Probing the Unfolding Pathway of α₁-Antitrypsin*

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Protein misfolding plays a role in the pathogenesis of many diseases. α₁-Antitrypsin misfolding leads to the accumulation of long chain polymers within the hepatocyte, reducing its plasma concentration and predisposing the patient to emphysema and liver disease. In order to understand the misfolding process, it is necessary to examine the folding of α₁-antitrypsin through the different structures involved in this process. In this study we have used a novel technique in which unique cysteine residues were introduced at various positions into α₁-antitrypsin and fluoroescence labeled with N,N'-dimethyl-N-(iodoacetyl)-N'-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-ethylenediamine. The fluorescence properties of each protein were studied in the native state and as a function of guanidine hydrochloride-mediated unfolding. The studies found that α₁-antitrypsin unfolded through a series of intermediate structures. From the position of the fluorescence probes, the fluorescence quenching data, and the molecular modeling, we show that unfolding of α₁-antitrypsin occurs via disruption of the A and C β-sheets followed by the B β-sheet. The implications of these data on both α₁-antitrypsin function and polymerization are discussed.

Protein folding is the process by which the primary sequence is translated into tertiary structure. Modification of this sequence via in vivo mutation or in vitro mutagenesis often alters the ability of the protein to fold correctly. Misfolding of proteins during their synthesis within the cell can lead to loss of protein function (1), an example being α₁-antitrypsin (α₁-AT) deficiency (2–4). Many well characterized α₁-AT variants are associated with an α₁-AT plasma deficiency; in these cases, the variants aggregate at their site of synthesis within the liver cell (5, 6). This aggregation is via an ordered polymerization process that is initiated by protein misfolding. In the case of the α₁-AT Z mutation (Glu-342 → Lys), the rate of protein folding is much slower than in the native state, leading to the accumulation of an intermediate, which then polymerizes (4). It has recently been shown that the heat-induced polymerization of α₁-AT also involves the formation of an unfolding intermediate (7). The actual process of polymerization has been well studied, and two mechanisms have been proposed: the loop-A-sheet and loop-C-sheet mechanisms (8–11). Both processes involve the insertion of the reactive center loop residues of the donating α₁-AT molecule into either the A β-sheet (loop-A-sheet) or the C β-sheet (loop-C-sheet) of the acceptor molecule (11). There is evidence for the occurrence of both of these mechanisms; indeed, in vitro α₁-AT has been shown to undergo both depending upon the buffer used (12–14).

α₁-AT is a member of the serine proteinase inhibitor (serpin) superfamily and is composed of 394 amino acid residues arranged into three β-sheets (A, B, and C) and nine α-helices (A–I). X-ray crystallographic and biochemical data have shown that α₁-AT can adopt a number of different structures (15–17). These data demonstrate that areas such as the reactive center loop and the A β-sheet of α₁-AT are extremely mobile, which is an essential requirement for inhibitory activity. Mutations that affect the mobility of α₁-AT cause it to become either a proteinase substrate or to undergo polymerization (16, 18–20). Although many studies have examined the structural changes of a serpin in relation to inhibitory mechanism, the conformational changes, which occur during folding and misfolding, have not yet been characterized.

The unfolding pathway of α₁-AT has been investigated using size exclusion chromatography, intrinsic fluorescence, circular dichroism, and urea gradient gel electrophoresis (21–23). These studies revealed the presence of multiple intermediates, the conformations of which have never been examined. A number of methods have been employed for other proteins to study the conformational changes during folding. The formation of disulfide bonds has been widely used to trap folding intermediates (24), but α₁-AT contains only a single cysteine residue. NMR methodology has been used to examine the rate of exchange between backbone amide protons of the folding protein and the solvent (25); however, due to the large size of α₁-AT, this method is not applicable. Another commonly adopted technique involves the insertion of unique tryptophan residues (26, 27). The changes in tryptophan residue fluorescence upon folding are then followed spectrophotometrically, yielding information about the events occurring at specific locations within the protein structure. α₁-AT possesses two tryptophan residues, one of which (Trp-194) is highly conserved and essential for activity (3). Therefore, the insertion of additional tryptophan residues would lead to a mixed population.

