Protein Science (2000), *9*:1497–1502. Cambridge University Press. Printed in the USA.

Bypassing the kinetic trap of serpin protein folding by loop extension

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(RECEIVED March 7, 2000; FINAL REVISION May 5, 2000; ACCEPTED May 26, 2000)

Abstract

The native form of some proteins such as strained plasma serpins (serine protease inhibitors) and the spring-loaded viral membrane fusion proteins are in a metastable state. The metastable native form is thought to be a folding intermediate in which conversion into the most stable state is blocked by a very high kinetic barrier. In an effort to understand how the spontaneous conversion of the metastable native form into the most stable state is prevented, we designed mutations of α_1-antitrypsin, a prototype serpin, which can bypass the folding barrier. Extending the reactive center loop of α_1-antitrypsin converts the molecule into a more stable state. Remarkably, a 30-residue loop extension allows conversion into an extremely stable state, which is comparable to the relaxed cleaved form. Biochemical data strongly suggest that the strain release is due to the insertion of the reactive center loop into the major β-sheet, A sheet, as in the known stable conformations of serpins. Our results clearly show that extending the reactive center loop is sufficient to bypass the folding barrier of α_1-antitrypsin and suggest that the constrain held by polypeptide connection prevents the conversion of the native form into the lowest energy state.

Keywords: α_1-antitrypsin; kinetic trap; loop extension; polypeptide connectivity; protein folding

The native form of most proteins is thermodynamically the most stable state, and protein folding is a spontaneous reaction (Anfinsen, 1973). However, the native form of some proteins are not in the most stable state, but in a metastable state. The strained native structure (Huber & Carrell, 1989) of serpins (serine protease inhibitors), the spring-loaded structure of the membrane fusion protein of influenza virus (Carr & Kim, 1993), heat-shock transcription factors (Orosz et al., 1996), and possibly the membrane fusion proteins of other viruses (Chan et al., 1997) are examples. The native metastability in these proteins is an important mechanism for regulating biological functions (Wiley & Skehel, 1987; Huber & Carrell, 1989; Stein & Carrell, 1995; Carr et al., 1997; Im et al., 1999). In the strained native form of inhibitory serpins (Ryu et al., 1996), the reactive center loop (RCL) is exposed at one end of the molecule for protease binding. Upon cleavage of the RCL by proteases, the N-terminal portion of the cleaved RCL is inserted into the central β-sheet, A sheet (Loebermann et al., 1984), resulting in a drastic increase in stability (Bruch et al., 1988). There exists another conformation of serpins called the "latent" form, in which the RCL is inserted into the β-sheet A without its cleavage (Mottonen et al., 1992). The "latent" form is more stable than the native form (Hekman & Loskutoff, 1985; Wang et al., 1996), but is inactive. The "latent" form can be produced from the native form under physiological conditions in 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., 1995). Existence of the stable "latent" form strongly supports that the metastable native state is a kinetically trapped folding intermediate (Creighton, 1992; Baker & Agard, 1994; Carr et al., 1997).

Two important questions arise about the design principles of the native serpin conformation: (1) what is the structural basis of the strain? and (2) why does the molecule not convert into its lowest energy state? We previously probed the structural basis of the native strain of α_1-antitrypsin (α_1AT, also known as α_1-protease inhibitor), a prototype inhibitory serpin, by characterizing various stabilizing single amino acid substitutions (Lee et al., 1996; Im et al., 1999). Unusual interactions, such as overpacking of hydrophobic side chains, buried polar groups, and cavities, were found as the structural basis of the metastability of α_1AT (Lee et al., 1996; Ryu et al., 1996; Im et al., 1999). However, it has not been clear what factors prevent the conversion of these types of unfavorable interactions into more favorable ones in a more stable state. In present study, we designed mutant α_1AT molecules that convert spontaneously into lower energy states. Results suggest that polypeptide connection is one critical factor for maintaining the metastable native conformation.

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Abbreviations: EDTA, ethylenediaminetetraacetic acid; α_1AT, α_1-antitrypsin; RCL, reactive center loop; SDS, sodium dodecyl sulfate; serpin, serine protease inhibitor.

