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Conformational Switch of the Strained Native Serpin Induced by Chemical Cleavage of the Reactive Center Loop

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The native conformation of serpins (*ser*ine *p*rotease *in*hibitors) is strained. Upon cleavage of the reactive center loop of serpins by a protease, the amino terminal portion of the cleaved loop is inserted into the central β-sheet, A sheet, as the fourth strand, with the concomitant release of the native strain. We questioned the role of protease in this conformational switch from the strained native form into a stable relaxed state. Chemical cleavage of the reactive center loop of α_1-antitrypsin, a prototype serpin, using hydroxylamine dramatically increased the stability of the serpin. A circular dichroism spectrum and peptide binding study suggests that the amino terminal portion of the reactive center loop is inserted into the A sheet in the chemically-cleaved α_1-antitrypsin, as in the enzymatically-cleaved molecule. These results indicate that the structural transformation of a serpin molecule does not require interaction with a protease. The results suggest that the serpin conformational switch that occurred during the complex formation with a target protease is induced by the cleavage of the reactive center loop *per se*.

Keywords: α_1-Antitrypsin, Chemical cleavage, Conformational switch, Native strain, Serpin

Introduction

The metastable native state has been recognized as an important mechanism for biological regulation (Wiley and Skehel, 1987; Huber and Carrell, 1989; Stein and Carrell, 1995; Carr *et al.*, 1997; Im *et al.*, 1999; Lee *et al.*, 2000). The strained native state (Huber and Carrell, 1989) of various serpins, the spring-loaded structure of the fusion protein of influenza virus (Carr and Kim, 1993) and possibly the membrane fusion protein of other viruses (Chan *et al.*, 1997), are examples. In the strained native form (Ryu *et al.*, 1996; Elliott *et al.*, 1998) of inhibitory serpins, the reactive center loop (RCL) is exposed at one end of the molecule for protease binding (Fig. 1A). Upon interaction with a non-cognate protease, the RCL is cleaved and the amino terminal portion of the cleaved RCL is inserted into the central β-sheet, A sheet (Fig. 1B; Loebermann *et al.*, 1984), with the concomitant release of the native strain (Bruch *et al.*, 1988). There is another form of serpins, called the latent form, in which the RCL is inserted into the A sheet without cleavage (Mottonen *et al.*, 1992). The latent form is much more stable than the native form (Hekman and Loskutoff, 1985; Wang *et al.*, 1996) and can be produced from the native form though slowly (Lawrence *et al.*, 1989). Existence of the latent form suggests that the native form of serpins is a kinetically trapped folding intermediate (Creighton, 1992; Baker and Agard, 1994; Carr *et al.*, 1997).

Our previous study showed that extending the RCL by flexible linker peptides could bypass the folding barrier of α_1-antitrypsin (α_1AT; also known as α_1-protease inhibitor) toward a more stable conformation (Im and Yu, 2000). This result suggests that the constrain held by the polypeptide connection prevents the conversion of the metastable native form into the lowest energy state. In order to test this proposal, in the present study we examined whether the interaction with a target protease is necessary to induce the conformational switch to a more stable form, or if the cleavage of the RCL itself is sufficient to induce the conformational change. We utilized the ability of hydroxylamine to preferentially cleave the Asn-X peptide bonds. A nucleophilic attack by the peptide -NH- group immediately following the labile asparagine residue causes deamidation and formation of a succinimide intermediate (Stephenson and Clarke, 1989). Peptide bond cleavage at asparagine residues has been observed and assumed to proceed through a succinimide intermediate by attacking the side-chain amide of asparagine on the backbone

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peptide carbonyl of asparagine (Geiger and Clarke, 1987; Lura and Schirch, 1988). These reactions are catalyzed by general bases (Tyler-Cross and Schirch, 1991) and dependent upon both sequence (Stephenson and Clarke, 1989) and local peptide chain conformation (Kossiakoff, 1988). Hydroxylamine has been known as a good catalyst of the reaction, and Asn-X sequences (particularly where X is Gly, Ser or Ala) were especially vulnerable (Wright, 1991; Tyler-Cross and Schirch, 1991). We introduced a hydroxylamine-sensitive site on the RCL and tested whether the chemical cleavage of the RCL can induce the conformational switch without an enzyme contact.

