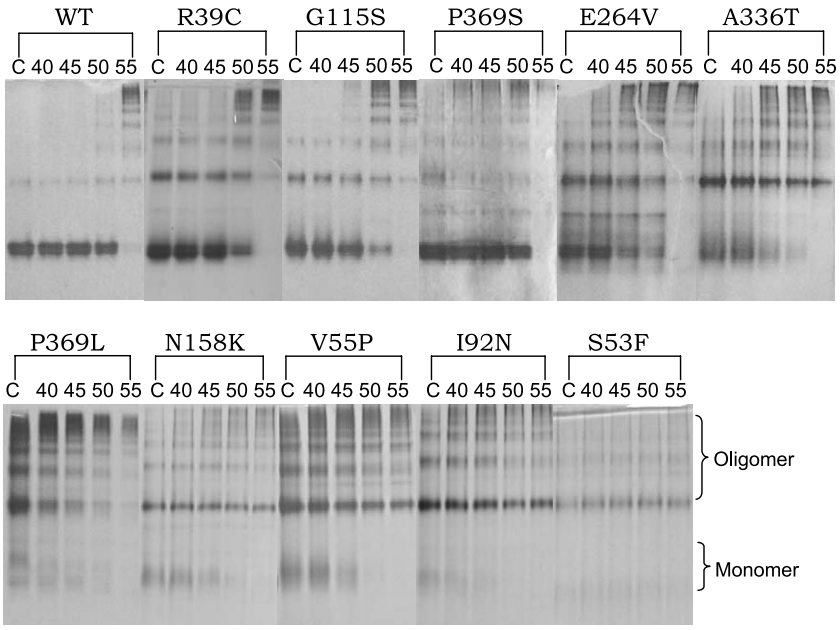

Fig. 3. Thermostability of the α_1AT variants. The α_1AT variant proteins were heat-treated for 30 min at various temperatures ranging from 40 to 55 °C, as shown on the top of the gel. The protein in each reaction was 4 μg in 30 μl buffer (10 mM phosphate, 50 mM NaCl, pH 6.5). The degree of polymerization was analyzed on non-denaturing gels containing 10% polyacrylamide. The protein bands were visualized by staining with Coomassie brilliant blue. Lane C contains α_1AT proteins before heat treatment. Migration positions of monomer and oligomers of α_1AT molecules are indicated.

temperature for the R39C, G115S, and P369S α_1AT variants. For the E264V, A336T, and P369L variant α_1ATs, substantial amounts of the respective polymers were produced by treatment at temperatures as low as 40 °C. For the severely destabilized variants, N158K, V55P, I92N, and S53F, neither the native forms were observed before any heat treatment, nor the molten globule-like intermediates remained stable during heat treatment.

The functionalities of the α_1AT variants were monitored based on the ability to form SDS-stable protease/inhibitor complexes with the target protease, elastase. The R39C, G115S, and P369S variants formed comparable levels of inhibitory complexes to that formed by the wild-type protein (Fig. 4). Low levels of complexes were formed for the E264V, A336T, and P369S variants, although most of these polypeptides were cleaved by the protease. The remaining variants (N158K, V55P, I92N, and S53F), which folded to loosely packed, molten globule-like intermediates, were vulnerable to protease attack; instead of forming a stable inhibitory complex, these proteins were degraded. It appears that there is a correlation between conformational stability and the ability to form inhibitory complexes.

The deficiencies in most of the α_1AT variants can be suppressed by the introduction of stabilizing mutations

Conformational liability, at least for most of the α_1AT variants, appears to affect α_1AT polypeptides so that they neither fold into the native form, nor retain the appropriate biological function. Therefore, we hypothesized that increasing the overall stabilities of the α_1AT variants would suppress the deficiencies associated with these variant molecules. We have previously identified single mutations that delay unfolding transitions during urea-dependent equilibrium unfolding, which indicates that they have increased conformational stability [14]. Of these, the M5 mutations (T68A, A70G, M374I, S381A, and K387R) have been utilized to suppress defects associated with the M_{malton} α_1AT variant [20]. The stabilized M_{malton} variant retained full inhibitory activity towards the target protease, and the defect of the M_{malton} variant, facile latency transition, was reduced significantly, although not completely blocked [20].

