Retarded protein folding of deficient human α_1-antitrypsin D256V and L41P variants

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Abstract

α_1-Antitrypsin is the most abundant protease inhibitor in plasma and is the archetype of the serine protease inhibitor superfamily. Genetic variants of human α_1-antitrypsin are associated with early-onset emphysema and liver cirrhosis. However, the detailed molecular mechanism for the pathogenicity of most variant α_1-antitrypsin molecules is not known. Here we examined the structural basis of a dozen deficient α_1-antitrypsin variants. Unlike most α_1-antitrypsin variants, which were unstable, D256V and L41P variants exhibited extremely retarded protein folding as compared with the wild-type molecule. Once folded, however, the stability and inhibitory activity of these variant proteins were comparable to those of the wild-type molecule. Retarded protein folding may promote protein aggregation by allowing the accumulation of aggregation-prone folding intermediates. Repeated observations of retarded protein folding indicate that it is an important mechanism causing α_1-antitrypsin deficiency by variant molecules, which have to fold into the metastable native form to be functional.

Keywords: α_1-antitrypsin; conformational disease; folding; metastability; serpin

α_1-Antitrypsin (α_1AT) is the archetype of the serine protease inhibitor (serpin) superfamily (Huber and Carrell 1989). Members of this family include protease inhibitors found in blood plasma, such as α_1-antichymotrypsin, antithrombin-III, C1 inhibitor, and plasminogen activator inhibitor-1, as well as noninhibitory proteins, such as ovalbumin and angiotensinogen. Serpins share a common tertiary structure based on a mobile reactive site loop (RSL), three β-sheets, and several α-helices (Elliott et al. 1996). Inhibitory serpins are unique in that the native form is metastable, and the RSL is mobile (Stratikos and Gettins 1997; Huntington et al. 2000). These features likely facilitate a conformational conversion during functional execution (Wiley and Skehel 1987; Huber and Carrell 1989; Stein and Carrell 1995; Carr et al. 1997; Im et al. 1999; Lee et al. 2000). In the active, native form of inhibitory serpins (Elliott et al. 1998), the RSL is exposed at one end of the molecule for protease binding (Fig. 1). Upon binding and cleavage of the RSL by the target protease, the acyl-enzyme intermediate is inserted into β-sheet A (Johnson and Travis 1978; Loebermann et al. 1984), the protease is translocated to the end of the serpin distal to the initial docking site (Huntington et al. 2000), and the catalytic triad of the target protease is distorted (Plotnick et al. 1996). These events markedly increase the stability of the serpin molecule (Bruch et al. 1988). In an alternate, "latent" serpin form, the RSL is inserted into β-sheet A without being cleaved (Mottonen et al. 1992). This latent form is more stable than the native form (Hekman and Loskutoff 1985; Wang et al. 1996), but is inactive. Another serpin conformation involves insertion of the RSL of one serpin molecule into a β-sheet of a second serpin molecule, resulting in the formation of loop–sheet serpin polymers (Lomas et al. 1992). Although the conformational versatility of serpins may be important for regulating their biological functions, it carries inherent disadvantages, such as vulnerability to protein misfolding and facile conformational conversion of the metastable native form into stable, inactive forms.

α_1AT is synthesized in the liver and secreted into plasma to protect tissues against indiscriminate proteolytic attack by neutrophil elastase (Carrell et al. 1982). An imbalance between serum proteases and their inhibitors due to dys-

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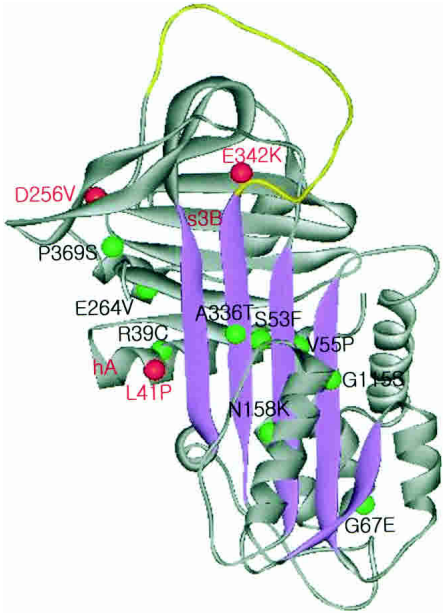

Figure 1. A schematic diagram of the native structure of α_1AT (2PSI.PDB; Elliott et al. 1998) showing mutation sites carried by deficient α_1AT variants. The β-sheet A is shown as purple strands, and the RSL is colored in yellow. Mutation sites that exhibited retarded protein folding are indicated with red beads, and other tested mutations that did not show retarded folding are indicated with green beads.

functional variant serpins or environmental factors causes serious clinical problems, such as tissue damage and bleeding disorders. Dozens of dysfunctional α_1AT genetic variants have been reported, but, for most pathogenic α_1AT molecules, the detailed structural basis for serpin deficiency has not been elucidated. They appear to form protein aggregates in the endoplasmic reticulum of hepatocytes, the site of α_1AT biosynthesis, which lead to liver cirrhosis, and subsequent α_1AT deficiency in plasma, causing pulmonary emphysema (Eriksson et al. 1986; Stein and Carrell 1995). The majority of dysfunctional α_1AT proteins are probably conformationally labile, and are prone to subsequent intermolecular loop–sheet polymerization, leading to hepatic inclusions and plasma deficiency of α_1AT (Lomas et al. 1993a, 1995). Indeed, loop–sheet polymers of α_1AT have been found in vivo in patients carrying defective α_1AT alleles, such as the I-type (Arg 39 → Cys) and S_{iiyama} (Ser 53 → Phe) variants (Mahadeva et al. 1999). However, the mechanisms by which these variants form aggregates remain mostly unknown.