Materials and Methods

Chemicals Ultrapure urea and guanidine hydrochloride were purchased from ICN Biomedicals, Inc. (Aurora, USA). Hydroxylamine and porcine pancreatic elastase were purchased from Sigma Chemical Co. (St. Louis, USA). All other chemicals were reagent grade.

Recombinant α_1AT proteins and plasmids pFEAT30, the plasmid for the expression of α_1AT cDNA (Lee and Yu, 1989) in *Esherichia coli*, was described previously (Kwon *et al.*, 1994). Recombinant α_1AT proteins were expressed in inclusion bodies and purified as described previously (Kwon *et al.*, 1994; Jeoung and Yu, 1999). The concentration of α_1AT were determined in 6 M guanidine hydrochloride using a value of $A^{1\%}_{1cm}$ = 4.3 at 280 nm.

Mutagenesis Oligonucleotide-directed mutagenesis of pFEAT30 was carried to introduce a hydroxylamine-sensitive Asn-Gly sequence on amino acid 359-360 (P2'-P3') of α_1AT. The mutagenic oligonucleotide was 39-mer of sequence 5'-GAGGCCATACCCATGAATGGCCCCCCCGAGGTCAAGTTC -3. Two hydroxylamine-sensitive sites of the wild-type α_1AT, Asn 46 and Asn 314, were also changed to Thr and Asp, respectively, by a site-directed mutagenesis using oligonucleotides of sequences 5'-CACCAGTCCACCAGCACCAAT-3' and 5'-GTCTTCAGCGAT GGGGCTGAC-3'. The mutant protein is named as 'HN α_1AT'.

Preparation of the cleaved α_1AT proteins To prepare the enzymatically-cleaved protein, HN α_1AT protein was incubated with porcine pancreatic elastase at a ratio of 1 : 0.1 (α_1AT: protease) for 30 min at 37°C in an assay buffer (30 mM phosphate, 160 mM NaCl, 0.1% PEG6000, 0.1% TritonX-100, pH 7.4). Phenylmethylsulfonyl fluoride at a final concentration of 1 mM was added to stop the reaction. To prepare the chemically-cleaved protein, HN α_1AT protein was incubated at 45°C for 7 h in 2 M hydroxylamine, 30 mM Tris-Cl, pH 10. Excess hydroxylamine was removed by dialysis overnight against 10 mM phosphate (pH 6.5), 1 mM EDTA, and 1 mM β-mercaptoethanol. After heat treatment of the sample at 70°C for 15 min to remove any remaining uncleaved molecule, the major cleavage product was purified by ion exchange chromatography on MonoQ column (Pharmacia LKB, Uppsala, Sweden) in the same buffer. Cleavage of α_1AT was confirmed by 10% SDS-polyacrylamide gel electrophoresis and Coomassie Brilliant Blue staining.

Preparation of transverse urea gradient gel Transverse urea gradient gels were prepared with a gradient of 0~8 M urea perpendicular to the direction of electrophoresis (Goldberg, 1989) 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, San Francisco, USA) using a gradient maker and a single-channel peristaltic pump.

Equilibrium unfolding Unfolding transition as a function of guanidine hydrochloride was monitored by fluorescence spectroscopy. The native protein was incubated in 10 mM potassium phosphate, 50 mM NaCl, 1 mM EDTA, 1 mM β-mercaptoethanol, and various concentrations of guanidine-HCl (pH 6.5) for 4 h at 25°C. The concentration of guanidine hydrochloride was calculated from the difference in refractive index between the guanidine hydrochloride solution and buffer, as described previously (Pace *et al.*, 1989). The protein concentration was 10 μg/ml. Fluorescence intensity was measured for each sample with excitation at 280 nm and emission at 360 nm using Shimadzu RF-5000 fluorescence spectrophotometer. Experimental data of the fluorescence spectroscopy measurement were fitted to a two-state unfolding model as described previously (Pace *et al.*, 1989).