In this study, we introduced M5 mutations into the α_1AT variant molecules to suppress the defects associated with the variants. The conformational stabilities of the α_1AT variants were drastically increased by the M5 mutations (Fig. 5A). The stabilized variant molecules not only acquired the tightly folded form, but remained resistant to higher concentrations of urea than the wild-type (compare Figs. 2 and 5). In contrast, in the case of the S53F α_1AT variant, about half of the molecules still folded into the molten globule-like intermediate, even after stabilization with the M5 mutations. The conformational stability increase was assessed quantitatively by equilibrium unfolding as a function of urea concentration using fluorescence spectroscopy. As expected, the wild-type α_1AT showed drastically increased conformational stability following the addition of the M5 mutations with the first unfolding transition shifting to 3.9 M from 1.8 M urea. The conformational stabilities of most of the α_1AT variant

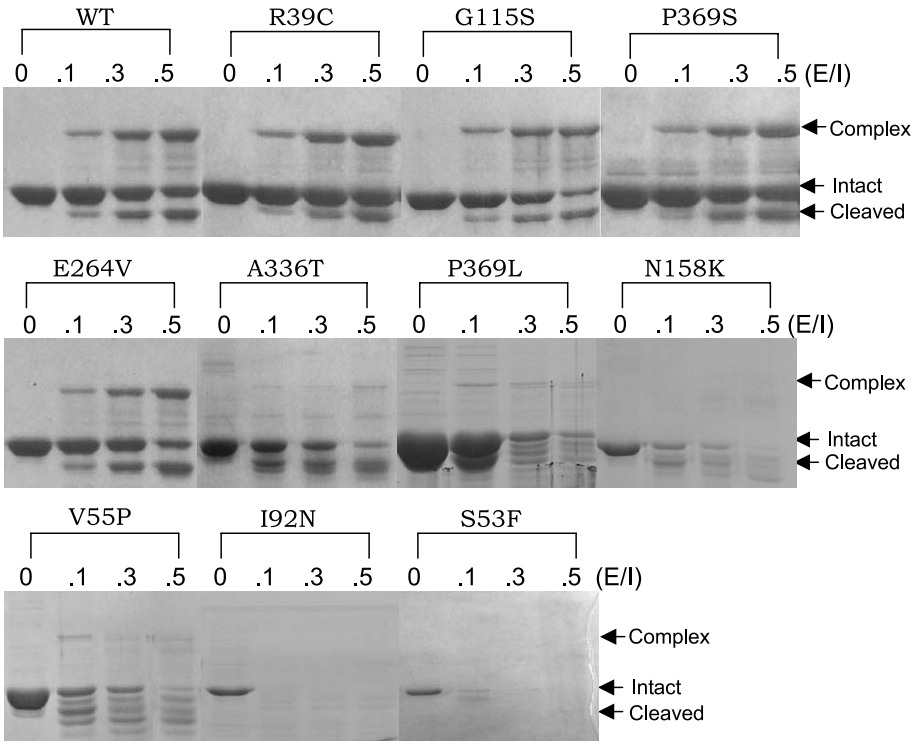

Fig. 4. Inhibitory complex formation with elastase by the α_1AT variants. The native wild-type (WT) and variant α_1AT proteins were incubated with porcine pancreatic elastase at the indicated molar ratios of the protease to α_1AT (E/I ratios). After incubation at 37 °C for 10 min in the assay buffer (30 mM phosphate, 160 mM NaCl, 0.1% PEG 8000, and 0.1% Triton X-100, pH 7.4), formation of the SDS-resistant α_1AT–elastase complex was analyzed by 10% SDS–polyacrylamide gel electrophoresis. The protein bands were visualized by staining with Coomassie brilliant blue. Migration positions of the inhibitory complex, intact α_1AT, and the RSL-cleaved α_1AT are indicated.