Results and discussion

Conversion of α_1AT into more stable conformations

We tested if simple extension of the RCL of the native α_1AT can bypass the folding barrier toward a more stable state. To insert additional amino acids on the RCL of α_1AT, a unique *Stu*I site was introduced at P4 (Ala355) and P3 (Ile356) position on the α_1AT-coding sequence, resulting in a substitution of Ile356 to Leu. Oligonucleotides encoding various lengths of flexible peptides were then incorporated at the unique *Stu*I site (Fig. 1A, green bead). Insertion mutations of GlyGlySer, $(GlyGlySer)_2$, $(GlyGlySer)_3$, $(GlyGlySer)_5$, and $(GlyGlySer)_{10}$ were designated as +3, +6, +9, +15, and +30, respectively. I356L substitution itself did not show any significant change in conformational stability and inhibitory activity. Guanidine hydrochloride-induced unfolding transition of RCL-extended α_1AT showed that all but +3 dramatically increased the stability (Fig. 2). The mutational effects of +6, +9, and +15 were substantial in that the transition midpoint was shifted from 0.7 M of the wild-type protein to 4.4, 4.5, and 4.7 M guanidine hydrochloride, respectively (Table 1). The increase in stabilization energy is equivalent to about 24 kcal mol^{-1}. Insertion of 30 amino acids, which was designed to increase the loop length more than 70 Å (the distance between P1 and P1′ residues in the cleaved form; Loebermann et al., 1984), shifted the transition midpoint to 5.9 M (equivalent to the increase in stability of 32 kcal mol^{-1}). Insertions of +9 or +15 amino acids at another site of the RCL, between P3′ and P4′ residues, also increased stability by 20 and 23 kcal mol^{-1}, respectively, suggesting that the stability increase by loop extension is independent of the precise location of insertion. None of the insertion mutants, including +3 α_1AT, formed a stable complex with a target protease, porcine pancreatic elastase; instead, they were cleaved at the RCL. Upon cleavage of the RCL by elastase, the transition midpoints of the cleaved mutant proteins were shifted to 5.9 M, the same degree to the uncleaved +30, in all cases. The results indicate that the loop extension in +30 allows the complete release of the native strain in α_1AT, and the stability increase in the other mutant proteins reflects a partial release of the strain.

