Probing the local conformational change of α_1 -antitrypsin

JE-HYUN BAEK,^{1,2} HANA IM,³ UN-BEOM KANG,^{2,4} KI MOON SEONG,² CHEOLJU LEE,⁴ JOON KIM,² AND MYEONG-HEE YU¹

¹Functional Proteomics Center, Korea Institute of Science and Technology, Seongbuk-gu, Seoul 136-791, Korea

²Laboratory of Biochemistry, School of Life Sciences and Biotechnology, Korea University, Seongbuk-gu, Seoul 136-701, Korea

³Department of Molecular Biology, Sejong University, Gwangjin-gu, Seoul 143-747, Korea

⁴Life Sciences Division, Korea Institute of Science and Technology, Seongbuk-gu, Seoul 136-791, Korea

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Abstract

The native form of serpins (serine protease inhibitors) is a metastable conformation, which converts into a more stable form upon complex formation with a target protease. It has been suggested that movement of helix-F (hF) and the following loop connecting to strand 3 of β -sheet A (thFs3A) is critical for such conformational change. Despite many speculations inferred from analysis of the serpin structure itself, direct experimental evidence for the mobilization of hF/thFs3A during the inhibition process is lacking. To probe the mechanistic role of hF and thFs3A during protease inhibition, a disulfide bond was engineered in α_1 -antitrypsin, which would lock the displacement of thFs3A from β -sheet A. We measured the inhibitory activity of each disulfide-locked mutant and its heat stability against loop-sheet polymerization. Presence of a disulfide between thFs3A and s5A but not between thFs3A and s3A caused loss of the inhibitory activity, suggesting that displacement of hF/thFs3A from strand 5A but not from strand 3A is required during the inhibition process. While showing little influence on the inhibitory activity, the disulfide between thFs3A and s3A retarded loop-sheet polymerization significantly. This successful protein engineering of α_1 -antitrypsin is expected to be of value in clinical applications. Based on our current studies, we propose that the reactive-site loop of a serpin glides through between s5A and thFs3A for the full insertion into β -sheet A while a substantial portion of the interactions between hF and s3A is kept intact.

Keywords: protein engineering; disulfide locking; α_1 -antitrypsin; serpin; conformational change; loop-sheet polymerization

Supplemental material: see www.proteinscience.org

The serpins (SERine Protease INhibitors) are a super-family of proteins that adopt a metastable conformation required for their inhibitory activity (Huber and Carrell 1989; Stein and Carrell 1995). As a member of serpins,

α_1 -antitrypsin (α_1 -AT) also has a metastable native conformation and plays a physiological role in modulating the activity of human leukocyte elastase in lung. Once the protease recognizes the reactive-site loop (RSL) exposed at one end of the α_1 -AT molecule, the scissile bond of the RSL is cleaved while the protease is covalently attached to the N-terminal part of the RSL (Loebermann et al. 1984; Wei et al. 1994). This event allows the RSL to insert into the central β -sheet A between strands 3 and 5 (s3A and s5A) (Fig. 1; Loebermann et al. 1984; Baumann et al. 1991), resulting in translocation of the target protease from one pole of α_1 -AT to the opposite side

Reprint requests to: Myeong-Hee Yu, Functional Proteomics Center, Korea Institute of Science and Technology, 39-1 Hawolgok-dong, Seongbuk-gu, Seoul 136-791, Korea; e-mail: mhyu@kist.re.kr; fax: +82-2-958-6919; or Joon Kim, School of Life Sciences and Biotechnology, Korea University, Anam-dong, Seongbuk-gu, Seoul 136-701, Korea; e-mail: joonkim@korea.ac.kr; fax: +82-2-927-9028.

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Figure 1. Structures of α_1 -AT. (A) The structure of native form (1QLP) is represented as a ribbon diagram with the position of six engineered disulfide bonds. *Left box*, focused side view showing three engineered disulfide bonds between thFs3A and s5A (dotted lines). *Right box*, focused side view showing two engineered disulfide bonds between thFs3A and s3A (dotted lines) and one engineered disulfide between s3A and s5A (solid line). The balls in the structure indicate mutated residues to cysteine. (B) Structure of α_1 -AT-trypsin complex (1EZK) showing that the RSL is inserted between s3A and s5A to form a new β -strand. Structures are color-coded: hF/thFs3A, yellow; s3A, red; s4A, pink; s5A, orange; α_1 -AT, blue; trypsin, cyan.

with concomitant distortion of the active site of the protease (Bruch et al. 1988; Stratikos and Gettins 1999; Huntington et al. 2000).