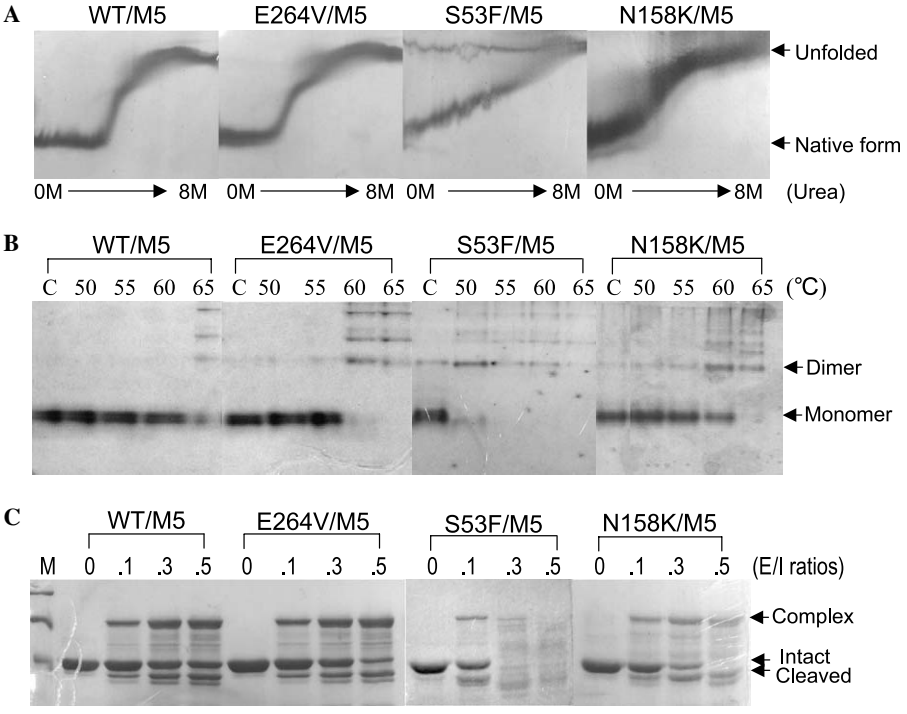

Fig. 5. Characteristics of the stabilized α_1AT variants. (A) TUG gel electrophoresis of the stabilized α_1AT variants. The stabilized wild-type (WT/M5) and variant α_1AT proteins were analyzed as described in Fig. 2. (B) Thermostability of the stabilized α_1AT variants. The purified α_1AT variant proteins were heat-treated for 30 min at various temperatures ranging from 50 to 65 °C, as shown on the top of the gel, and analyzed on non-denaturing gels as described in Fig. 3. Lane C contains α_1AT proteins before heat treatment. (C) Inhibitory complex formation by the stabilized α_1AT variants with elastase. The stabilized wild-type (WT/M5) and variant α_1AT proteins were incubated with porcine pancreatic elastase at the indicated molar ratios of the protease to α_1AT (*E*/*I* ratios), as described in Fig. 4. Migration positions of the inhibitory complex, intact α_1AT, and the RSL-cleaved α_1AT are indicated.

molecules, with the sole exception of the S53F/M5 variant, were increased sufficiently by M5 mutations such that the unfolding transition midpoint was shifted to >2.6 M urea (Table 1). The thermostabilities of the variant proteins were also dramatically increased, and temperatures >57 °C were necessary to induce polymerization of about half of the α_1AT molecules within 30 min, again with the exception of S53F/M5, which was polymerized at temperatures <50 °C (Fig. 5B). Given that the wild-type α_1AT molecules exhibited unfolding transitions at 1.8 M urea and that 50% of the wild-type molecules polymerized at approximately 52 °C, we conclude that most of the variants acquired sufficient conformational stability following the introduction of the M5 mutations.