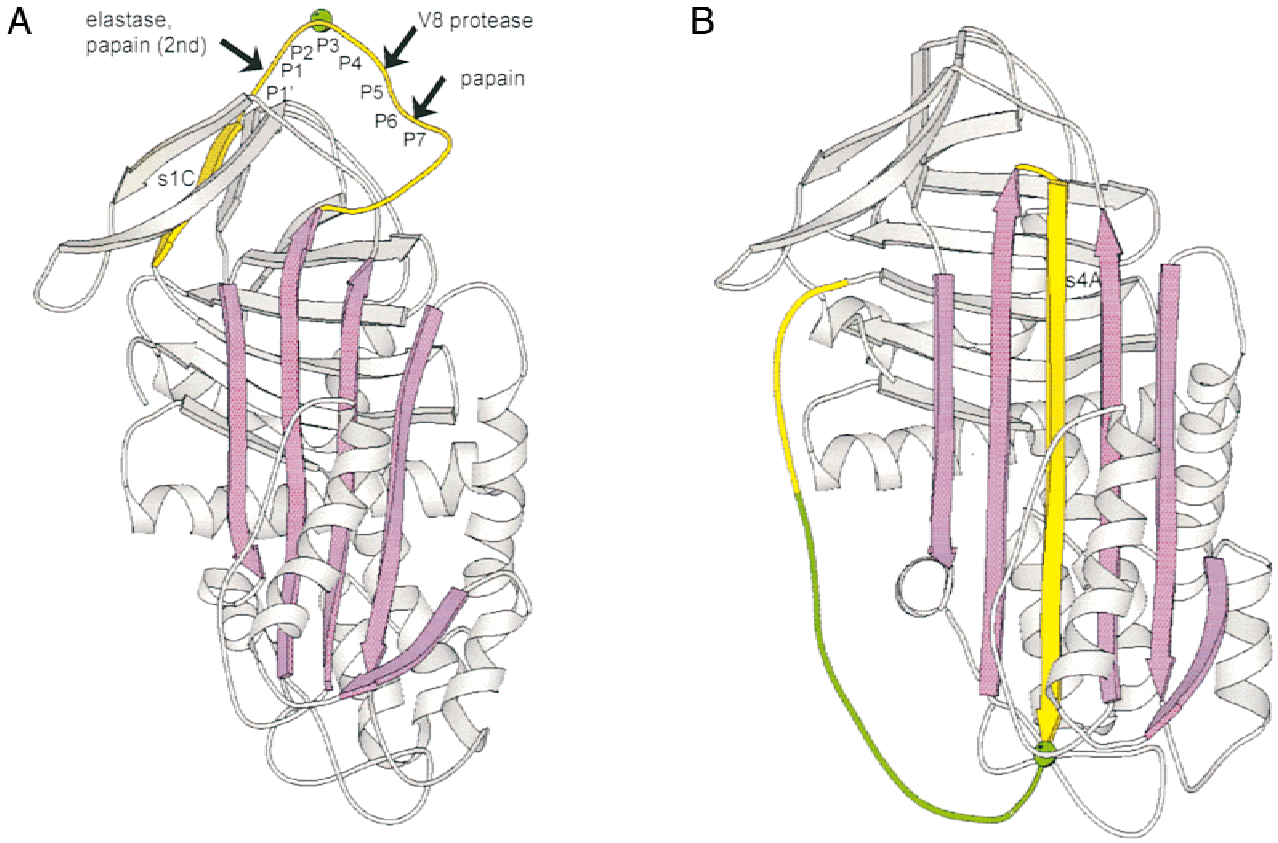

Fig. 1. A schematic diagram of α_1AT. **A:** The native structure (Ryu et al., 1996; 1atu.pdb). The β-sheets A is indicated as purple strands, and the RCL and the first stand of β-sheet C (s1C) are indicated in yellow. The residues in N- and C-terminal position of the scissile peptide bond (designated as P1, P2, P3 . . . , and P1′, P2′, P3′, respectively) are labeled. The cleavage sites for elastase, V8 protease, and papain are indicated by black arrows. Ile356, the insertion site for the additional flexible amino acids, is also shown as a green bead. **B:** The proposed structure of the loop-extended mutant α_1AT. The cleaved α_1AT structure (Loebermann et al., 1984; 7api.pdb) is referred, and the "latent-like" structures are suggested for +6, +9, and +15 proteins. Experimental data support that the N-terminal portion of the scissile peptide bond is inserted into β-sheet A without cleavage, forming the fourth strand of the β-sheet (s4A; indicated in yellow). Inserted amino acids are shown in green, and the s1C (yellow) is likely peeled off from the β-sheet C. A structure similar to the cleaved form, in which the s1C is attached to the β-sheet C, is suggested for +30 protein (diagram is not shown). These figures were prepared using MOLSCRIPT program.

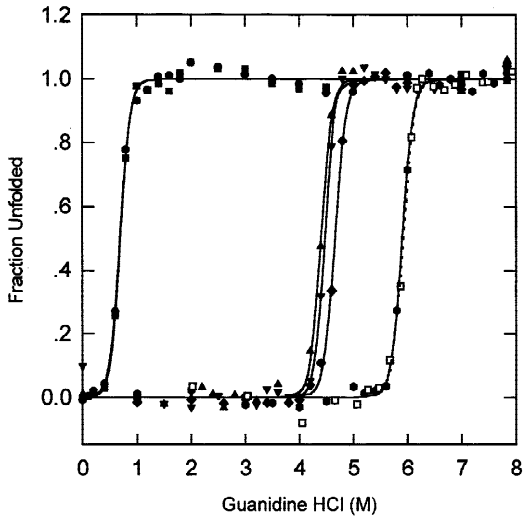

Fig. 2. Guanidine hydrochloride-induced unfolding transition. Unfolding transitions were measured by the increase in fluorescence emission intensity at 360 nm (λ_{ex} = 280 nm). Samples were incubated in each guanidine hydrochloride solution containing 10 mM potassium phosphate, 50 mM NaCl, 1 mM EDTA, 1 mM β-mercaptoethanol (pH 6.5) at 25 °C for 4 h. The protein concentration was 10 μg/mL. Symbols: ●, wild-type; ■, +3; ▲, +6; ▼, +9; ♦, +15; ⬢, +30; □, +3 α_1AT cleaved by porcine pancreatic elastase.

