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NOTE

A Recombinant Human α_1-Antitrypsin Variant, M_{malton}, Undergoes a Spontaneous Conformational Conversion into a Latent Form

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Many genetic variants of α_1-antitrypsin have been associated with early onset emphysema and liver cirrhosis. However, the detailed structural basis of pathogenic α_1-antitrypsin molecules is rarely known. Here we found that a recombinant M_{malton} variant (Phe52-deleted) lost inhibitory activity by spontaneous conformational conversion into a more stable, inactive form under physiological conditions. Biochemical and spectroscopic data suggested that the variant converts into a reactive center loop-inserted conformation, resembling the latent form of plasminogen activator inhibitor-1.

Key words: antitrypsin, conformational change, latent form, serine protease inhibitors

α_1-Antitrypsin (α_1AT) is archetypal of the serine protease inhibitor (serpin) superfamily (Huber and Carrell, 1989), which includes protease inhibitors in blood plasma, such as α_1-antichymotrypsin, antithrombin-III, C1 inhibitor and plasminogen activator inhibitor-1. Serpins share a common tertiary structure, which is based on a mobile reactive center loop (RCL), three β-sheets and several α-helices (Elliot *et al.*, 1996). The mobility of the RCL may allow serpin molecules to adopt a variety of conformations. In the active native form of inhibitory serpins (Ryu *et al.*, 1996), the RCL is exposed at one end of the molecule for protease binding. Since the exposed RCL of serpins fits the active site of protease, the residues at the P1-P1' sites of serpins (the residue position before and after the scissile peptide bond, respectively) determine target specificity. Upon cleavage of the RCL by proteases, the acyl-enzyme intermediate formed is inserted into the β-sheet A (Johnson and Travis, 1978; Loebermann *et al.*, 1984). This change results in a drastic increase in the stability of the serpin molecule (Bruch *et al.*, 1988). There also exists a conformation called the 'latent' form of serpin, in which the RCL is inserted into β-sheet A without the RCL cleavage (Mottonen *et al.*, 1992). The 'latent' form of serpin is more stable than the native form, but is inactive (Hekman and Loskutoff, 1985; Wang *et al.*, 1996). This 'latent' form can be produced from the native form under physiological conditions, in a matter of hours in the case of plasminogen activator inhibitor-1 (Lawrence *et al.*, 1989), while in other serpins, it can be induced under mild denaturing conditions (Carrell *et al.*, 1991; Lomas *et al.*, 1995a). Another conformation exists in the loop-sheet polymers, in which the RCL of one molecule is inserted into a β-sheet of a second molecule.

Dysfunctional genetic variants of serpins have serious clinical consequences, such as emphysema and thrombosis, by breaking the balance between serum proteases and their inhibitors. α_1AT is synthesized in the liver and secreted into the plasma to protect tissues against indiscriminate proteolytic attack by neutrophil elastase (Carrell *et al.*, 1982). The majority of deficient genetic variants of human α_1AT cause its aggregation in the endoplasmic reticulum of the liver, the location of α_1AT biosynthesis (Eriksson *et al.*, 1986), and a subsequent deficiency in plasma, leading to liver cirrhosis and pulmonary emphysema. Even though several dozens of α_1AT genetic variants are known, the detailed structural basis for the inhibitory deficiency of most pathogenic α_1AT molecules remains substantially unknown. Most dysfunctional α_1AT proteins are likely to have conformational liability, which leads to consequent intermolecular loop-sheet polymerization (Stein and Carrell, 1995). In case of the best studied Z-type variant (Glu342→Lys), found in the Northern European population at an allelic frequency of 0.04, extremely retarded α_1AT protein folding has been identified to be the structural basis for the accumulation of a folding intermediate with a high tendency to aggregate (Yu *et al.*, 1995). Once folded, the native Z-type protein exhibits substantial stability and a comparable inhibitory

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activity toward target proteases (Yu *et al.*, 1995). Meanwhile, some variants show impaired protease inhibitory activity. α_1 AT Pittsburgh has an amino acid substitution at the P1 site on the RCL (Met358→Arg), and shows a shifted target specificity from neutrophil elastase to thrombin, causing a bleeding disorder (Owen *et al.*, 1983). Various biochemical (Wright and Scarsdale, 1995; Gils *et al.*, 1996; Huntington *et al.*, 1997) and structural (Aertgeerts *et al.*, 1995; Lukas *et al.*, 1996) studies suggest that the rate of loop insertion is critical for inhibitory function. Hence, bulky substitutions on the inserted RCL interfere with the kinetics of the inhibitory function of α_1 AT, shifting the serpins into substrates rather than inhibitors of the target proteases. A recent study of α_1-antichymotrypsin showed that a variant (Leu55→Pro) in the 'shutter' domain, which participates in the opening of β-sheet A, can adopt not only the native conformation, but also form an inactive latent conformation, and an inactive, stable intermediate on the α_1 AT polymerization pathway (Gooptu *et al.*, 2000). In this study, we examined the conformational versatility of dysfunctional shutter-domain mutants of α_1 AT, such as Val55→Pro and M_{malton} (Phe52-deleted) variants.