However, it is not known whether this tightly folded species is actually the native form with native biological functions or another misfolded conformation. To address this question, the abilities of the stabilized α_1AT variants to form SDS-stable protease/inhibitor complexes were monitored using SDS–polyacrylamide gels (Fig. 5C). Most of the stabilized α_1AT variants formed similar levels of inhibitory complexes, although the S53F/M5 polypeptides were fragmented into smaller pieces, rather than forming an inhibitory complex with the target protease. This was not surprising, considering that the stability of this variant was not recovered to a degree comparable to that of the wild-type protein. When the ratios of the α_1AT levels present in the inhibitor–protease complexes to the α_1AT levels cleaved by the protease were compared, there were noticeable differences in inhibitory activity between the wild-type (WT/M5) and the N158K α_1AT variant molecules. This variant showed increased cleavage, as compared to the wild-type protein. When the inhibitory activity was quantified as the stoichiometry of inhibition (SI; the moles of inhibitors required to completely inhibit one mole of a target protease), the SI values of most of the variants, with the exceptions of the S53F and N158K variants, were close to that of the wild-type molecule (Table 1). This result confirms that the deficiencies of destabilized α_1AT variant molecules can be suppressed by stabilizing mutations, with exception of variant(s) carrying a mutation in a region critical for functionality.

Table 1
Stability and activity of the stabilized α_1AT variants

α_1AT variants	Equilibrium unfolding (C_m)[a]	Thermostability (T_m)[b]	Relative activity[c]
WT	1.8	52	1.00
WT/M5	3.9	64	1.00
R39C/M5	3.7	62	0.95
S53F/M5	0.0[d]	45	0.27
V55P/M5	2.7	57	0.94
I92N/M5	3.8	63	0.97
G115S/M5	3.3	61	0.89
N158K/M5	2.8	58	0.66
E264V/M5	3.0	59	0.90
A336T/M5	2.7	58	0.93
P369S/M5	3.3	62	0.92
P369L/M5	3.1	61	0.88

[a] C_m is the transition midpoint of urea-induced equilibrium unfolding in M. The experimental errors are ±0.1 M.
[b] T_m is the temperature in °C at which half of α_1AT monomers are polymerized for 30 min heat-treatment. The experimental errors are ±1 °C.
[c] Relative activity against protease is indicated as SI_{wt}/SI_{mut}, where SI_{wt} is the SI value of the wild-type α_1AT and SI_{mut} is the SI value of the mutant α_1AT.
[d] Since about half of S53F/M5 molecules were unfolded at 0 M urea in TUG gel, C_m of the variant is estimated to be about 0 M.

Discussion

Structural basis for destabilized α_1AT variants

The molecular characteristics of the deficient α_1AT variants were defined by the precise site and nature of the mutations. Most of the dysfunctional α_1AT variants examined in this study folded into a loosely packed intermediate (Fig. 2), which was prone to protein aggregation. Therefore, conformational instability is the primary defect of these dysfunctional α_1AT variants. The stabilities of the variant proteins were decreased marginally to severely, as compared to that of the wild-type α_1AT. The conformational stability results were consistent with the thermostability measurements for these variant molecules (Fig. 3). Reduced thermostability probably exacerbates α_1AT polymerization, especially during inflammation owing to the elevated body temperature, which leads to the development of the clinical symptoms of α_1AT deficiency [31].

In the severely destabilized variants, the functional integrity of the native form could not be addressed, since the native α_1AT molecules were difficult to obtain. Therefore, we decided to increase the overall stability by the addition of thermostable mutations to the deficient α_1AT molecules. Since direct interactions between the introduced stabilizing mutations and the deficiency-causing substitutions were considered to be unlikely, the molecular characteristics of the deficient α_1AT variants could be revealed. Indeed, we have previously increased the stability of the M_{malton} (Phe52-deleted) α_1AT variant by the addition of the same M5 mutations, and found that the facile latency conversion of M_{malton} α_1AT was retained, albeit at decreased rates [20]. Most of the stabilized variants (R39C, V55P, I92N, G115S, E264V, A336T, P369S, and P369L) showed recovery of inhibitory activities to levels comparable to that of the wild-type molecule, which suggests that the major defect is conformational destabilization. The destabilizing effects of these substitutions can be explained by looking at the native structure [2] of wild-type α_1AT (2psi.pdb); the side-chain of Arg39 (located on helix A) is projected backwards from the molecule, and substitution of this residue causes only marginal destabilization. Val55 (located on helix B) is buried in the hydrophobic core of α_1AT, and substitution to proline may promote a different configuration to the molecule. Ile92 (located on helix D) displays a hydrophobic interaction