Probing the conformation of loop-extended α_1AT

Stability increase in the mutant α_1AT may be due to the insertion of the RCL into β-sheet A as in the known stable conformations such as the "latent" or the cleaved form. This was investigated by probing the accessibility of the RCL to *Staphylococcus aureus* V8 protease and papain, which are known to cleave the exposed RCL of the native α_1AT at the sites (Mast et al., 1992) indicated in Fig. 1A. Incubation of wild-type α_1AT or +3 α_1AT with V8 protease at 37 °C for 1 h produced a species with a molecular mass of 40 kDa on a polyacrylamide gel (Fig. 3A). Mass spectroscopic analysis shows that the RCL of the wild-type α_1AT is cleaved at P5–P4 peptide bond as described (Fig. 1A). However, other mutant α_1AT proteins remained intact, indicating that the V8 target site (P5–P4 peptide bond) is not accessible in these mutant proteins. Likewise, incubation of the wild-type or +3 α_1AT protein with papain at 37 °C resulted in a species with a molecular mass of 38.5 kDa on a polyacrylamide gel (Fig. 3B). The N-terminal sequencing of the cleavage products showed that both primary and secondary target sites (Fig. 1A, P7–P6 and P1–P1′ peptide bonds) were cleaved. The incubation of +6 and +9 α_1AT produced a fragment, but the size of the fragment was larger (Fig. 3B). The molecular mass and the N-terminal sequencing of the cleavage products of +6 α_1AT showed that the cleavage occurred only at P1′–P2′ peptide bond. The results indicate that even the secondary target site (P1–P1′ peptide bond) was not accessible to proteolytic attack in the +6 α_1AT. Instead, the next peptide bond (P1′–P2′) was attacked at lower frequency in this mutant protein. The +15 and +30 α_1AT were more resistant to papain attack. The results suggest that almost the entire portion of RCL is unavailable for proteolytic attack in all the mutant proteins besides +3 α_1AT.

The native serpin molecule binds a peptide that mimics the RCL sequence (Carrell et al., 1991), and the peptide is inserted into β-sheet A in an X-ray crystal structure (Lawrence, 1997). If the RCL were already inserted into β-sheet A, the molecule would not bind the peptide. As shown in Figure 3C, both wild-type and +3 α_1AT bound the peptide with a half-time of 2 h at 40 °C, but the other mutants of α_1AT did not bind the peptide under the same experimental conditions. Extension of the RCL seems to allow the insertion of the RCL into β-sheet A and preclude an additional insertion of the peptide. These results suggest that the stability increase of the mutant α_1AT is due to the insertion of RCL into β-sheet A as the fourth strand (Fig. 1B).

Table 1. *Unfolding transition of the loop-extended mutant α_1AT*

α_1AT protein	C_m[a] (M)	*m*-Value[a] (kcal mol^{-1} M^{-1})	$\Delta\Delta G$[b] (kcal mol^{-1})
wt (I356L)	0.7 ± 0.1	6.3 ± 0.9	0
+3	0.7 ± 0.1	6.5 ± 0.9	0
+6	4.4 ± 0.1	6.0 ± 0.7	22.9
+9	4.5 ± 0.1	6.1 ± 0.7	23.6
+15	4.7 ± 0.1	6.1 ± 0.5	24.8
+30	5.9 ± 0.1	6.0 ± 0.6	32.2
Cleaved α_1AT[c]	5.9 ± 0.1	6.1 ± 0.3	32.2

[a] Experimental data of guanidine hydrochloride-induced unfolding transition of α_1AT were fitted to a two-state unfolding model to determine the transition midpoint (C_m) and *m*-values as described previously (Lee et al., 1996; Im et al., 1999).

[b] Stability increase ($\Delta\Delta G$) by mutations were calculated as $\Delta C_m(Cm_{mut} - Cm_{wt}) \times$ average *m*-value (6.2 kcal mol^{-1} M^{-1}), as described (Pace et al., 1989). $\Delta\Delta G$ values obtained by this method were similar to the values obtained by subtracting the wild-type $\Delta G(H_2O)$ from the mutant $\Delta G(H_2O)$ (within ±1.2 kcal mol^{-1}), which was obtained by extrapolating a plot of ΔG vs. guanidine to zero concentration of the denaturant.