Spontaneous conversion of M_{malton} α_1AT to a stable conformation under physiological conditions

There is a possibility that α_1 AT mutations in the shutter domain may somehow influence the insertion of the RCL into β-sheet A, as is the case for the α_1-antichymotrypsin Leu55→Pro variant. Thus, we introduced shutter domain mutations (M_{malton}, V55P, and S53F) into recombinant α_1 AT by oligonucleotide-directed mutagenesis. Recombinant α_1AT was expressed as inclusion bodies in *Escherichia coli* and was refolded as described previously (Kwon *et al.*, 1994). Monomeric forms were purified by ion exchange chromatography on a MonoQ column (Pharmacia Co., USA). Conformational changes of the serpin molecules were monitored by transverse urea gradient gel electrophoresis. The native M_{malton} α_1AT (Phe52-deleted) protein was slightly more unstable than the wild-type native form, as it was unfolded at a lower urea concentration (Fig. 1). However, prolonged incubation at 30°C converted M_{malton} α_1AT into a very stable form that was resistant to urea-induced protein unfolding (Fig. 1). Other shutter domain variants, such as V55P (Fig. 1) and S53F (data not shown), were very unstable and accumulated a low-mobility folding intermediate. These variants did not convert to stable species on prolonged incubation at 30°C, instead the mutant proteins formed protein aggregates.

The inhibitory activity of the native and the stable forms of M_{malton} α_1 AT were examined by monitoring the formation of a covalently-bound inhibitory complex with a target protease, elastase (Fig. 2). To purify the stable form of M_{malton} α_1 AT, thermostable mutations (T68A, A70G, M374I, S381A, and K387R) were introduced into this

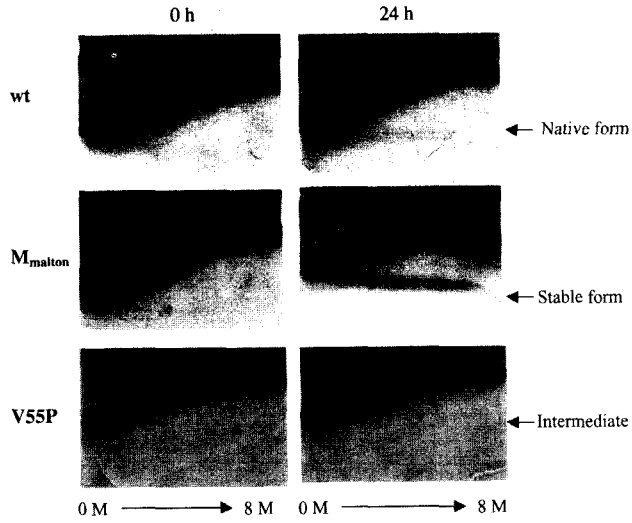

Fig. 1. Conversion of M_{malton} α_1 AT into a stable conformation. Soluble monomeric forms of wild-type (wt), M_{malton}, and V55P α_1 AT were incubated at 30°C for 24 h in 10 mM phosphate, 50 mM NaCl, 1 mM EDTA, 1 mM β-mercaptoethanol, pH 6.5. Conformational change was monitored by transverse urea gradient gel electrophoresis (Goldenberg, 1989). Transverse urea gradient gels contains a gradient of 0~8 M urea perpendicular to the direction of electrophoresis with an opposing acrylamide gradient from 15 to 11%. Four slab gels (100×80 mm) were prepared simultaneously in a multigel caster (Hoefer Scientific Instruments, USA) by using a gradient maker and a single-channel peristaltic pump. The native protein (20 μg in 100 μl) was applied across the top of the gel. The electrode buffer used was 50 mM Tris-acetate/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 25°C. Protein bands were visualized by Coomassie Brilliant Blue staining. The migration positions of the native, stable, and intermediate forms of α_1 AT are indicated accordingly.