In order to study the folding of α₁-AT, we propose a novel approach in which we have fluorescently labeled unique cysteine residues that have been inserted at positions throughout the tertiary structure of the molecule. The fluorescence properties of these unique groups were then examined as a function of denaturant concentration. The results demonstrate that the equilibrium unfolding pathway of α₁-AT consists of at least one stable intermediate structure, which we have begun to characterize.
EXPERIMENTAL PROCEDURES

Materials—1,1’-Bis-4-anilino-5-naphthalenesulfonic acid (dipotassium salt) and N,N’-dimethyl-N-(iodoacetyl)-N’-7-nitrobenz-2-oxa-1,3-diazol-4-ylidenediamine (IANBD) were purchased from Molecular Probes Inc. (Eugene, OR). Ultrapure grade guanidine hydrochloride (Gdn HCl) was obtained from ICN Biochemicals.

Production of Proteins—The recombinant α1-AT proteins were expressed in E. coli strain BL21 (DE3) as described previously (28). The proteins were purified from cell inclusion bodies using Gdn HCl denaturation and a continual dilution procedure as recently described (29). In this study recombinant α1-AT-P1-Pro-Arg was used as the control and the construction of α1-AT(S313C), and α1-AT(P3Ser) have been described (12). The protein concentration was determined by Bradford assay using a standard curve of purified recombinant α1-AT; the concentration of which had been determined by amino acid analysis. The association rate constant (k_on) and stoichiometry of inhibition were measured using thiorham for both the unlabeled and labeled α1-AT forms, as described previously (30). Fluorescent labeling of the proteins with IANBD was performed as described previously (12). The extent of labeling was determined using the extinction coefficient of IANBD (ε = 25,000 M⁻¹ cm⁻¹).

Spectroscopic Methods—Fluorescence emission spectra were recorded on a Perkin-Elmer LS50B spectrophotometer, using a thermostatted cuvette holder at 25 °C in a 1-cm path length quartz cell. Excitation and emission slits were set at 2.5 nm for all spectra, and a scan speed of 10 nm/min was used. The absorbance at the excitation wavelengths was monitored in all experiments and remained below 0.05. Circular dichroism spectra were measured on a Jasco 720S spectropolarimeter at 25 °C. Far UV spectra from 190 to 250 nm were collected with 5°/point signal averaging; baseline measurements were made with the signal averaged over 60 s. The concentration of α1-AT used was 0.1 mg/ml with a 0.1-cm path length cell.

Denaturation Transitions—Unfolding curves were determined by either measuring the fluorescence emission spectra of a 200 μM protein solution as a function of denaturant concentration or the change in signal at 222 nm in the far UV as a function of denaturant concentration. Attempts at measuring the near UV CD unfolding curve proved impractical due to protein precipitation at the concentrations required. Previous studies (31) and our own preliminary data have shown that unfolding in Gdn HCl reaches equilibrium within a matter of minutes at all concentrations of Gdn HCl used. Therefore, the samples were allowed to equilibrate at 25 °C for 2 h before measurement. For both the labeled and unlabeled proteins, the reversibility of the unfolding process was established by adjusting buffer to samples of unfolded protein, allowing each to re-equilibrate at a lower Gdn HCl concentration. The fluorescence emission spectra acquired for these samples, when corrected for dilution, were identical with corresponding samples that had reached equilibrium from the folded state, thus confirming the reversibility of the systems. The inhibitory activity of these refolded proteins was also analyzed, and they were found to be fully active.

Unfolding Data Analysis—The Gdn HCl denaturation curves were fitted to either a two-state or three-state unfolding model, using a nonlinear least-squares fitting algorithm. Raw equilibrium data were fitted directly to a two-state model of denaturation in which the baseline lines are fitted simultaneously with the unfolding transition (32). A three-state model was also employed for the analysis of the denaturation curves based upon the mechanism F = I + U, where F and U are the fully folded and unfolded forms of the protein, respectively, and I is an intermediate that is populated at equilibrium (33).