Over the last two decades, the structural bases for the inhibitory mechanism of α_1 -AT have been extensively studied by various approaches. Through crystal structure analysis, Loebermann et al. (1984) suggested that helix-F (hF) and the following loop connecting to strand 3 of β -sheet A (thFs3A) facilitate the insertion of RSL by loosening their interactions with sheet A. Other biochemical and crystallographic studies have also suggested that a temporal opening of hF/thFs3A from β -sheet A may be required for complete insertion of RSL (Bijnens et al. 2000; Goptu et al. 2000; Wind et al. 2001). More recently, thermodynamic analysis on α_1 -AT has shown that hF/thFs3A has sufficient flexibility for insertion of RSL (Cabrita et al. 2004; Tsutsui et al. 2006). Such a conformational change of α_1 -AT occurring during inhibition process can also be observed without proteolytic cleavage in the process of loop-sheet polymerization in which RSL of one molecule inserts into the β -sheet A of another molecule (Lomas et al. 1992; Skinner et al. 1998). Plasminogen activator inhibitor type 2, whose regulatory mechanism depends on redox status, shows polymerogenic behavior at the physiological state, possibly through the displacement of hF/thFs3A induced by an intrinsic disulfide bond (Wilczynska et al. 2003). However, reducing the disulfide bond, which may allow the return of hF/thFs3A to the position covering β -sheet A, abrogates loop-sheet polymerization. The results indicate

that hF/thFs3A in the native state plays a gatekeeping role for preventing loop-sheet polymerization.

Although there are many suggestions, there is little direct experimental evidence for the temporal displacement of hF/thFs3A during the inhibition of protease. Rather, it has mostly been inferred from the analysis of serpin structure itself. To understand the mechanistic role of hF/thFs3A, we have engineered several double-cysteine variants of α_1 -AT and introduced a disulfide bond to each mutant, which was expected to restrict the movement of hF/thFs3A from β -sheet A. We investigated how the designed disulfide locking influenced the inhibitory activity and the loop-sheet polymerization.

Materials and Methods

Chemicals

Porcine pancreatic elastase (PPE), *N*-succinyl-(Ala)₃-*p*-nitroanilide, sodium iodoacetate, dithiothreitol, bis-ANS, and glutathione (oxidized form) were purchased from Sigma. Thiol and sulfide quantitation kit (T-6060) was purchased from Molecular Probes. All other chemicals were reagent-grade.

Recombinant α_1 -AT proteins and site-directed mutagenesis

Site-specific mutations were induced by oligonucleotide-directed mutagenesis at the target region and confirmed

by DNA sequencing of the plasmid. All variants carried the C232S mutation. Plasmids for α_1 -AT expression in *Escherichia coli* and purification of recombinant α_1 -AT protein were described previously (Kwon et al. 1995; Kang et al. 2004). Protein concentration was determined by measuring A_{280} in 6 M guanidine hydrochloride and calculated from tyrosine and tryptophan content of the α_1 -AT protein (Edelhoch 1967).

Disulfide bond formation and purification of α_1 -AT

Protein solution was loaded on a Q-Sepharose column and washed with 50 mM tris-HCl buffer (pH 8.0). The oxidation of double-cysteines to a disulfide was carried out on column for 48 h at 4°C after buffer exchange with the same buffer containing 1 mM oxidized glutathione (GSSG). Protein was eluted with a linear-gradient of 0–0.6 M NaCl. In case of low oxidation yield (K168C-V337C), the eluted protein was treated with 10 mM sodium iodoacetate (ICH₂COONa) in 10 mM KH₂PO₄ buffer (pH 6.8) for 1 h at room temperature to add two more negative charges onto the free thiols in reduced form. The oxidized protein was purified by NaCl salt gradients using a Mono-Q column on an ÄKTA Explorer 100 instrument (Amersham Pharmacia). At the end of all purification steps, samples were desalted using Sephadex G-25 to exchange the buffer with 10 mM KH₂PO₄ buffer (pH 6.5).