In the case of the best-studied Z-type (Glu 342 → Lys) α_1AT variant, which is found at an allele frequency of 0.04 in Northern European populations, extremely retarded protein folding leads to the accumulation of an intermediate with a high tendency to aggregate. Once folded, the native Z-type protein is quite stable and shows inhibitory activity toward target proteases comparable to that of the wild-type molecule (Yu et al. 1995). Some variants show impaired protease inhibitory activity. Various biochemical (Wright and Scarsdale 1995; Gils et al. 1996; Huntington et al. 1997) and structural (Aertgeerts et al. 1995; Lukacs et al. 1996) studies have suggested that the rate of RSL insertion into β-sheet A is critical for inhibitory function. Hence, bulky substitutions on the inserted RSL would interfere with the kinetics of the inhibitory function of α_1AT, converting the serpins into substrates rather than inhibitors of target proteases. Because the exposed RSL of serpins fits into a catalytic cleft on the protease, the serpin residues at sites P1 and P1′ (the residues before and after the scissile peptide bond, respectively) determine target protease specificity. $\alpha_1AT_{Pittsburgh}$ has an amino acid substitution at site P1 on the RSL (Met 358 → Arg), and its target specificity is shifted from neutrophil elastase to thrombin, causing a bleeding disorder (Owen et al. 1983). Some variants may adopt conformations different from the native form. A variant α_1-antichymotrypsin (Leu 55 → Pro) has a mutation in the so-called shutter domain underlying the opening of β-sheet A, which has to be mobilized on complex formation with the target protease. This variant is found not only in the native conformation, but also in the inactive latent conformation and in an inactive stable conformation that is an intermediate in the polymerization pathway (Gooptu et al. 2000). Several naturally occurring dysfunctional serpin mutations, such as S_{iiyama} (53 Ser → Phe) and M_{malton} (Phe 52-deleted) α_1AT variants, are also clustered in the shutter domain. Perturbation of this region probably increases the flexibility of β-sheet A and favors the formation of different conformers. Indeed, the M_{malton} α_1AT variant spontaneously transforms into a latent-like conformation (Jung and Im 2003).

In this study, we examined the structural basis for the deficiency of a dozen α_1AT variants with substitutions outside the RSL. Most variant α_1AT proteins studied exhibited decreased stability as compared with the wild-type molecule. Conspicuously, protein folding of the D256V and L41P variants was markedly retarded, but, once folded, their stabilities and inhibitory activities were comparable to those of the wild-type molecule.

Results

Folding of some dysfunctional α_1AT variants is markedly retarded

To study the structural bases for α_1AT deficiency of genetic variants, a dozen mutant alleles 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 conformation of the mutant serpins was analyzed by transverse urea gradient gel electrophoresis (Fig. 2). In this gel system, electrophoretic mobility of proteins depends on the hydrodynamic volumes of different conformational states, induced