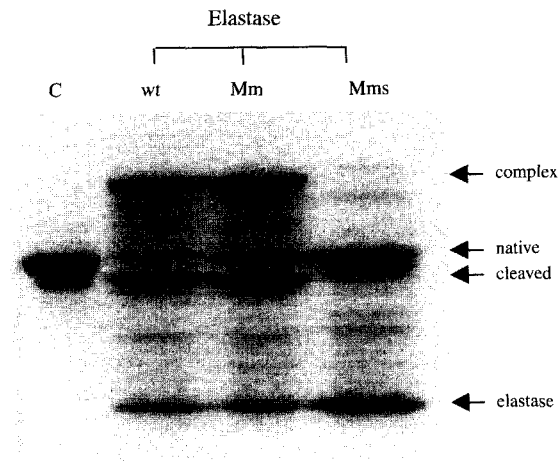

Fig. 2. The inhibitory activity of M_{malton} α_1 AT protein was lost in its stable form. The native wild-type (wt), native M_{malton} (Mm), and stable forms of M_{malton} α_1 AT (MmS) were incubated with porcine pancreatic elastase (Sigma, USA) at a molar ratio of 1:0.5 (α_1 AT : protease) at 37°C for 10 min. Untreated native wild-type protein (C) is also shown. The buffer used was 30 mM phosphate, 160 mM NaCl, 0.1% PEG6000, 0.1% Triton X-100, pH 7.4. The formation of the SDS-resistant α_1 AT-elastase complex was analyzed on 10% SDS-polyacrylamide gels. Protein bands were visualized by Coomassie Brilliant Blue staining.

variant to reduce loop-sheet polymerization. The stable form was enriched by incubating at 42°C for 3 days, and any remaining native conformation was removed by heat treatment at 65°C for 1 h. The stable form was purified by FPLC on a MonoQ column in 10 mM phosphate, 1 mM β-mercaptoethanol, and 1 mM EDTA, pH 6.5. The native wild-type, M_{malton}, and a stable form of M_{malton} α_1 AT were incubated with porcine pancreatic elastase, and the reaction products were analyzed by 10% SDS-polyacrylamide gel electrophoresis. The native form of M_{malton} α_1 AT formed an SDS-resistant inhibitory complex, as does wild-type α_1 AT. When the ratio of native M_{malton} α_1 AT molecules forming a tight SDS-resistant inhibitor-protease complex to M_{malton} molecules cleaved by the target protease was determined by densitometrically scanning polyacrylamide gel, it was found that the ratio of native M_{malton} α_1 AT molecules was similar to that of the wild-type protein. However, the stable form of M_{malton} variant did not produce an inhibitory complex, instead it remained intact upon treatment with elastase, suggesting that the P1-P1' site is not accessible for protease binding.