Gel electrophoresis

Nonreducing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a 10% gel as described previously (Laemmli 1970). Prior to SDS-PAGE, samples were boiled for 5 min in loading buffer containing 2% SDS except reducing reagent. Non-denaturing PAGE was performed at 4°C without SDS in a 10% gel using the method described previously (Goldenberg and Creighton 1989). The gels were visualized by staining with Coomassie Brilliant Blue.

Determination of the yield of disulfide bond formation

Free thiols in engineered proteins were quantitated with a colorimetric assay using the Thiol and Sulfide Quantitation Kit (Molecular Probes) according to the manufacturer's instructions. Briefly, 0.6 nmol of engineered proteins was incubated with 2 mM cystamine followed by 0.6 mg/mL papain-SSCH₃ for 1 h incubation at room temperature. The reaction mixture was then incubated with 2.5 mM L-BAPNA for 1 h at room temperature, and absorbance was measured at 410 nm. Thiol contents were calculated from a calibration curve using standardized cysteine solutions. Alternatively, the percentage of ox-

dized form was measured by densitometric analysis for both oxidized and reduced forms in nonreducing SDS-PAGE using ImageQuant software (Molecular Dynamics).

Identification of disulfide bond by mass spectrometry

The protein band was excised, diced to small pieces, washed with H₂O, and destained with 50% acetonitrile. After dehydration with acetonitrile, the gel pieces were dried in the air and the protein was digested with 12.5 ng/ μ L trypsin (Promega) in 25 mM ammonium bicarbonate buffer (pH 8.0) at 37°C for overnight. To check the existence of target mass for disulfide bonded tryptic peptide, mass spectrum of tryptic digest was acquired using MALDI-TOF instrument (Voyager DE-STR, Applied Biosystems). For determination of disulfide position, tryptic digest was loaded on a nanospray tip-coupled capillary column (75 μ m $\times$ 16 cm) packed in-house with Magic C18aq (Michrom BioResources) by helium pressure cells. Peptides were eluted with a linear gradient of 5%–40% solvent B over 30 min at 300 nL/min (solvent A, 0.1% formic acid in water; solvent B, 0.1% formic acid in 100% acetonitrile) using an Agilent Nano-flow 1200 series HPLC system. We used a mass acquisition method in which MS survey scan from 300 to 2000 m/z is followed by targeted MS/MS scans for the disulfide-bonded tryptic peptide (888.73 m/z for the +3 charge state and 1332.59 m/z for the +2 charge state) on an LTQ linear ion trap mass spectrometer (ThermoFinnigan). Mass spectra were inspected manually.

Activity assay

Inhibitory activity of α_1 -AT was assessed by determining the amount of α_1 -AT to completely inhibit 1 mol of PPE (Seo et al. 2000). Various amounts of purified recombinant α_1 -AT proteins were incubated in 50 μ L of assay buffer (30 mM phosphate, 160 mM NaCl, 0.1% polyethylene glycol 8000, and 0.1% Triton X-100, pH 7.4) with 100 nM PPE. After incubation at 37°C for 10 min, the reaction mixture was diluted 10-fold with the assay buffer and the residual protease activity was determined using 1 mM *N*-succinyl-(Ala)₃-*p*-nitroanilide as a substrate.

Analysis of heat stability

α_1 -AT proteins were incubated at 0.2 mg/mL in 10 mM KH₂PO₄ buffer (pH 6.5) for 8 h at 60°C. Aliquots were removed at each time point (0, 0.25, 0.5, 1, 2, 3, 4, 6, and 8 h) and were rapidly cooled on ice. Samples were analyzed by non-denaturing PAGE. The remaining inhibitory activity of each aliquot was also determined by the above-mentioned activity assay.

Determination of association rate constants

The association rate constant for the interaction of α_1 -AT with porcine pancreatic elastase was measured under second-order conditions in a reaction mixture containing equimolar concentrations (14 nM) of enzyme and inhibitor as previously described (Beatty et al. 1980).

Bis-ANS binding assay

Binding of bis-ANS was carried out with 1 μ M α_1 -ATs for effective analysis of biphasic change in bis-ANS fluorescence as previously described (James and Bottomley 1998). The kinetic fluorescence measurements were performed in a PerkinElmer SynergyHT-I spectrofluorimeter at 50°C and in an LS50B spectrofluorimeter at 65°C. The rates of each phase in fluorescence change were determined by fitting to a single-exponential function.