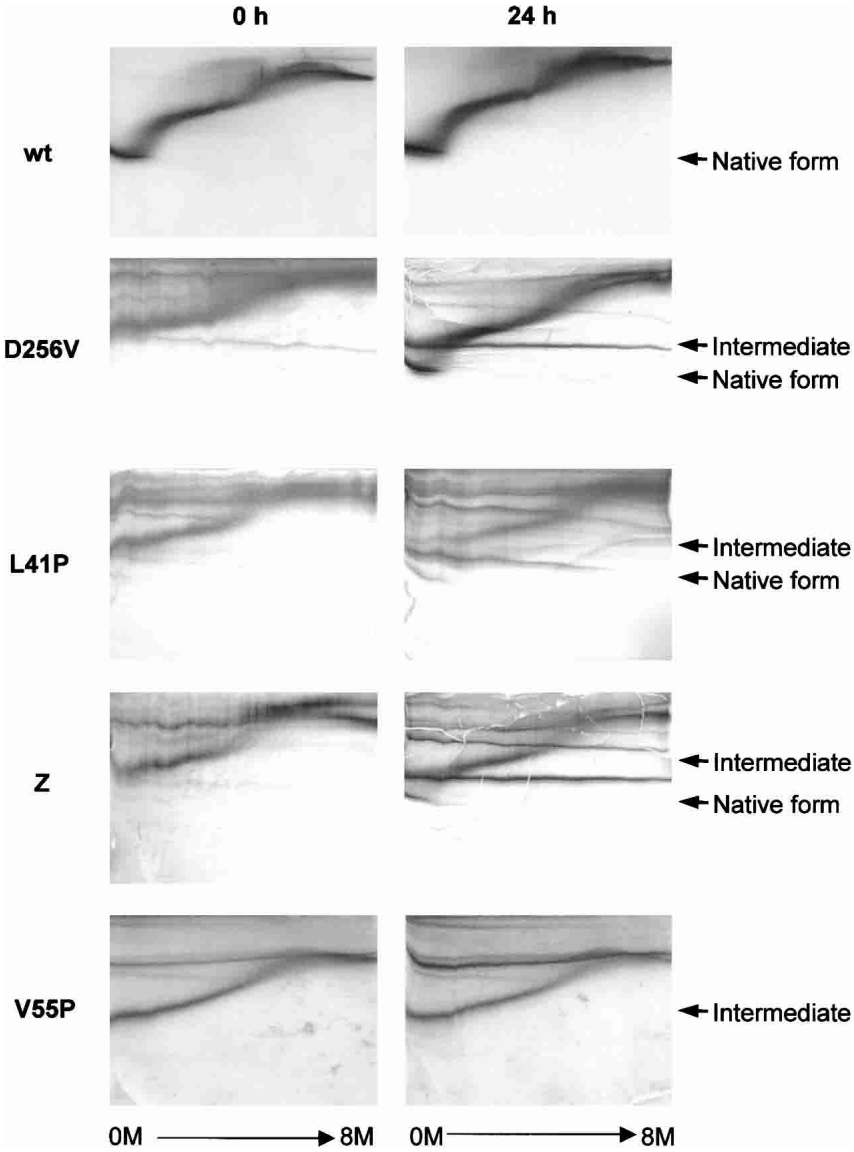

Figure 2. Transverse urea gradient gel electrophoresis showing retarded protein folding of α_1AT variants. The wild-type (wt), D256V, L41P, Z-type (Z), and V55P α_1AT were incubated in a buffer (10 mM phosphate at pH 6.5, 50 mM NaCl, 1 mM EDTA, and 1 mM β-mercaptoethanol) at 30°C for 24 h, and conformational changes were followed by transverse urea gradient gel electrophoresis (Goldenberg 1989). The transverse urea gradient gels contained a gradient of 0~8 M urea perpendicular to the direction of electrophoresis. Proteins were visualized by staining with Coomassie brilliant blue. Positions of the native and intermediate conformers are indicated.

by urea-dependent denaturation. As urea concentration gradually increased, wild-type α_1AT exhibited two cooperative unfolding transitions (Fig. 2). Most of the α_1AT variants tested (R39C, S53F, V55P, G67E, G115S, N158K, E264V, A336T, and P369S) exhibited decreased stability, as shown by unfolding of their native forms at lower urea concentrations than that required to unfold wild-type α_1AT (Fig. 2 for V55P; data not shown). In contrast, the D256V and L41P α_1AT polypeptides folded into a molten globule-like intermediate that had a lower mobility than the native form. However, prolonged incubation of these species at 30°C induced a conformational change to a tightly folded form, which migrated to the same position as the native form on electrophoresis (Fig. 2). These results indicate that these α_1AT variants can be folded into a native-like conformation, although the folding rate is very slow. A previously reported Z-type α_1AT variant (Glu 342 $\rightarrow$ Lys) also exhibited retarded folding (Yu et al. 1995; Fig. 2). Although certain α_1AT variants tested accumulate as low-mobility species, they do not convert to the tightly folded form on prolonged incubation; instead, they tend to aggregate (Fig. 2, V55P), possibly by facile loop insertion into a β-sheet of

a neighboring molecule owing to destabilization of the native form.

Slow folding to the native form with inhibitory activity

The results of transverse urea gradient gel electrophoresis showed that, for some α_1AT variants, a tightly folded conformation with electrophoretic mobility similar to that of the native form is generated upon prolonged incubation. However, it is not known whether this species is indeed the native form, with native biological functions, or is another misfolded conformation. To address this question, the ability of refolded α_1AT samples to form an SDS-stable protease/inhibitor complex was monitored over time. Immediately after folding (0 h), very little of the D256V or L41P α_1AT polypeptides was tightly folded (Fig. 3A), and they were unable to form inhibitory complexes with the target protease, human neutrophil elastase (Fig. 3B). Instead, these protein samples were fragmented into smaller pieces. This result is consistent with a loosely packed, molten globule-like conformation for the folding intermediate, which is vulnerable to protease attacks. On incubation at 30°C, however, the abilities of these variant proteins to form inhibitory complexes increased (Fig. 3B) coincidently with the formation of the tightly folded form (Fig. 3A). The Z-type variant behaved similarly, whereas wild-type α_1AT folded immediately on the initiation of protein folding (Fig. 3). When the folding rates were quantitatively measured by acquisition of inhibitory activity (Fig. 3C), the folding rates for the D256V, L41P, and Z-type variants were 0.48, 0.03, and 0.36 h^{-1}, respectively. On the other hand, the wild-type molecules immediately obtained full inhibitory activity upon initiation of folding. When the folding rate of the wild-type α_1AT molecules was measured by stopped-flow CD and fluorescence spectroscopy, most of the secondary structures were formed within a dead time of 2 msec (data not shown), and inhibitory activity was obtained within 210 sec (Kim and Yu 1996). The kinetics of inhibitory activity gain (Fig. 3C) was compatible with the conversion rates from the folding intermediate into the tightly folded form shown in Figure 3A. The results show that protein folding of these α_1AT variants was, indeed, extremely retarded, but they could ultimately fold into their native, functional conformations.