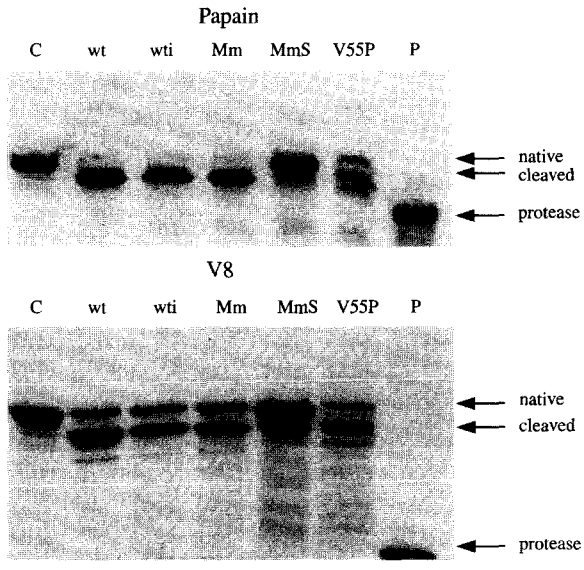

Fig. 3. Protease-accessibility of the RCL in stable M_{malton} α_1 AT. The native wild-type, native M_{malton}, or the stable form of M_{malton} α_1 AT were incubated with papain (Sigma, USA) at a molar ratio of 1000 : 1 (α_1 AT to protease) at 37°C for 15 min (upper figure). The buffer used was 20 mM phosphate, pH 7.0, 5 mM cysteine, 10 mM EDTA, and 5 mM NaCl. 4 μg of α_1 AT protein in a total volume of 30 μl was used in all reactions. *Staphylococcus aureus* V8 protease (Sigma, USA) was incubated with the native wild-type, native M_{malton}, or the stable form of M_{malton} α_1 AT at 37°C for 1 h (lower figure), at a molar ratio of 50 : 1 (α_1 AT to protease) in a buffer containing 0.1 M Tris-HCl, pH 7.8. Reaction products were analyzed by 10% SDS-polyacrylamide gel electrophoresis, and protein bands were stained with Coomassie Brilliant Blue. Samples: C, untreated native wild-type protein; wt, protease-treated native wild-type protein; wti, protease-treated wild-type protein after incubation at 30°C for 3 days; Mm, protease-treated native M_{malton} protein; MmS, protease-treated stable form of M_{malton} α_1 AT; V55P, protease-treated native V55P protein; P, protease only.

Characteristics of the stable conformation

The stability increase in the urea-resistant form of M_{malton} α_1 AT may be due to the insertion of the RCL into β-sheet A as in the 'latent' form of plasminogen activator inhibitor-1 (Mottonen *et al.*, 1992). This possibility was investigated by probing the accessibility of the RCL to proteases known to cleave the exposed RCL of the native α_1 AT (Mast *et al.*, 1992). The native wild-type and native M_{malton} α_1 AT produced a species with a molecular mass of ~40 kDa when interacted with *Staphylococcus aureus* V8 protease or papain (Fig. 3). The incubated wild-type α_1 AT remained vulnerable to the protease attacks, but the stable form of M_{malton} α_1 AT became inaccessible to *Staphylococcus aureus* V8 protease and papain (Fig. 3). Unstable V55P protein was very fragile to protease attacks and fragmented into smaller pieces. The results suggest that the RCL of the stable form of M_{malton} α_1 AT is not available for protease binding.

Fluorescence spectrum of the native form of M_{malton} α_1 AT was very similar to that of the native wild-type protein. However, the fluorescence spectrum of the stable form of α_1 AT was quite different from the native form in that its intrinsic fluorescence emission intensity was much higher and its maximum wavelength was slightly shifted versus those of native α_1 ATs (Fig. 4). The result indicates that some changes occur in the stable form in microenvironment near Trp194, which is located underneath β-sheet A. However, the fluorescence spectrum of the stable

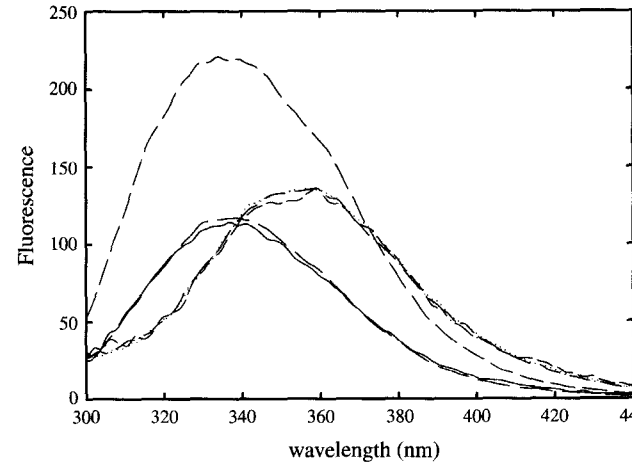

Fig. 4. Fluorescence spectrum of the stable form of M_{malton} α_1 AT. Fluorescence emission spectra were measured by exciting at 280 nm (excitation and emission slit widths=5 nm for both), using a Shimadzu RF-5301PC spectrophotometer. 10 μg/ml of protein in 10 mM phosphate, 50 mM NaCl (pH 6.5) was used. Intrinsic tryptophan fluorescence spectra of unfolded α_1 AT proteins were monitored at 25°C in the buffer containing 6 M guanidine hydrochloride (ICN Biomedicals, Inc., USA), 10 mM phosphate, and 50 mM NaCl, pH 6.5. Scans were repeated three times for each sample at a scan speed of 20 nm per min. Spectra were background subtracted. Symbols: ——, the native wild-type α_1 AT; – – – –, the native M_{malton} α_1 AT; - - - - -, the stable form of M_{malton} α_1 AT; ·····, the denatured wild-type α_1 AT; —·—·—, the denatured M_{malton} α_1 AT; ——·——·——, the denatured protein of the stable M_{malton} α_1 AT.

form was indistinguishable from that of the structurally defined-latent form of α_1 AT (Im *et al.*, 2002; unpublished data). These results also suggest that the RCL is inserted into β-sheet A, as it is in the latent form.