Results

Selecting sites for disulfide bonds

To investigate the role of hF/thFs3A for inhibitory mechanism, several disulfide bonds were designed and separately introduced, through which the hF-thFs3A structure would be constrained to the main body of α_1 -AT, β -sheet A. The X-ray crystallographic structure for the native form of α_1 -AT (PDB entry 1QLP); (Elliott et al. 2000) was examined to select suitable sites. The sites were selected according to the following criteria: (1) pairs of residues with one located at hF or thFs3A and the other at s3A or s5A; (2) pairs of residues whose C_α - C_α distances are within 5.0–9.5 Å; and (3) if possible, the residues for which single amino acid substitution has been shown by previous investigations to have minor or beneficial effects on the stability of α_1 -AT. In our previous studies, the inhibitory activities for single amino acid substitutions in the sites of K168, I169, L172, V173, and F189 have been determined. Typically, K168I, I169V, L172V, L172A, V173I, F189I, and F189V mutations had a marginal effect on the stability, and some of the mutations even stabilized the protein. As determined by denaturant-induced equilibrium unfolding, the stability change was $\sim$ 0.0–1.9 kcal/mol, and the activity loss by the mutations was $<$ 30% (Im et al. 1999; Seo et al. 2000; Lee et al. 2001). On the basis of the criteria given, seven disulfide bond sites were chosen. We added to the list one more mutant, V185C–V333C, linking s5A and s3A, as a control for detrimental effect on the activity. However, we excluded the two double-mutant constructs, V161C–Y187C and T165C–F189C, because of low-level expression in *E. coli*. Finally, six disulfide bond sites were chosen as listed in Table 1. There is no native disulfide bond in human α_1 -AT, and the single free cysteine at residue site 232 was replaced with

Table 1. Properties of double-cysteine mutants of α_1 -AT

Group	Mutants ^a	C_α - C_α ^b	Location ^c	Yield (%) ^d	Relative activity ^e	
					2SH	S–S
1	K168C–F189C	8.02	thFs3A–s3A	97	0.93	0.94
1	I169C–Y187C	8.36	thFs3A–s3A	99	0.36	0.38
2	K168C–V337C	8.47	thFs3A–s5A	98	0.56 (0.55)	0.01
2	L172C–V333C	8.25	thFs3A–s5A	80	0.79	0.16
2	V173C–K331C	9.13	thFs3A–s5A	95	0.73	0.04
3	V185C–V333C	5.08	s3A–s5A	90	0.34	0.03

^aOne-letter codes for amino acids are used for mutations, which are designated by the code for the wild-type residue followed by the residue number and the code for the replacement residue.

^bThe distance (Å) between two α -carbons in the crystal structure of native α_1 -AT (1QLP).

^cThe nomenclature of Huber and Carrell was followed (Huber and Carrell 1989).

^dPurity of disulfide-bonded form.

^eRelative activity compared to the wild type. Activity in the parentheses was measured after the oxidized protein was re-reduced with DTT. 2SH, reduced form; S–S, oxidized form.

serine prior to engineering the disulfide bond at the designated sites.

Protein engineering for disulfide bond

The plasmids for the double-cysteine mutants were constructed by site-directed mutagenesis, and the recombinant mutant proteins were expressed in *E. coli*. The six proteins were expressed in a sufficient quantity for further purification. Oxidation of the two cysteines in each mutant protein was carried out in the presence of 1 mM GSSG. When a double-mutant was oxidized and analyzed by nonreducing SDS-PAGE, another protein band was detected that migrated faster than the reduced form. For all double-mutants, the oxidized form migrated faster than the corresponding free thiol form in non-reducing SDS-PAGE (Fig. 2). The yield of disulfide bond formation was also determined by densitometric analysis and titration of free thiols using the thiol and sulfide quantitation kit (Molecular Probes). The yield of the oxidized form was $>$ 80% for all the constructs except K168C–V337C, which yielded $\sim$ 50% of the disulfide-bonded form (Table 1). The oxidized form of K168C–V337C was separated from the reduced form through ion-exchange chromatography with a yield of 98% purity. Mass-spectrometric analysis was carried out to confirm that the fast-migrating species truly contained the engineered disulfide bond. The sequence and the position of disulfide bond were confirmed by tandem mass spectrometry (results are in the Supplemental material).