Stability of folded α_1AT variants is comparable to the wild type

Because the inhibitory activity of the above α_1AT variants increased gradually with time, a slow folding rate was implicated as the primary defect of these dysfunctional variants, and the stability and function of the folded native α_1AT proteins might not be seriously impaired. To enrich the native form of α_1AT variants sufficiently for detailed characterization, refolded α_1AT proteins were incubated at 30°C for 1 d (for D256V and Z-type variants) or 2 d (for L41P). The native forms of the variant α_1ATs were purified by FPLC and compared with that of the wild-type molecule. The thermostability of each variant during heat treatment was followed by nondenaturing gel electrophoresis. Surprisingly, the thermostability of these α_1AT variants was comparable to that of the wild-type α_1AT (Fig. 4). Neither the variant α_1ATs nor the wild-type protein were observed to polymerize upon heat treatment to 40°C. Noticeable amounts of polymers were first detected upon heat treatment at 45°C for variant α_1ATs and at 50°C for the wild-type protein.

The conformational stability of the folded α_1AT variants was quantified by equilibrium unfolding in the presence of urea. When monitored at 360 nm by fluorescence spectroscopy, emission fluorescence intensity drastically increases upon transition from the native form to an unfolding intermediate (corresponding to the first unfolding transition on transverse urea gradient gels). The unfolding transition midpoint was 1.8 M urea for the wild-type protein. The native D256V and L41P α_1AT proteins showed unfolding transition midpoints at 1.2 M and 1.3 M urea, corresponding to a free energy change ($\Delta\Delta G$) of 1.0 and 0.9 kcal/mole, respectively. The Z-type variant has a mutation (Glu 342 $\rightarrow$ Lys) located at the head of β-sheet A, very close to Trp 194, which is responsible for the intrinsic fluorescence of native α_1AT (Tew and Bottomley 2001). Because of the effect of the E342K mutation on the microenvironment near Trp 194, the fluorescence intensity of the native Z-type α_1AT was almost as high as that of the unfolding intermediate, and unfolding transition of the native Z-type protein could not be monitored by intrinsic fluorescence changes. Therefore, the conformational stability of this variant was measured by equilibrium unfolding using circular dichroism spectroscopy. The native Z-type protein unfolded with a transition midpoint of 1.2 M urea, compared with 1.8 M urea for the wild-type α_1AT. The results of the thermostability and the conformational stability examinations were consistent with each other and indicate that the stability of the folded variant proteins were only marginally decreased as compared with that of the wild-type α_1AT.

Inhibitory activity of folded α_1AT variants is comparable to the wild type

The inhibitory activities of the native forms of the α_1AT variants were examined by monitoring formation of a covalently bound, inhibitory complex with a target protease, porcine pancreatic elastase. The folded α_1AT variants formed amounts of SDS-resistant inhibitory complexes comparable to that of wild-type α_1AT (Fig. 5). Consistent with previous studies (Lee et al. 1998; Im and Yu 2000), slightly more portions of α_1AT molecules partitioned into the cleaved form when incubated with porcine pancreatic elastase, compared with those incubated with human neu-