Peptide binding The native α_1AT, enzymatically-cleaved α_1AT,

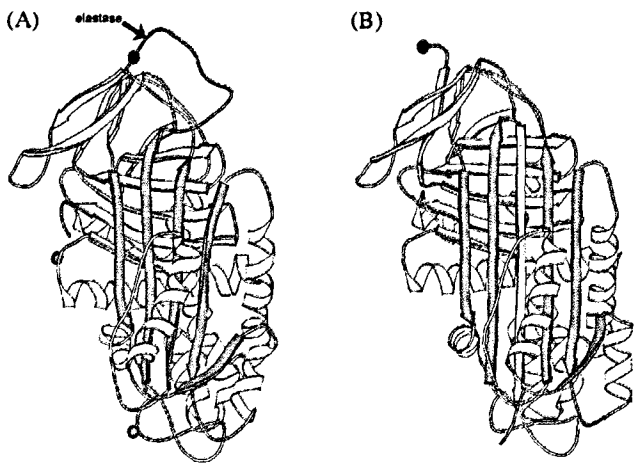

Fig. 1. (A) A schematic diagram of the native structure of α_1AT (Ryu *et al.*, 1995; 1atu.pdb). The β-sheet A is indicated as purple strands, and the RCL is indicated in yellow. The original cleavage sites (Asn 46 and Asn 314) of the wild-type α_1AT for hydroxylamine are indicated by green beads. The newly inserted hydroxylamine sensitive site (Asn 359) is also shown as a purple bead. The cleavage sites for porcine pancreatic elastase is indicated by a black arrow. (B) The enzymatically-cleaved structure of α_1AT (Loebermann *et al.*, 1984; 7api.pdb). The *N*-terminal portion of the scissile peptide bond is inserted into β-sheet A, forming the fourth strand of the β-sheet (s4A; indicated in yellow). These figures were prepared using a Molscript program.

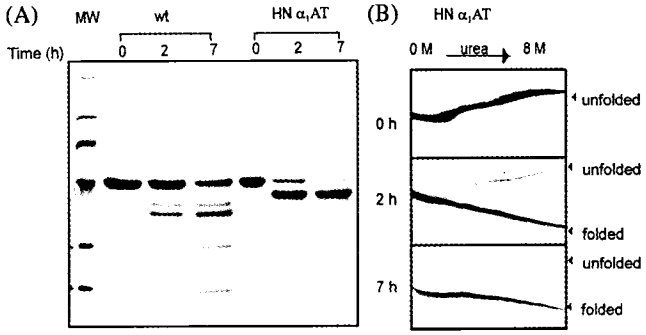

Fig. 2. Chemical cleavage of the RCL by hydroxylamine. (A) SDS-polyacrylamide gel electrophoresis showing specific cleavage of α_1AT by hydroxylamine. The α_1AT protein (at a concentration of 3 mg/ml) was incubated in 2 M hydroxylamine, 30 mM Tris-Cl, pH 10, at 45°C for up to 7 h. MW represents the molecular mass standard (Gibco BRL, Gaitherburg, USA; from the top, 200 kDa, 97.4 kDa, 68 kDa, 43 kDa, 29 kDa, and 18.4 kDa). The gel contained 10% polyacrylamide, and the protein bands were visualized by Coomassie Brilliant Blue staining. (B) Transverse urea gradient gel electrophoresis of HN α_1AT protein cleaved by hydroxylamine. The same HN α_1AT samples as in Fig. 2A, which were treated with hydroxylamine for 0 (top), 2 (middle), or 7 h (bottom), were analyzed on transverse urea gradient gel electrophoresis. The native protein (20 µg in 100 µl) was applied across the top of the gel. The electrode buffer 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 15°C. The protein bands were visualized by Coomassie Brilliant Blue staining.