Activity assay of disulfide-locked α_1 -ATs

To analyze the effect of disulfide locking on the function of α_1 -AT, we measured the inhibitory activities for all six double-mutants in both reduced and oxidized states.

Figure 2. Nonreducing SDS-PAGE analysis of α_1 -AT variants. Four representative double-cysteine mutants were analyzed on nonreducing SDS-polyacrylamide gel. Lanes 1,2, K168C–F189C; lanes 3,4, I169C–Y187C; lanes 5,6, K168C–V337C; lanes 7,8, V185C–V333C; lanes 1,3,5,7, reduced form; lanes 2,4,6,8, oxidized form.

According to the position of disulfide bond, the disulfide pairs can be grouped into three categories: (1) K168C–F189C and I169C–Y187C, in which disulfide connects thFs3A to s3A; (2) K168C–V337C, L172C–V333C, and V173C–K331C, in which disulfide connects thFs3A to s5A; and (3) V185C–V333C, in which disulfide links s3A and s5A (Table 1).

The first group of proteins showed no significant changes in inhibitory activities upon formation of the disulfide bond (Fig. 3; Table 1). The association rate (k_{ass}) of the inhibitors with porcine pancreatic elastase was not affected significantly either by the amino acid substitutions or by disulfide formation (WT, $2.4 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$; reduced K168C–F189C, $2.9 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$; oxidized K168C–F189C, $2.7 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$; reduced I169C–Y187C, $2.3 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$; oxidized I169C–Y187C, $2.5 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$). However, the second group of proteins showed significant loss of inhibitory activity by disulfide locking, as revealed by the completely substrate-like behavior of the oxidized form (Fig. 3; Table 1). For example, the K168C–V337C protein in the reduced state had 56% activity compared to the wild type, but its activity decreased significantly to <1% after disulfide locking. When the disulfide bond was reduced with dithiothreitol, the inhibitory activity was recovered to the original level (55% of the wild-type activity), suggesting that loss of activity is mainly attributed to disulfide locking and not to some other effect such as protein denaturation (Fig. 3; Table 1). Disulfide locking also impaired the inhibitory activity of the third group, V185C–V333C; the reduced form already showed activity loss (34% of the wild-type activity), but the activity decreased even further by disulfide locking (Table 1).

Heat stability of the disulfide-locked α_1 -ATs

In order to test the role of hF/thFs3A region in gate-keeping against loop-sheet polymerization, stability

against heat-induced aggregation was measured. Based on the peptide insertion experiment that mimics the sequence of RSL, previous studies showed that the molecular mechanism of heat-induced aggregation of α_1 -AT was loop-sheet polymerization (Lomas et al. 1992; Kwon et al. 1994; Kim et al. 1995; Dafforn et al. 1999). With the two proteins whose functional activity was not affected by disulfide locking, heat-induced aggregation was measured after incubation at 60°C for a defined time by monitoring disappearance of monomeric form in non-denaturing PAGE. As shown in Figure 4, disulfide locking in the two mutant forms retarded the disappearance of the monomeric form significantly. In particular, the monomeric band of the oxidized K168C–F189C remained up to 240 min, while that of the reduced K168C–F189C was nearly gone after 30 min. The measured remaining activity (Fig. 5) correlated well with the amount of remaining monomeric form observed in non-denaturing PAGE.

Serpin polymerization was shown to consist of two steps: a monomeric conformational change followed by self-association (James and Bottomley 1998; Dafforn et al. 1999). In order to assess the effect of disulfide locking on the individual steps during polymerization, binding of bis-ANS by α_1 -AT was measured under heat-induced denaturing conditions. Since the two oxidized mutant proteins have

Figure 3. Activity assay of α_1 -AT variants. A fixed amount of PPE was mixed with varying concentrations of each α_1 -AT mutant, and the residual protease activity was measured with *N*-succinyl-(Ala)₃-*p*-nitroanilide as a substrate. Inhibited fraction of the protease activity vs. molar ratio of α_1 -AT to PPE is plotted. (Inset) Plot of inhibitory activity of K168C–V337C with disulfide bond shown with extended x-axis. Slope values were derived from the plot by linear regression analysis, and the inhibitory activity of each mutant was calculated as $\text{slope}_{\text{mutant}}/\text{slope}_{\text{wild-type}}$. The resultant activity values are summarized in Table 1. $\diamond$, wild type; $\bullet$, reduced form of K168C–F189C; $\circ$, oxidized form of K168C–F189C; ∇ , reduced I169C–Y187C; $\square$, oxidized I169C–Y187C; $\blacksquare$, reduced K168C–V337C; $\square$, oxidized K168C–V337C; $\triangle$, oxidized K168C–V337C that was re-reduced with DTT.