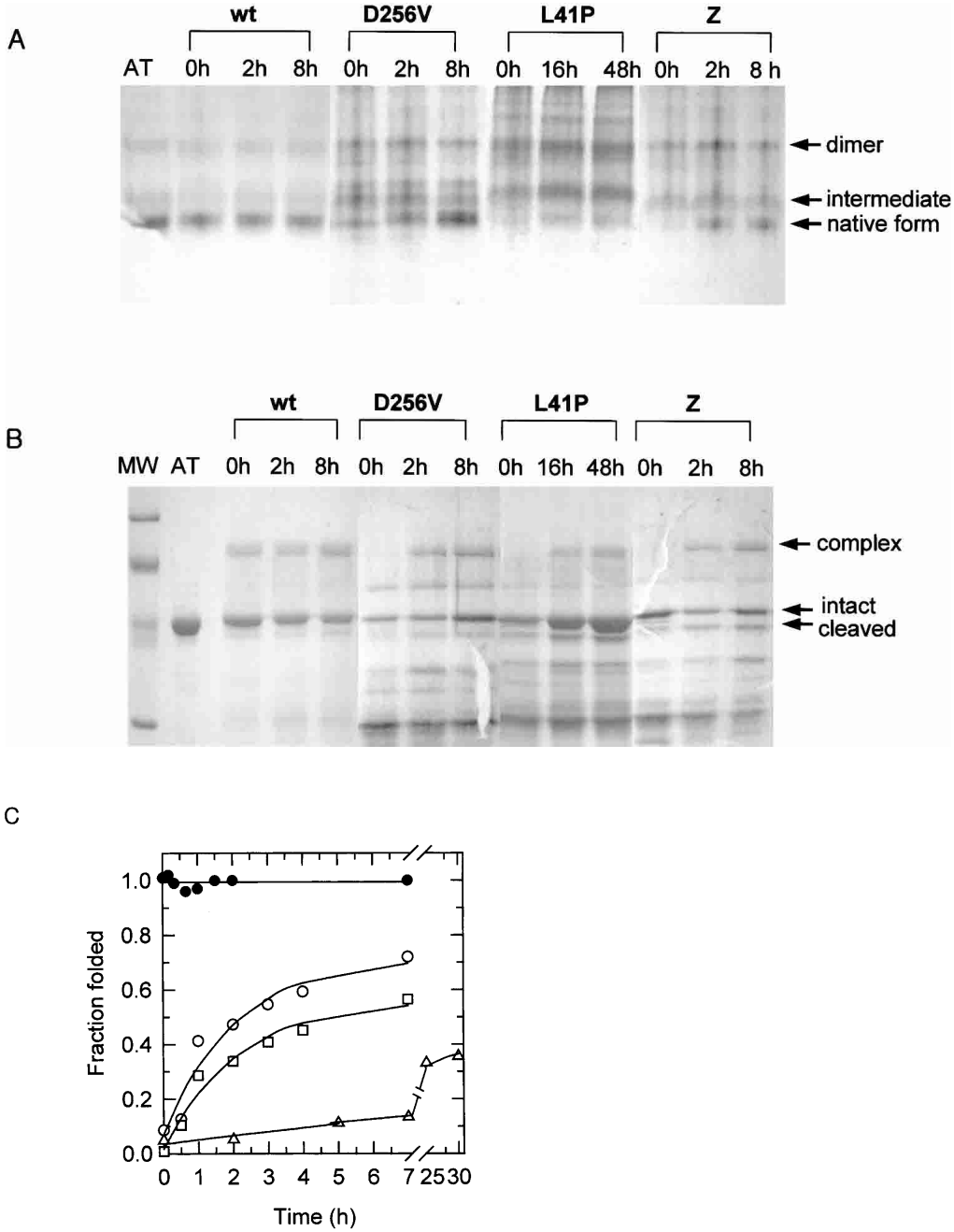

Figure 3. Protein folding time course of the α_1AT variants. (*A*) The nondenaturing polyacrylamide gel electrophoresis showing retarded folding of α_1AT variant proteins. Unfolded α_1AT polypeptides were refolded at 30°C for the indicated times as described in Materials and Methods, and analyzed by nondenaturing gel electrophoresis. Proteins were visualized by staining with Coomassie brilliant blue. Migration positions of the native form, folding intermediate, and dimer are indicated. (*B*) Inhibitory complex formation during incubation of the refolded α_1AT variants. Refolded α_1AT variant proteins were sampled during the folding time course and incubated with human leukocyte elastase at a molar ratio of 1 : 0.2 (α_1AT proteins to protease) for 10 min at 37°C. The reaction products were analyzed by 10% SDS-polyacrylamide gel electrophoresis, and proteins 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. MW, Molecular weight makers (Bio-Rad Co.; low range; 97.4, 66.2, 45, and 31 kD from the *top*); AT, the purified wild-type α_1AT protein; wt, the refolded wild-type α_1AT incubated for various times. (*C*) Measurement of refolding rates. The refolding rates to the native form during incubation of α_1AT variants at 30°C were followed by the gain of inhibitory activity. Refolded α_1AT variant proteins were sampled during the folding time course and incubated with 1 pmole of porcine pancreatic elastase for 10 min at 37°C. The residual enzyme activity was determined using 1 mM *N*-succinyl-Ala-Ala-Ala-*p*-nitroanilide as a substrate. The experimental data were fitted to a single exponential rise. (●) The wild-type α_1AT, (○) D256V α_1AT, (□) Z-type α_1AT, (△) L41P α_1AT.

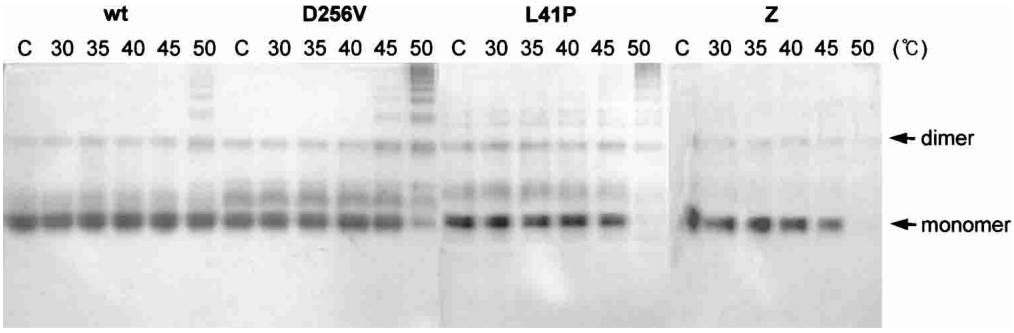

Figure 4. Thermostability of the native α_1AT variants. The folded α_1AT variant proteins were purified and heat-treated for 30 min at various temperatures ranging from 30°C to 50°C. The protein in each reaction was 4 μg in 30 μL of buffer (10 mM phosphate, 50 mM NaCl at pH 6.5). The degree of polymerization was analyzed on nondenaturing gels containing 10% polyacrylamide. Lane *C* contains nonheated α_1AT proteins. Migration positions of the monomer and dimer of α_1AT molecules are indicated.