Fluorescence Quenching Experiments—Fluorescence quenching measurements were performed in 50 mM Tris, pH 8.0, at 0 and 1 mM Gdn HCl in a solution of KI (2 M stock) containing 1 mM Na₂S₂O₃. IANBD was added to protein solutions (200 μM), and the change in fluorescence emission intensity of the tryptophan residues (λ_em = 290 nm) and covalently bound IANBD (λ_em = 480 nm) were measured. Measurements in the presence of I KI or Gdn HCl were made on individual samples, so there was no dilution of the Gdn HCl. The data were analyzed as described previously by Lehrer (34). All data were corrected for inner filter effects where necessary.

RESULTS

Fluorescence Properties of α1-AT and Its Variants—The aim of this study was to monitor the structural changes of α1-AT as it unfolds. To achieve this, we generated α1-AT variants, which were fluorescently labeled at specific sites on the protein. The structural changes occurring in the unfolding process were then characterized using the site-specific fluorescence changes.

α1-Antitrypsin Unfolding

FIG. 1. A schematic of native α1-AT in two orientations. A, front view of antitrypsin, with Trp-194, Trp-238, Pro-361 (P3′), Cys-232, and Ser-313 in Van der Waals spheres and labeled. B, side view, highlighting the position of Cys-232. In both A and B, the P1/P′1 is shown in ball and stick form. This figure was produced using Molscript (47).

α1-AT possesses a unique cysteine residue (Cys-232), which is situated on strand s2B (Fig. 1). Two further cysteine residues were introduced into α1-AT at positions P3′ (P3′) and Ser-313 in Van der Waals spheres and labeled. The substitutions were made using an α1-AT variant, in which Cys-232 had been replaced by a serine residue. This variant has previously been shown to have the same activity as the native protein (12, 28). The native and mutant constructs were efficiently expressed as inclusion bodies and purified as described previously (16). The two mutations (P3′ Cys and S313C) had no effect upon the inhibitory properties of the proteins (Table I). These cysteine residues were then selectively labeled with the fluorescent probe IANBD. The extent of modification was measured using the extinction coefficient of the probe and was found to be 1:1 in all cases; this also confirmed the insertion of one unique cysteine residue per monomer. The presence of the fluorescent label had no effect upon the inhibitory properties of the protein (Table I).

The addition of the IANBD label affords the protein new fluorescence properties. The fluorescence emission spectra of the three labeled proteins show distinct differences depending upon the location of the probe within the α1-AT structure (Fig. 2). The fluorescently labeled wild type protein (α1-AT, Cys-232-IANBD) had an emission maximum at 531 nm, whereas the two other proteins exhibited emission maxima at 527 nm (α1-AT, S313C-IANBD) and 532 nm (α1-AT, P3′ Cys-IANBD) (Fig. 2). These spectra indicate that the environment of the fluorescent probes at Cys-232 and P3′ Cys are more solvent-exposed than the probe at Cys-313. Although the labeled proteins were fully active (Table I), there was the possibility that the presence of the IANBD altered the structure of the native state. To examine this, we carefully measured the tryptophan emission spectra and far UV CD spectra for all labeled and unlabeled proteins. Both the tryptophan emission spectra and far UV CD spectra for the three labeled proteins were identical to the unlabeled fluorescence and CD spectra (data not shown). These data, in conjunction with the full inhibitory activity of the proteins, demonstrate that the mutations and the covalent modification had no significant effect upon the native structure of the proteins.

Unfolding Studies—The fluorescence characteristic of these novel α1-AT variants allows the solvent induced denaturation of α1-AT to be followed spectrofluorometrically. The fluorescence properties of both the tryptophan residues and the covalently attached probe were monitored following treatment

The nomenclature for residues within the reactive center loop is based on that described by Schechter and Berger (46) for the substrates of proteases.
with various concentrations of Gdn HCl. Incubation of the proteins in 6 mM Gdn HCl resulted in the emission maximum of the tryptophan residues (for all three proteins) becoming red shifted from 337 to 352 nm, concomitant with an increase in fluorescence emission intensity (Fig. 3A). This demonstrates that the tryptophan residue fluorescence is heavily quenched in the native structure. The emission spectra of the probes under the same conditions showed a red shift to 538 nm and a decrease in fluorescence intensity (Fig. 3B).