[c] The RCL-extended mutant α_1AT was cleaved by porcine pancreatic elastase and the cleaved form was purified as described in Materials and methods.

The kinetic trap and biological implications

Our results show that extension of the RCL of α_1AT induced a very stable conformation in which the RCL appears to be inserted in the β-sheet A (Fig. 1B). Stability increase by +6 α_1AT is comparable to the effects of +9 or +15, but not as great as +30 α_1AT, which is as stable as the cleaved form. This may be due to that the RCL insertion into β-sheet A without cleavage or long enough extension would enforce detachment of the strand 1 of C sheet (s1C) from β-sheet C as in the "latent" form. The current results show that the extension of the RCL is sufficient to bypass the folding trap of native α_1AT and indicate that the constrain of the RCL held by polypeptide connection is one critical factor preventing the conversion into the lowest energy state. It was suggested that native interactions in metastable proteins somehow prevent the conversion into a more stable structure (Carr et al., 1997). Native interactions are considered to be an important factor in guiding polypeptide to fold into right tertiary conformations: early folding intermediates contain native secondary structures (Ptitsyn et al., 1990); subdomains of some proteins fold into native structures (Oas & Kim, 1988; Staley & Kim, 1990); and native disulfide-bonded intermediates predominate in the folding of bovine pancreatic trypsin inhibitor (Weissman & Kim, 1991). In the case of serpins,