and hydroxylamine-cleaved α_1AT proteins were incubated with a 14-mer peptide (Ac-Thr-Glu-Ala-Ala-Gly-Ala-Met-Phe-Leu-Glu-Ala-Ile-Val-Met-OH; Genemed Synthesis, Inc., South San Francisco, USA) at 42°C for 0-16 h. The molar ratio of the α_1AT protein to the peptide was 1 : 500 (protein concentration was 0.125 mg/ml; total volume of 20 µl). The buffer was 10 mM phosphate (pH 6.5), 50 mM NaCl, and 1 mM EDTA. The reaction samples were analyzed on a native gel containing 13.5% polyacrylamide. The gel electrophoresis buffer was 50 mM Tris-acetate, 1 mM EDTA (pH 7.5). The protein bands were visualized by Coomassie Brilliant Blue staining.

Results

Conversion of α_1AT into a very stable state by chemical cleavage of the RCL We tested whether or not a simple cleavage of the RCL of the native a_1AT by a chemical reagent can overcome the energy barrier in a conformational change to a more stable state. A hydroxylamine treatment of the wild-type α_1AT generated two smaller fragments (35 and 30 kDa bands) detected on SDS-PAGE (Fig. 2A). *N*-terminal amino acid sequencing identified two hydroxylamine-sensitive sites, Asn 46 and Asn 314 (Fig. 1A, green beads). These two hydroxylamine-sensitive sites of the wild-type α_1AT were substituted to non-sensitive sites by site-directed mutagenesis

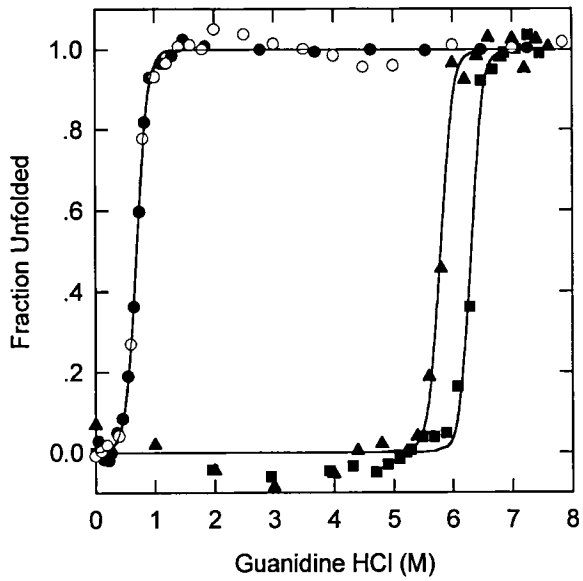

Fig. 3. Guanidine-induced unfolding transition of the chemically-cleaved α_1AT. Unfolding transition as a function of guanidine hydrochloride was monitored by fluorescence spectroscopy as described in Materials and Methods. Symbols: ○, the native wild-type α_1AT protein; ●, the native HN α_1AT protein; ■, HN α_1AT protein cleaved by hydroxylamine; ▲, HN α_1AT protein cleaved by porcine pancreatic elastase.