Biological implications

Our results elucidate the structural basis for the α_1 AT deficiency of the M_{malton} variant. The native M_{malton} molecules were found to retain an essentially normal inhibitory activity with a target protease, i.e., elastase (Fig. 2). However, the native form of M_{malton} α_1 AT was found to be not only slightly unstable compared to the wild-type molecules, but to spontaneously convert into a urea-stable conformation under physiological conditions (Fig. 1). Since the stable form did not show inhibitory activity, the variant gradually lost its activity at clinically significant rates. Various biochemical and spectroscopic results strongly suggest that the RCL is inserted into β-sheet A, as occurs in the latent plasminogen activator inhibitor-1 structure (Fig. 3 and Fig. 4). Thus, our results reasonably explain the clinical observations made in patients carrying this variant. M_{malton} α_1 AT molecules form inclusions in the liver and aggregates in the plasma, as most deficient α_1 AT variants do. However, α_1 AT polymers found in the plasma of an M_{malton}/null heterozygote were much shorter than those of other deficient variants, such as Z (Glu342 →Lys) and S_{iiyama} (Ser53→Phe) variants (Lomas *et al.*, 1995b). This phenomenon may be partially due to a mild destabilization of M_{malton} α_1 AT molecules, compared to other variants. It has been reported that M_{malton} polymers contain RCL-cleaved molecules as well as intact ones (Lomas *et al.*, 1995b). It was suggested that the incorporation of the cleaved molecule into the growing α_1AT polymer terminates chain elongation, by making the RCL unavailable for insertion into the β-sheet of the next molecule. However, cleaved molecules having both s1C (the first strand in β-sheet C) and s4A (the 4th strand in β-sheet A) attached to their own β-sheets, are very resistant to further peptide loop insertion, which is required for loop-sheet polymerization. We observed that the stable form obtained from M_{malton} α_1 AT is not a cleaved form, since it retained the molecular mass of the native form, as monitored by SDS-polyacrylamide gel electrophoresis (Fig. 2). The stable form, like the latent form, was also partially resistant to heat treatment (data not shown), while the cleaved form did not polymerize even after heating at 100°C for 2 h (Lomas *et al.*, 1995a). Therefore, it is likely that the latent form of M_{malton} observed in this study, is first inserted into the growing end of the polymer along its empty s1C site, and stops polymer-chain elongation. The latent form may be then cleaved by extraneous plasma proteases. Similar findings have been described for a variant of antithrombin, Rouen VI (Asn187→Asp; a residue substitution in helix F), which forms short-chain polymers. These polymers were truncated by 2-3 units by the complete conversion of the terminal molecule to the latent form (Bruce *et al.*, 1994).

Other destabilizing α_1 AT mutations at the shutter domain, such as Val55→Pro and Ser53→Phe, did not promote conversion into the stable state (Fig. 1), but rather induced aggregation. A previous study suggested that the hydrophobic region underneath β-sheet A is overpacked (Kwon *et al.*, 1994). Therefore, S_{iiyama} (Ser53→Phe), which increases the volume of the side chain in this region, is very likely to induce facile opening of β-sheet A and subsequent polymerization. A genetic variation (Leu55→Pro) in the shutter domain of α_1-antichymotrypsin also accumulates an intermediate on the conformational change to the latent state, as well as the native and the latent α_1-antichymotrypsin (Gooptu *et al.*, 2000). However, the equivalent substitution in α_1AT, Val55→Pro, did not promote the conversion into an intermediate or a latent form, suggesting that subtle structural differences exist among inhibitory serpin members. In the case of M_{malton}, deletion of a large residue might induce rearrangement of the side chains in the shutter domain, either distorting the gap between strands 3 and 5 of β-sheet A or modifying the RCL configuration for facile insertion into its own β-sheet.

Our present study provides a significant understanding of structure-function relationships of serpins. The native state of serpins is not the thermodynamically most stable state, but rather a strained metastable state. The native metastability of these proteins is an important aspect of regulating their biological function, and the final stable state of these proteins is normally reached only when the function (complex formation with a protease) has been executed (Huber and Carrell, 1989; Stein and Carrell, 1995; Wright and Scardale, 1995). Meanwhile, this metastability promotes serpins to easily adopt more stable conformations, such as the RCL-inserted latent form and loop-sheet polymers. Many deficient variants of α_1 AT facilitate such structural conversions at physiologically significant rates. The locations and detailed structural changes of the α_1 AT variations are likely to determine the clinical phenomena associated with each variant serpins. It will be of interest to identify further variant serpins that adopt versatile conformations.

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