Figure 4. Heat stability of wild-type and engineered proteins monitored by non-denaturing PAGE. K168C-F189C and I169C-Y187C proteins in their reduced and oxidized forms as well as the wild-type protein were incubated at 60°C. At each designated time point, an aliquot was removed and analyzed by non-denaturing PAGE. Wild-type and reduced proteins show two bands on non-denaturing PAGE, while oxidized proteins that have no free sulfhydryl group showed one band. This can be explained by the fact that the pK_a value of the sulfhydryl group on the cysteine residue ($\sim$ pH 8.3) is close to the pH value of the separating condition (pH 8.8).

quite different heat stability at 60°C (Fig. 4), two distinct denaturation temperatures were applied for each mutant (50°C for I169C-Y187C and 65°C for K168C-F189C). Figure 6A shows that bis-ANS fluorescence of the wild type and mutant α_1 -AT showed biphasic changes during heat denaturation. In comparison to the wild-type kinetics, I169C and Y187C substitutions by themselves did not alter

the kinetics of both phases significantly (Fig. 6A; $0.023 \text{ min}^{-1} \rightarrow 0.028 \text{ min}^{-1}$ for the first phase and $3.31 \times 10^{-3} \text{ min}^{-1} \rightarrow 2.59 \times 10^{-3} \text{ min}^{-1}$ for the second phase; data summarized in Table 2). The disulfide locking in I169C-Y187C retarded the first phase only slightly ($0.028 \text{ min}^{-1} \rightarrow 0.024 \text{ min}^{-1}$) and retarded the second phase by twofold ($2.59 \times 10^{-3} \text{ min}^{-1} \rightarrow 1.37 \times 10^{-3} \text{ min}^{-1}$). The K168C and F189C substitutions by themselves did not alter the kinetics of both phases significantly at 65°C (Fig. 6B; Table 2; $0.738 \text{ min}^{-1} \rightarrow 0.708 \text{ min}^{-1}$ for the first phase and $1.27 \times 10^{-2} \text{ min}^{-1} \rightarrow 9.87 \times 10^{-3} \text{ min}^{-1}$ for the second phase). The disulfide locking in K168C-F189C, however, retarded both phases significantly ($0.708 \text{ min}^{-1} \rightarrow 0.051 \text{ min}^{-1}$ for the first phase and $9.87 \times 10^{-3} \text{ min}^{-1} \rightarrow 3.87 \times 10^{-4} \text{ min}^{-1}$ for the second phase).

Discussion

Conformational change of the proteins associated with functional regulation has been of great interest in the studies of protein structure and design. Serpins have provided insights into the process because the metastable native form undergoes a dramatic conformational transition to a stable state when they form a tight complex with a target protease (Huber and Carrell 1989; Stein and Carrell 1995; Huntington et al. 2000). Our previous studies on stabilizing mutations of α_1 -AT revealed that, while the metastability, caused by side-chain overpacking, cavities, and surface hydrophobic pockets, is distributed throughout the molecule, the interactions critical to the activity were confined in the regions that are mobilized during the complex formation with a target

Figure 5. Heat stability of wild-type and engineered proteins monitored by inhibitory activity. The same aliquot as in Fig. 4 was used for activity measurement. Inhibitory activity at each time point relative to the activity at zero time of each protein is plotted as a function of incubation time: $\diamond$, wild type; $\bullet$, reduced form of K168C-F189C; $\circ$, oxidized form of K168C-F189C; $\blacktriangledown$, reduced I169C-Y187C; $\triangledown$, oxidized I169C-Y187C.