(Asn 46→Thr and Asn 314→Asp, respectively). Then, a new hydroxylamine-sensitive Asn-Gly sequence was introduced on the RCL in place of amino acids Ser 359 and Ile 360 of α_1AT (Fig. 1, purple bead), two residues that follow the cleavage site by a target protease, Met 358 (Fig. 1A, black arrow). This mutant protein, designated 'HN α_1AT', generated only a 40 kDa fragment on SDS-PAGE upon hydroxylamine treatment (Fig. 2A). *N*-terminal sequence of the large fragment was Met-Asp-Gln-Asp-His-Pro, the same as the original *N*-terminal sequence. The small fragment, 4 kDa in size, which was separated by tricine-gel system (Schägger and von Jagow, 1987), was Gly-Pro-Pro/Cys-Glu-Val-Lys-Phe (corresponding residues 360 to 366), identifying the newly created Asn-Gly bond on the RCL as the cleavage site. The stability of the hydroxylamine-treated HN α_1AT was monitored by transverse urea gradient gel electrophoresis (Fig. 2B). While the native HN α_1AT unfolded at about 2 M urea as the native wild-type protein did (effect of the amino acid substitutions on the stability was not significant), the cleaved form did not undergo unfolding transition in presence of up to 8 M urea. The portions of the cleaved HN α_1AT in Fig. 2A were well correlated with the urea-stable portions in Fig. 2B. The stability of the chemically-cleaved α_1AT was quantitatively measured with purified proteins by unfolding in the presence of guanidine hydrochloride (Fig. 3). The unfolding transition midpoint for the hydroxylamine-treated a_1AT is 6.2 M, which is similar to the value (5.9 M) for the same molecule cleaved by porcine pancreatic elastase. The unfolding midpoint of the uncleaved native α_1AT is 0.7 M for both wild-type and HN

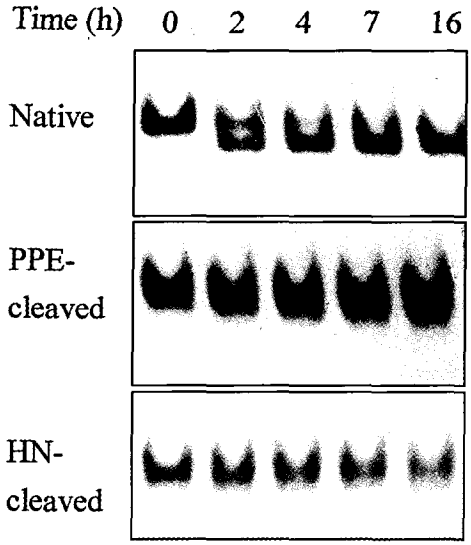

Fig. 4. Binding of a peptide of the reactive center loop sequence to α_1AT proteins. The native α_1AT, enzymatically (PPE)-cleaved α_1AT protein, and hydroxylamine (HN)-cleaved α_1AT protein were incubated with the 14-mer peptide mimicking the RCL sequences at 42°C for 0-16 h. Due to two negative charges on the peptide, the α_1AT-peptide complex migrates faster than the α_1AT molecule on electrophoresis in native conditions.

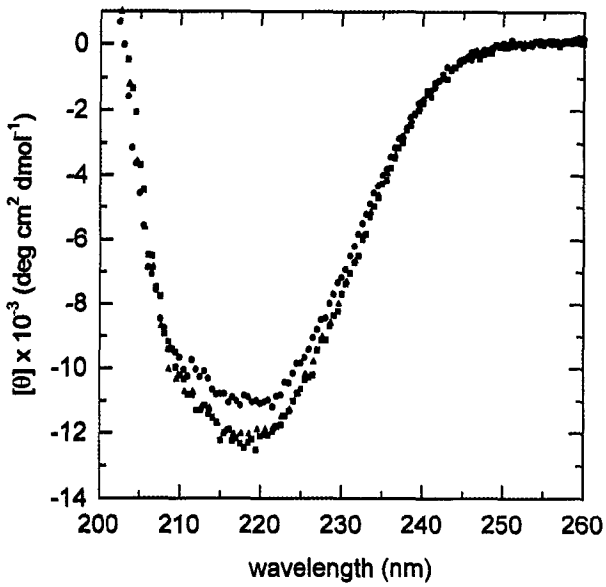

Fig. 5. CD spectra of the native, enzymatically- and chemically-cleaved mutant α_1AT. Circular dichroism spectra were measured on a Jasco-720 spectropolarimeter in a 1 mm path-length cell as previously described (Shin *et al.*, 2000). Temperature was controlled at 25°C by a Jasco PTC343 connected on a water bath. Concentration of α_1AT was 5 μM in 10 mM phosphate, 50 mM NaCl, pH 6.5. Scans were repeated seven times for each sample at a scan speed of 20 nm per min. The mean of two separate readings for each sample is shown. Symbol: black circles, the native HN α_1AT; red squares, HN α_1AT cleaved by porcine pancreatic elastase; green triangles, HN α_1AT cleaved by hydroxylamine.