trophil elastase. When the ratios of α_1AT present in inhibitor–protease complexes to α_1AT cleaved by the protease were compared, the variant α_1AT molecules showed a slight increase in cleavage as compared with the wild-type protein, especially for Z-type variant proteins. These results may have been caused by the slightly diminished inhibitory activities of the variant proteins or by small amounts of residual folding intermediates remaining in the preparation. However, these results confirm that the inhibitory activity of the native conformation, once formed, is not significantly affected by these mutations.

Discussion

Retarded folding of D256V and L41P variants may cause α_1AT deficiency

We have examined the detailed structural basis for the deficiency of a dozen α_1AT variants. Unlike most α_1AT variants, the D256V and L41P α_1AT polypeptides initially fold into a loosely packed intermediate, and then convert spontaneously into the native conformation under physiological conditions (Figs. 2, 3). Because the native form of these variants, once folded, has comparable stability and inhibitory activity (Figs. 4, 5), extremely slow folding is indicated as a cause for α_1AT deficiency by D256V and L41P variants.

Although the L41P α_1AT protein level in plasma is not sufficient to protect the lower respiratory tract, the variant protein in plasma exhibits inhibitory activity comparable to that of wild-type α_1AT (association rate constants of 7.0×10^6 and 9.3×10^6 M^{-1} sec^{-1}, respectively, for human neutrophil elastase), and the half-life of this variant in plasma is similar to that of the wild-type α_1AT (Takahashi et al. 1988). The low plasma level of this protein was puzzling, given the normal behavior of plasma L41P α_1AT protein and its normal promoter region regulating α_1AT

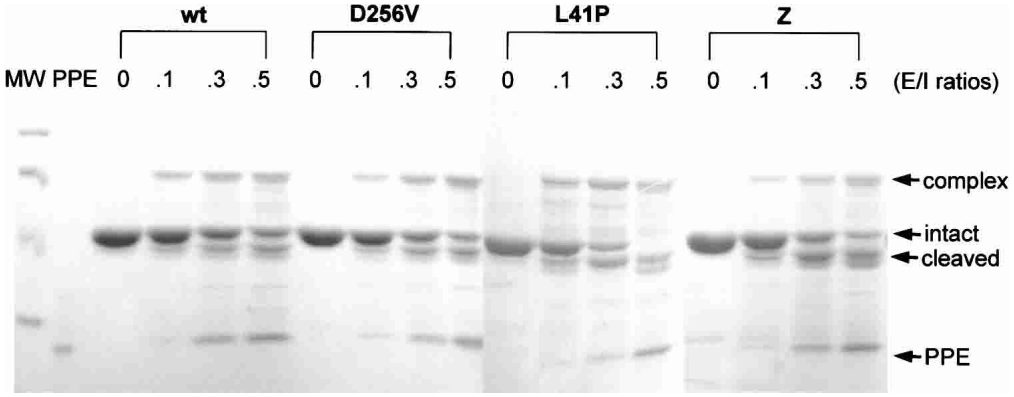

Figure 5. Inhibitory complex formation by the folded α_1AT variant proteins with elastase. The native wild-type (wt), D256V, L41P, and Z-type (Z) α_1AT proteins were incubated with porcine pancreatic elastase at the indicated molar ratios of the protease to α_1AT (*E*/*I* ratios). After incubation for 10 min at 37°C in the assay buffer (30 mM phosphate, 160 mM NaCl, 0.1% PEG 8000, 0.1% Triton X-100 at 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, the RSL-cleaved α_1AT, and elastase are indicated. MW, Molecular weight makers (Bio-Rad Co.; low range; 97.4, 66.2, 45, and 31 kD from the *top*); PPE, porcine pancreatic elastase.