Figure 6. Bis-ANS binding of wild-type and engineered proteins. (A) The fluorescence change of bis-ANS followed by loop-sheet polymerization process of α_1 -AT was monitored. (B) The excitation (bandwidth 5 nm) and emission (bandwidth 5 nm) wavelengths used were 420 and 485 nm, respectively. All proteins were incubated in 50 mM Tris-HCl, 50 mM NaCl (pH 8.0), containing bis-ANS.

enzyme (Lee et al. 1996; Seo et al. 2000). One of the regions various studies have focused on is the hF/thFs3A region, which appears to be an obstacle during the RSL insertion into the β -sheet A. In the present study, the mechanistic role of hF/thFs3A during protease inhibition was investigated by introducing several disulfide bonds that could restrict the movement of thFs3A away from s3A or s5A of β -sheet A.

The disulfide locking of thFs3A to s5A (group 2 mutants, K168C–V337C, L172C–V333C, and V173C–K331C) impaired the inhibitory activity, but the release of the lock restored the original activity (Fig. 3; Table 1). These results suggest that restriction of the movement of hF/thFs3A relative to s5A blocks the insertion of RSL into β -sheet A. In contrast, the engineered disulfide bonds between thFs3A and s3A (group 1 mutants, K168C–F189C and I169C–Y187C) showed little effect on inhibitory activity of α_1 -AT (Table 1). Locking the β -sheet A between s3A and s5A by disulfide (group 3 mutant,

V185C–V333C) affected inhibitory activity severely (Table 1), which was expected because RSL has to be inserted between the two strands to form a new strand, s4A. The result shows that the disulfide locking method is an effective way of preventing conformational change. Taken together, our results suggest that temporal opening of hF/thFs3A from s5A, but not from s3A, is necessary for inhibition.

Previous studies have suggested that hF/thFs3A plays an essential role in the protease inhibition mechanism (Gooptu et al. 2000; Cabrita et al. 2002, 2004). It was speculated that the movement of hF/thFs3A controls the early conformational change (Cabrita et al. 2002, 2004; Tsutsui et al. 2006). These proposals were based on the interpretation of serpin structures, which were probed by crystallography, denaturant-induced equilibrium unfolding, or hydrogen-deuterium exchange under unfolding conditions. In addition, Gettins hypothesized that full displacement of the whole hF/thFs3A region and its subsequent return during protease translocation were essential for RSL insertion and the final protease crushing (Gettins 2002). In our current study, however, we showed through a direct experimental approach that such a drastic detachment of hF is seemingly unnecessary for protease inhibition, although the possibility of local unfolding could not be excluded.

The current results are consistent with our previous data on the structure–activity relationship of single-point mutations. For the stabilizing mutations located at s5A and facing toward thFs3A (substitutions at Lys331 and Lys335), their stabilizing effect was correlated with activity loss (average relative activity = 0.65 ± 0.11 of the wild-type activity; Im and Yu 2000). Tighter interactions between s5A and thFs3A caused by the mutations seem to interfere with the insertion of RSL. However, stabilizing mutations located on hF (K163T, G164V, and T165S) and s3A

Table 2. Rate constants for the kinetic parameters of bis-ANS binding during heat-induced denaturation

Protein	Rate (min^{-1}) ^a			
	First phase	Folds retardation ^b	Second phase	Folds retardation ^b
Temperature (50°C)				
Wild type	0.023	—	3.31×10^{-3}	—
Reduced I169C–Y187C	0.028	1.0	2.59×10^{-3}	1.0
Oxidized I169C–Y187C	0.024	1.2	1.37×10^{-3}	1.9
Temperature (65°C)				
Wild type	0.738	—	1.27×10^{-2}	—
Reduced K168C–F189C	0.708	1.0	9.87×10^{-3}	1.0
Oxidized K168C–F189C	0.051	13.9	3.87×10^{-4}	25.5

^aRate constants were determined by kinetic analysis described as previously reported (James and Bottomley 1998).

^bRetardation by disulfide locking.

(A183V, A183I, F189I, and F189V) did not cause activity loss (average relative activity = 0.98 ± 0.07 of the wild-type activity); (Im et al. 1999; Seo et al. 2000). These results suggest that mobilization between hF and s3A is not critical in the inhibitory activity. The average *B* factor of thFs3A (E166–D179) is 47.0, while that of hF (D149–T165) is 39.5 (PDB entry 1QLP). In addition, it was reported that the hydrogen/deuterium exchange rate of thFs3A, which is comparable to that of RSL, is faster than that of hF (Tsutsui et al. 2006). Our results are consistent with all of these previous structural studies. It is likely that RSL glides through the gap between s5A and thFs3A during complex formation with a target protease.