α_1AT. These results indicate that the chemical cleavage of the RCL also produced a molecule that is at least as stable as the enzymatically-cleaved molecule. The results show that cleavage of the RCL, without any interaction with a target enzyme, is sufficient to convert the native conformation of α_1AT to a very stable relaxed form.

Probing the conformation of the chemically-cleaved α_1AT

The stability increase by loop cleavage using hydroxylamine may be due to the insertion of the RCL into β-sheet A as in the enzymatically-cleaved form. The native serpin molecule binds a peptide that mimics the RCL sequence (Carrell *et al.*, 1991). The peptide is inserted into β-sheet A in an X-ray crystal structure (Lawrence, 1997). If the RCL had already been inserted into β-sheet A, as in the enzymatically-cleaved form, the molecule would not bind the peptide. As shown in Fig. 4, the wild-type α_1AT bound the peptide with a half-time of 2 h at 42°C, but neither the enzymatically-cleaved nor the chemically-cleaved α_1AT bound the peptide under the same experimental conditions. Furthermore, circular dichroism spectrum of the chemically-cleaved protein shows increased negative molar ellipticity. This indicates an increased secondary structure formation, as in the case of the enzymatically-cleaved structure (Fig. 5). These results suggest that the stability increase by chemical cleavage of the RCL is due to the insertion of the RCL into β-sheet A, as the fourth strand and as in the enzymatically-cleaved α_1AT.

Discussion

In some proteins, the polypeptides fold initially into a metastable state and internal cleavage induces rearrangement into a presumably more stable form: the precursor of the surface glycoprotein of influenza virus, hemagglutinin (Chen *et al.*, 1998), the precursor of α-lytic protease (Sauter *et al.*, 1998), and the precursor of subtilisin (Gallagher *et al.*, 1995). In all of these cases, the cleavage induces a separation of the residues next to the scissile bond over 20 Å. Cleavage of the RCL of α_1AT also separates Met 358 and Ser 359 by 70 Å (Loebermann *et al.*, 1984). The RCL insertion in serpins would result in reorganization of the five-stranded mixed β-sheet into a six-stranded, antiparallel β-sheet (Fig. 1). Formation of new hydrogen-bonds between adjacent antiparallel β-strands explains the drastic stability increase in the cleaved molecules (Loebermann *et al.*, 1984). We found that chemical cleavage of the RCL by hydroxylamine alone lowered the kinetic barrier of the conformational change of the native form into a very stable relaxed form. Also, the interaction with a protease is not required for the conformational switch. The results, bypassing the folding barrier of α_1-antitrypsin by chemical cleavage or extension of the RCL (Im and Yu, 2000), strongly support that the major force blocking the conversion of the metastable native α_1AT into the lowest energy is the polypeptide connection of the

RCL between A sheet and C sheet. Our results show that release of the constrain held by the RCL is sufficient to trigger the switch of native α_1AT.

Our results also reveal the simplicity of nature's design for releasing energy at the stage of functional execution, where a facile conformational switch is critical (Engh *et al.*, 1995; Wright and Scarsdale, 1995; Carr *et al.*, 1997; Im *et al.*, 1999). During formation of a complex between an inhibitory serpin and its target protease, it is possible that cleavage of the RCL *per se* lowers the kinetic barrier substantially and allows conversion into the lowest energy state, which accompanies a spontaneous insertion of the RCL into A sheet (Lawrence, 1997). It appears that nature explores the protein folding steps as a mechanism of functional regulation by trapping the molecules in a metastable state first and then by releasing the remaining energy to carry out discrete conformational switches.

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