gene expression (Takahashi et al. 1988). There is a possibility that the extremely retarded folding of L41P polypeptide causes accumulation of folding intermediates, which are prone to aggregation and clearance at the site of biosynthesis, rather than being secreted into plasma as the functional, native form. The comparable stability and activity of the folded D256V protein indicate that slow folding may cause the deficiency of this variant as well. Retarded protein folding has also been suggested as the predominant cause of aggregation of Z-type α_1AT in liver cells (Yu et al. 1995). However, the plasma form of Z-type α_1AT protein exhibited a reduced association rate constant with neutrophil elastase (1.2×10^7 M^{-1} sec^{-1} for Z-type versus 5.3×10^7 M^{-1} sec^{-1} for wild-type α_1AT; Lomas et al. 1993b). The increased cleavage of Z-type variant molecules as compared with wild-type α_1AT molecules (Fig. 5) is consistent with this observation. Many dysfunctional α_1AT proteins easily adopt a loop–sheet polymeric conformation, and especially a high body temperature during inflammation is likely to exacerbate α_1AT polymerization and develop the clinical symptoms of α_1AT deficiency (Lomas et al. 1992). However, the native form of slow-folding α_1AT variants has comparable thermostability to the wild-type molecule, and remains intact for 30 min even at 40°C (Fig. 4). Therefore, these variants are likely to have a different mechanism for α_1AT deficiency; that is, retarded folding accumulates folding intermediates prone to polymerization prior to being secreted into plasma.

Structural basis for retarded folding

In the native structure of wild-type α_1AT (2psi.pdb; Elliott et al. 1998), Leu 41 is located on helix A (Fig. 1). Distortion of helix A by the L41P substitution may delay compact packing of secondary structures into a tightly folded tertiary structure, the last step in α_1AT folding. Interestingly, the $C_{\delta 1}$ atom of Leu 41 interacts with the $C_{\delta 2}$ atom of Phe 52 at a distance of 3.5 Å, and deletion of Phe 52 also causes α_1AT deficiency (Lomas et al. 1995). Asp 256 is located in the turn between s3B (the third strand in β-sheet B) and helix G, and forms a salt bridge to His 231 in s1B. Loss of the salt bridge by the D256V substitution might affect the folding rate. Likewise, in the Z-type variant (Glu 342 → Lys), a salt bridge between Glu 342 and Lys 290 is broken, and the charge repulsion between the newly introduced Lys 342 and Lys 290 is likely to interfere with normal packing.

Conformational versatility and disease

This study has provided significant insight into the structure–function relationships of serpins. The native state of serpins is not their most thermodynamically stable state, but is a strained metastable state. The native metastability of these proteins is important for regulating their biological functions (Wiley and Skehel 1987; Huber and Carrell 1989; Stein and Carrell 1995; Wright and Scarsdale 1995; Carr et al. 1997), and the final, stable state of these proteins is normally reached only when the function is executed. Therefore, the metastability is probably needed for facile conformational conversion during formation of the inhibitory complex with a protease (Lee et al. 2000). However, the structural aspects that cause the native metastability of inhibitory serpins also expose serpin proteins to easily adopt several abnormal conformations, such as the RSL-inserted latent form and loop–sheet polymers. Structural conversion to these inactive, stable conformations seems to occur at physiologically significant rates in many deficient serpin variants.

The precise site and nature of the mutations determine etiology of the serpin variants. Destabilizing α_1AT substitutions in the "shutter" domain, such as V55P and S53F, also accumulated as intermediate-like species with low mobility, as determined by transverse urea-gradient gel electrophoresis. However, they did not convert into the native state upon prolonged incubation; rather, they formed protein aggregates (Fig. 2). Therefore, instability of the native proteins appears to be the main cause of inhibitory deficiency for these α_1AT variants. A previous study suggested that the hydrophobic region underneath β-sheet A is overpacked, because mutations that reduce the size of the side chain in this region, for example, F51L, stabilize the α_1AT molecule (Kwon et al. 1994). Indeed, S_{iiyama} (Ser 53 → Phe), which increases the volume of the side chain in this region, induces conformational instability and subsequent polymerization. A genetic variant of α_1-antichymotrypsin (L55P) accumulated as a species that may be an intermediate in the formation of the latent state (Gooptu et al. 2000), as well as in the native and latent forms. However, V55P variation of α_1AT, which is equivalent to L55P substitution of α_1-antichymotrypsin, did not accumulate as a similar intermediate or as the latent form, indicating that subtle structural differences among inhibitory serpins affect the etiology of serpin deficiency. Unlike most α_1AT variants, whose major defect may be conformational instability of the native form, leading to exacerbated protein polymerization, the D256V, L41P, and Z-type variants suffer from retarded folding, although once folded, their native form behaves quite normally. Retarded folding arrests the polypeptide at the stage of an incompletely folded intermediate that cannot be properly channeled through the secretory pathways. This phenomenon may lead to accumulation of the intermediate, which has a high tendency to polymerize, leading to hepatic inclusions. Retarded protein folding seems to be a recurrent theme associated with dysfunctional α_1AT variants, because three of 12 α_1AT variants tested displayed retarded folding (Fig. 1). It will be of interest in future studies to identify other types of variant proteins that adopt versatile conformations.