The hF/thFs3A region was considered critical in blocking the loop–sheet polymerization (Bruce et al. 1994; Vleugels et al. 2000; Wilczynska et al. 2003; Cabrita et al. 2004). Loop–sheet polymerization, which has been reported for several natural mutants of serpin, is a unique molecular interaction between RSL of one molecule and the gap in the β -sheet A of another (Aulak et al. 1993; Bruce et al. 1994; Gooptu et al. 2000). Typically, the Z mutation of α_1 -AT makes the protein very prone to the loop–sheet polymerization, which impedes the folding and secretion of α_1 -AT from liver cells into blood (Lomas et al. 1992; Yu et al. 1995). Various studies showed that molecular mechanism of heat-induced aggregation of serpins is indeed loop–sheet polymerization (Lomas et al. 1992; Kim et al. 1995; Chang et al. 1997; Dafforn et al. 1999). Interestingly, the two disulfide mutants (K168C–F189C and I169C–Y187C), whose activities and association rate constants are little affected by disulfide locking (Fig. 3; Table 1), showed a substantial enhancement in heat stability upon disulfide locking (Figs. 4, 5). The results indicate that disulfide locking between s3A and thFs3A retards loop–sheet polymerization while it has little effect on loop insertion into its own β -sheet during inhibition process.

Several studies further suggested that, based on the biphasic changes of bis-ANS fluorescence during heat-induced denaturation, the process of loop–sheet polymerization consists of two steps: an initial opening of β -sheet A of the molecule itself, and a following step of loop insertion into β -sheet A of another molecule (James and Bottomley 1998; Dafforn et al. 1999). Disulfide locking of K168C–F189C and I169C–Y187C showed stabilization effects in both phases but more prominently toward the second phase (Table 2). The stabilization effects of disulfide locking as measured by bis-ANS binding kinetics (Fig. 6; Table 2) are qualitatively consistent with those monitored by non-denaturing PAGE (Fig. 4). Interestingly, the disulfide locking of K168C–F189C, which showed a substantial stabilization effect on the first phase of bis-ANS binding (Fig. 6B), did not affect the activity (Fig. 3; Table 1). These results indicate that the first phase of hydrophobic dye binding in heat denaturation does not reflect the conforma-

tional change of the molecule during the inhibition process. The results also imply that the basis of loop insertion during the complex formation is different from that during loop–sheet polymerization, as previously suggested (Cabrita et al. 2004). These results are consistent with hF/thFs3A exerting an obstructing effect on insertion of RSL from another molecule but being ready for opening from s5A for the RSL insertion during inhibitory complex formation.

The disulfide engineering of K168C–F189C mutant α_1 -AT turned out to be a very useful outcome in practical respects. Protein engineering through mutational approaches, such as single amino acid substitutions and disulfide bridges, has been effectively applied to improve protein properties for industrial or medical purposes (Reiter et al. 1996; Hugo et al. 2002; Eijnsink et al. 2004; McHugh et al. 2004). Patients with α_1 -AT deficiency require replacement therapy with available protein (Abusriwil and Stockley 2006). The oxidized form of K168C–F189C turned out to be a very successful engineering result, in the sense that detrimental loop–sheet polymerization was abrogated while inhibitory activity was affected little. This case of protein engineering is expected to be of considerable value in replacement therapy of α_1 -AT without risk of possible viral contaminations.

In conclusion, our results suggest that displacement of hF/thFs3A from s5A but not from s3A is required for formation of a stable complex of a serpin with a target protease in inhibitory processes. It is likely that the conformational change of hF/thFs3A involves a full detachment of thFs3A away from s5A while keeping a substantial portion of the interactions between hF and s3A intact. Restricting the movement of thFs3A from strand s3A appears to reinforce its gatekeeping role against loop–sheet polymerization. It may be that the RSL of α_1 -AT glides from the side through the gap between s5A and thFs3A for its full insertion into β -sheet A during inhibitory complex formation.

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