with Phe82 (located at the end of helix C) at a distance of 4.0 Å, and substitution to a polar asparagine should destabilize the protein. Gly115 and Ala336 are located at s2A and s5A, respectively. Gly115 is covered by the side-chain of Tyr187 (located on s3A underneath helix F) at a distance of 3.5 Å, and substitution to a larger, polar residue of serine would push Tyr187 upwards, push away helix F, and lose some favorable interactions. The side-chain of Ala336, which is located at the center of β-sheet A, points towards the hydrophobic core of the α_1AT molecule and substitution to a polar threonine residue would introduce a devastating buried polar group. Glu264 is located on the short helix G and forms a salt bridge to Lys387 in s5B (the fifth strand of β-sheet B). Loss of the salt bridge due to E264V substitution may affect stability. Pro369 is located at the turn between s1C and s4B; this proline residue may be necessary to induce a turn between these two β-strands. These mutations are located in regions that are not directly involved in functionality, and they could be rescued by overall stabilization of the α_1AT molecules.

S53F was not sufficiently stabilized to fold into the native form by the M5 mutations, which increased the conformational stability of the wild-type α_1AT molecule by 6.7 kcal mol^{-1}. Since some native molecules were observed on Tug gels of the S53F/M5 variant, the urea-induced unfolding transition midpoint was approximately 0 M urea, which indicates that the S53F polypeptide is destabilized by more than 10 kcal mol^{-1}. Ser53 is located in the "shutter" domain, which is the hydrophobic region underneath β-sheet A and should be mobilized upon RSL insertion during inhibitory complex formation. A previous study has suggested that this region is over-packed, and the S53F substitution, which increases the volume of the side-chain in this region, is postulated to induce drastic conformational instability [27]. Since the S53F variant molecules were not stabilized sufficiently to fold into the native form following the addition of the M5 mutations, we could not determine whether the locus is also critical for functional execution.

Structural basis for the activity-modulating α_1AT variant

The N158K variant had a defect other than protein destabilization. Upon addition of the M5 mutations, the levels of conformational stability and thermostability of the variant were higher than those of the wild-type molecule, whereas the inhibitory function of this variant was not fully recovered. In the native structure of the α_1AT molecule [2], helix F sits on top of β-sheet A, and the gap between s3A and s5A (the third and fifth strands of β-sheet A) is closed. Upon RSL insertion into β-sheet A as s4A during inhibitory complex formation, helix F should be lifted and strands 1, 2, and 3 of β-sheet A should slide open with respect to strands 5 and 6. Asn158 is located on helix F and points towards the turn that connects helix F and s5A. The $N_{\delta 2}$ atom of Asn158 is hydrogen-bonded to the backbone oxygen atom of Val173 at a distance of 3 Å. The native interactions at these loci may be important for the regulation of RSL insertion during inhibitory complex formation, and the introduction of different residue(s) should modify the native configuration of α_1AT, which is critical for inhibitory activity. An extensive search for stabilizing mutations in the α_1AT molecule has demonstrated that most of the stabilizing substitutions distributed throughout the molecule do not affect inhibitory activity [14]. Those mutations that do affect the inhibitory activity are confined to those regions that are mobilized during inhibitory complex formation: for example, K168I at helix F affects the inhibitory function.

This study has provided significant insights into the structure–function relationships of serpins. Aberrant folding caused by conformational destabilization due to mutations can be compensated for by increasing the overall stability of the α_1-antitrypsin molecule, with exception of a mutation in the highly localized region critical for functional execution. Unlike most globular proteins, the native forms of inhibitory serpins are thought to be metastable states rather than the thermodynamically most stable forms. The energy saved in the protein folding process is utilized for a facile conformational switch during functioning [12]. Although the native metastability may be important for function, it makes the serpin molecules vulnerable to protein misfolding and spontaneous conformational switches, even in the absence of protease binding [32]. The approach adopted in this study, the analysis of stabilized variants by releasing native strains in functionally non-critical regions, is useful in clarifying the major defects in deficient serpin molecules, which may involve conformational instability or other dysfunctions, such as aberrant ligand binding and/or conformational changes.

Acknowledgments

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