Materials and methods

Chemicals

Ultrapure urea was purchased from ICN Biochemicals. Porcine pancreatic elastase, human leukocyte elastase, and *N*-succinyl-$(Ala)_3$-*p*-nitroanilide were purchased from Sigma. All other chemicals were 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* (Kwon et al. 1994), by oligonucleotide-directed mutagenesis (Kunkel et al. 1987). Recombinant α_1AT was expressed as inclusion bodies in *E. coli* and refolded as described previously (Kwon et al. 1994). Either immediately or after incubation at 30°C for the indicated time, α_1AT proteins were quickly purified on a Q Sepharose Fast Flow column (2.5 × 0.7 cm; Amersham Bioscience Ltd.) in 10 mM phosphate, 1 mM β-mercaptoethanol, and 1 mM EDTA (pH 6.5). This purification process takes less than 1 h at 4°C. To characterize the native conformation of α_1AT variants, it was enriched by incubation at 30°C, and the folded conformation was purified by ion exchange chromatography by FPLC on a ResourceQ column (Amersham Bioscience Ltd.) in 10 mM phosphate, 1 mM β-mercaptoethanol, and 1 mM EDTA (pH 6.5). Concentrations of α_1AT were determined in 6 M guanidine hydrochloride using a value of $A_{1\ cm}^{1\%} = 4.3$ at 280 nm, calculated from the tyrosine and tryptophan content of the α_1AT protein (Edelhoch 1967) and based upon $Mr = 44{,}250$ (Kwon et al. 1994).

Conformational analysis by gel electrophoresis

Conformation of α_1AT proteins was analyzed by transverse urea gradient gel electrophoresis (Goldenberg 1989). Transverse urea gradient 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.

Folding of α_1AT proteins was followed using 10% nondenaturing polyacrylamide gel with Tris-glycine buffer system. Inclusion bodies from *E. coli* cells overexpressing α_1AT proteins were dissolved in 8 M urea in a buffer (50 mM Tris-Cl at pH 8, 50 mM NaCl, 1 mM EDTA, and 1 mM β-mercaptoethanol), and refolded by dilution 10-fold in 10 mM phosphate (pH 6.5), 1 mM EDTA, and 1 mM β-mercaptoethanol. Refolded α_1AT protein was incubated at 30°C for various times, and their conformation was analyzed by nondenaturing gel electrophoresis.

Thermostability

To measure thermostability of α_1AT variants, purified α_1AT protein was incubated for 30 min at various temperatures ranging from 30°C to 50°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 at pH 6.5). Disappearance of monomers and formation of oligomers of α_1AT molecules were monitored on 10% nondenaturing gels containing Tris-glycine buffer system.

Denaturant-induced unfolding transition

To measure the stability of α_1AT variants, 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 at pH 6.5). The native protein was incubated in the buffer containing various concentrations of urea for 2 h at 25°C. Equilibrium unfolding was monitored by fluorescence spectroscopy ($\lambda_{ex} = 280$ nm and $\lambda_{em} = 360$ nm, excitation and emission slit widths = 5 nm for both), using a Shimadzu RF-5301PC spectrophotometer as described previously (Kwon et al. 1994; Lee et al. 1996). Intrinsic fluorescence emission at 360 nm markedly increases upon transition from the native form to an unfolding intermediate (the first transition), but remains almost constant during transition from the unfolding intermediate to the unfolded state (the second transition). Therefore, experimental data of the fluorescence measurement at 360 nm were fitted to a two-state unfolding model to measure stability of the native form. For the Z-type variant, unfolding of the native form was monitored at 25°C by circular dichroism spectroscopy at 220 nm using a Jasco J715 spectropolarimeter. The protein concentration was 10 μg/mL in a buffer (10 mM phosphate, 50 mM NaCl at pH 6.5) containing various concentrations of urea.

Determination of the inhibitory activity

During the refolding time course, inhibitory activity of α_1AT variants was followed by monitoring the ability to form SDS-stable complexes with human leukocyte elastase. Active concentration of human leukocyte elastase was determined by trypsin-titration reactions as described (Hopkins et al. 1993). For this, 4 μg of α_1AT samples from refolding time courses was incubated with human leukocyte elastase at a molar ratio of 1 : 0.2 (α_1AT : protease) for 10 min at 37°C. The buffer was 30 mM phosphate (pH 7.4), 160 mM NaCl, 0.1% PEG 6000, and 0.1% Triton X-100. The inhibitory complex formation of α_1AT proteins with target proteases was examined by 10% SDS-polyacrylamide gel electrophoresis.

The refolding rates to the native form during incubation of α_1AT variants at 30°C were followed by the gain of inhibitory activity. 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 (Bieth et al. 1974). α_1AT samples were taken at various time points and incubated with 1 pmole of the protease in 50 μL of elastase assay buffer (30 mM phosphate, 160 mM NaCl, 0.1% PEG 6000, and 0.1% Triton X-100 at pH 7.4). After incubation with the protease for 10 min at 37°C, the reaction mixture was diluted 20-fold with the assay buffer, and the residual enzyme activity was determined. The experimental data were fitted to a single exponential rise.

To measure inhibitory activity of the native form, 4 μg of purified folded α_1AT protein was incubated with porcine pancreatic elastase at various molar ratios for 10 min at 37°C. Appearance of the SDS-resistant α_1AT–proteinase complex was monitored by 10% SDS-polyacrylamide gel electrophoresis. The protein bands were visualized by staining with Coomassie brilliant blue.

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