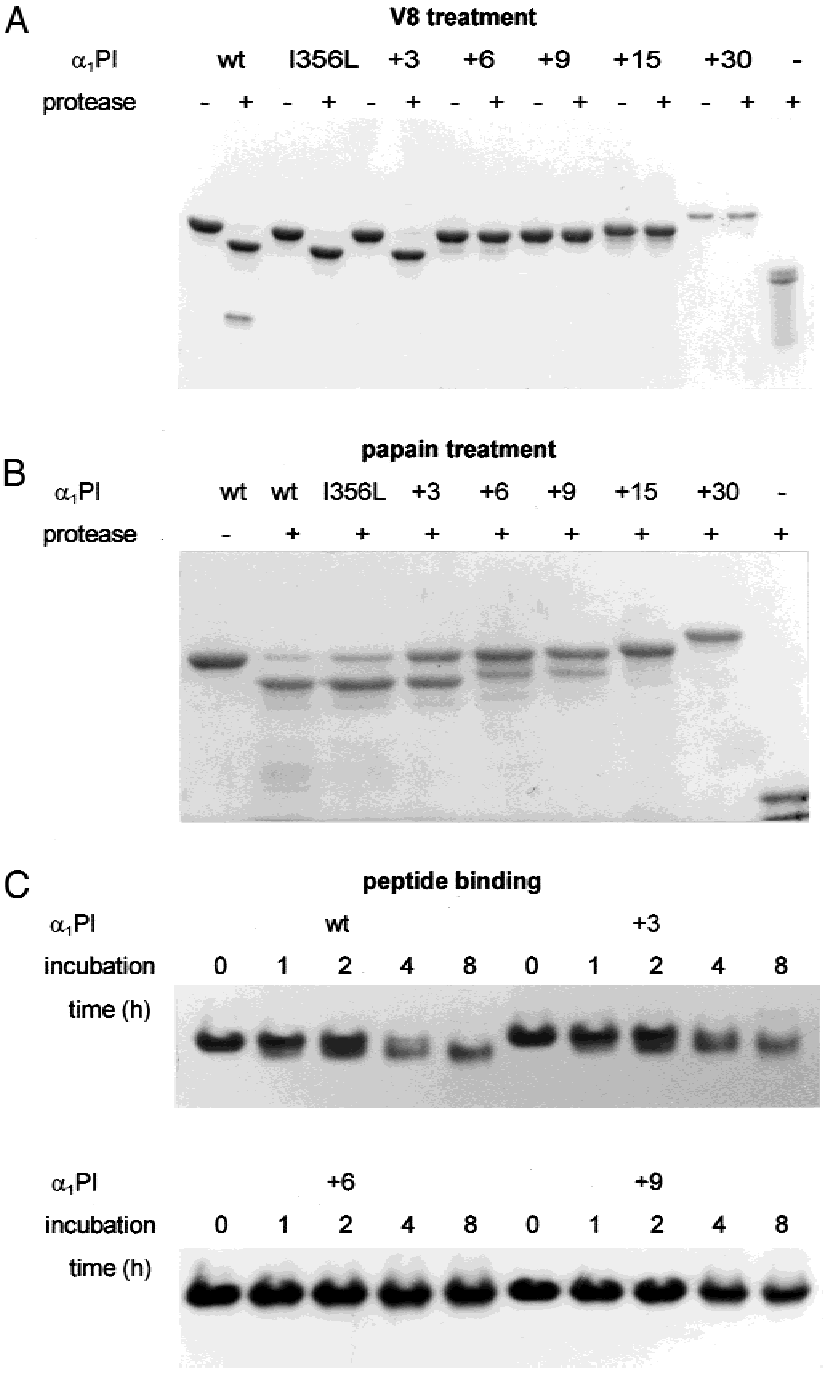

Fig. 3. Probing the conformation of the RCL in the loop-extended mutant α_1AT. **A:** Limited proteolysis of α_1AT with *S. aureus* V8 protease. Various α_1AT mutant proteins were incubated with (+) and without (−) V8 protease, as indicated on top of the figure. *S. aureus* V8 protease (Sigma, St. Louis, Missouri) was incubated with α_1AT at a ratio of 1:50 (w/w) in 0.1M Tris-HCl, pH 7.8, at 37 °C for 1 h. **B:** Limited proteolysis of α_1AT with papain. Various α_1AT mutant proteins were incubated with (+) and without (−) papain, as indicated on top of the figure. Papain was incubated with α_1AT protein at a ratio of 1:1000 (w/w) at 37 °C for 15 min. The buffer was 20 mM phosphate, pH 7.0, 5 mM cysteine, 10 mM EDTA, and 5 mM NaCl. **C:** Binding of a peptide mimicking the RCL sequences to native α_1AT proteins. Various native α_1AT proteins, labeled on top of the figure, were incubated with a 14-mer peptide (Ac-Thr-Glu-Ala-Ala-Gly-Ala-Met-Phe-Leu-Glu-Ala-Ile-Val-Met-OH) at 40 °C for 0–8 h. The molar ratio of α_1AT protein to peptide was 1:500 in 10 mM phosphate (pH 6.5), 50 mM NaCl, and 1 mM EDTA. Protein concentration was 0.25 mg/mL in total volume of 10 μL. Peptide binding to α_1AT was analyzed on 13.5% polyacrylamide gel electrophoresis in a native condition (50 mM Tris-acetate, 1 mM EDTA, pH 7.5).

our results strongly suggest that the native interactions in β-sheet A do not prevent the conversion into a more stable state. Instead, β-sheet A, due to the metastability, readily accepts the RCL when it is available. The RCL of serpins connect β-sheet A and β-sheet C (Fig. 1A). It was hypothesized that formation of β-sheet C ahead of formation of β-sheet A excludes the RCL from being incorporated into β-sheet A and thus from folding into the more stable latent conformation (Patston & Gettins, 1996). Although we could not test this hypothesis directly because most of the secondary and tertiary structure formations occurred within a dead time of stopped-flow circular dichroism and fluorescence spectroscopy (1 ms), it is possible that the native interactions in β-sheet C or the β-barrel composed of β-sheets B and C (Huber & Carrell, 1989) do not allow the spontaneous conversion into a more stable form. Further work is needed to elucidate the exact nature of the kinetic trap in the folding of metastable native forms.

There are other examples where the polypeptides fold 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 cases, the cleavage

induces a separation of the residues next to the scissile bond over 20 Å. We found that the native form of α_1AT is suboptimally folded and can be stabilized at many sites by single amino acid substitutions (Lee et al., 1996; Im et al., 1999). The precursor structure of hemagglutinin also revealed unusual interactions, such as cavities and a strained surface loop (Chen et al., 1998). In the case of α-lytic protease and subtilisin, the structure of the precursor protein is not known, but it is suspected to be distorted (Baker, 1998; Sauter et al., 1998). The driving force for the conformational switch is likely to be mobilization of such unfavorable interactions in the metastable state into more favorable ones in the cleaved structure. 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. It will be interesting to examine if the loop extension at the cleavage site in these other proteins also releases the strain held in their precursor forms.

Materials and methods

Mutagenesis and expression of mutant α_1AT in Escherichia coli

A unique *Stu*I site was introduced on pFEAT30 (Lee et al., 1996), the plasmid for the expression of recombinant α_1AT, at P4 (Ala355) and P3 (Ile356) position on the α_1AT-coding sequence. This resulted in a substitution of P3 residue (Ile356) by leucine. To generate the loop-extended mutations of α_1AT, each pair of synthetic oligonucleotides coding for GlyGlySer, $(GlyGlySer)_2$, $(GlyGlySer)_3$, $(GlyGlySer)_5$, and $(GlyGlySer)_{10}$, were phosphorylated at the 5′-termini, mutually annealed, and then inserted into the unique *Stu*I site. Recombinant α_1AT was expressed as inclusion bodies in *E. coli* as described previously (Lee et al., 1996). Inclusion bodies were dissolved in 0.5% SDS, 10 mM phosphate, 1 mM EDTA, 1 mM β-mercaptoethanol, pH 6.5, and refolded in the same buffer without SDS by diluting 20-fold. The remaining SDS was removed by extensive dialysis. Monomeric forms were purified by ion exchange chromatography on MonoQ column (Pharmacia, Uppsala, Sweden), and concentrations of α_1AT were determined as described previously (Kwon et al., 1994).

Guanidine hydrochloride-induced unfolding transition

Unfolding as a function of guanidine hydrochloride (ICN Biomedicals, Inc., Costa Mesa, California) was monitored by fluorescence spectroscopy (λ_{ex} = 280 nm and λ_{em} = 360 nm, excitation and emission slit widths = 5 nm for both), details of which were described previously (Lee et al., 1996). The buffer was 10 mM phosphate, pH 6.5, 50 mM NaCl, 1 mM EDTA, and 1 mM β-mercaptoethanol. The protein concentration was 10 μg mL^{-1}. The native protein was incubated in the buffer containing various concentrations of guanidine hydrochloride at 25 °C for 4 h. Experimental data of the fluorescence intensity measurement were fitted to a two-state unfolding model.

Preparation of cleaved α_1AT

To prepare enzymatically cleaved protein, mutant α_1AT was incubated with porcine pancreatic elastase at a molar ratio of 1:0.2 (α_1AT: protease) at 37 °C for 1 h. The buffer contains 30 mM phosphate, 160 mM NaCl, 0.1% PEG6000, 0.1% Triton X-100, pH 7.4. Phenylmethylsulfonyl fluoride, at a final concentration of 1 mM, was added to stop the reaction. After heat treatment at 70 °C for 15 min to remove any remaining uncleaved molecule, the major cleavage product was purified by ion exchange chromatography on a MonoQ column in 10 mM phosphate, 1 mM β-mercaptoethanol, 1 mM EDTA, pH 6.5. Cleavage of α_1AT was confirmed by 10% SDS-polyacrylamide gel electrophoresis and Coomassie Brilliant Blue staining.

Binding of a peptide mimicking the RCL sequence to α_1AT

A 14-mer peptide mimicking the reactive center loop sequence (Ac-Thr-Glu-Ala-Ala-Gly-Ala-Met-Phe-Leu-Glu-Ala-Ile-Val-Met-OH; Genemed Synthesis, Inc., South San Francisco, California) was incubated with the native α_1AT protein (0.25 mg/mL) at 40 °C for various periods up to 8 h. The peptide was 500-fold molar excess to α_1AT, and the buffer was 10 mM phosphate, 50 mM NaCl, and 1 mM EDTA, pH 6.5. The degree of complex formation was determined by 13.5% polyacrylamide gel electrophoresis in the native condition. The electrophoresis buffer was 50 mM Tris-acetate, and 1 mM EDTA, pH 7.5. The protein bands were visualized by staining with Coomassie Brilliant Blue.

Acknowledgments

We thank S.-E. Ryu and S.-H. Park for helpful comments. This research was supported by the National Creative Research Initiatives Project of the Korean Ministry of Science and Technology.

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