Table I: Association rate constants, stoichiometry of inhibition and Stern-Volmer constants for the α1-AT variants

<table>
<thead>
<tr>
<th></th>
<th>k_{SV} × 10^4</th>
<th>SI</th>
<th>Native</th>
<th>Intermediate</th>
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<tr>
<td>α1-AT</td>
<td>3.3 ± 0.1</td>
<td>0.5</td>
<td>3.24</td>
<td>2.70</td>
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<td>α1-AT(P1→Arg)</td>
<td>4.8 ± 0.1</td>
<td>1.1</td>
<td>4.24</td>
<td>6.78</td>
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<tr>
<td>α1-AT(P3→Cys)</td>
<td>3.2 ± 0.1</td>
<td>0.5</td>
<td>3.18</td>
<td>10.95</td>
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<tr>
<td>α1-AT(P3→Cys-IANBD)</td>
<td>5.0 ± 0.1</td>
<td>1.2</td>
<td>4.54</td>
<td>8.51</td>
</tr>
</tbody>
</table>

Fig. 3. Native and denatured fluorescence emission spectra of α1-AT variants. A, Trp emission spectra (λ_{ex} = 290 nm) of native (-----) and denatured (-------) (6 M Gdn HCl) α1-AT(S313C-IANBD), probe emission spectra λ_{ex} = 480 nm of the native (-----) and denatured (-------) α1-AT(S313C-IANBD). B, Fluorescence emission spectra of the labeled variants and unlabeled α1-AT with various concentrations of Gdn HCl. Incubation of the proteins in 6 mM Gdn HCl resulted in the emission maximum of the tryptophan residues (for all three proteins) becoming red shifted from 337 to 352 nm, concomitant with an increase in fluorescence emission intensity (Fig. 3A). This demonstrates that the tryptophan residue fluorescence is heavily quenched in the native structure. The emission spectra of the probes under the same conditions showed a red shift to 538 nm and a decrease in fluorescence intensity (Fig. 3B).

Fig. 3 shows the normalized changes in λ_{max} as a function of denaturant concentration for both the tryptophan residues and the probes. There was little change in the λ_{max} from the tryptophan residues below 1 M Gdn HCl for all the variants. Between 1 and 3.5 M Gdn HCl, λ_{max} increased (Fig. 4A), indicating an increase in solvent exposure of the tryptophan residues as the protein unfolds. The spectral changes associated with denaturation of the modified proteins were unique to the probe position (Fig. 4B). α1-AT(P3→Cys-IANBD) showed no change in emission maximum λ_{max} = 531 nm) until the concentration of Gdn HCl reached 2 M. This is a 2-fold increase in Gdn HCl concentration compared with the concentration required to initiate changes in the tryptophan residue fluorescence. The fluorescence changes of α1-AT(P3→Cys-IANBD) and α1-AT(S313C-IANBD) occurred at much lower Gdn HCl concentrations (< 0.5 M).

The labeled variants and unlabeled α1-AT exhibited the same tryptophan fluorescence change as a function of Gdn HCl concentration, with a midpoint of 2.5 M Gdn HCl (Fig. 4A, Table II). Analysis of these data using a two-state folding model allows calculation of ΔG_{U→F}, (the free energy of unfolding in water) and m (a constant that is proportional to the increase in degree of exposure of the protein on denaturation) (Table II). It can be seen that ΔG_{U→F} is the same for both the unlabeled and labeled proteins, with an average value of 2.22 ± 0.03 kcal/mol. In addition, there is no significant difference in m with an average value of 0.885 ± 0.02 kcal/mol. The coincidence of these labeled and unlabeled data suggests that the fluorescent probes do not perturb the equilibrium-folding pathway.

The probe fluorescence changes are nonsuperimposable (Fig. 4B), implying that the probes are reporting site specific conformational changes. The data in Fig. 4B were analyzed by either a two-state or three-state mechanism as described previously (25, 35). It was found that the data for α1-AT(S313C-IANBD) and α1-AT(Cys-232-IANBD) could be adequately described by the two-state analysis, whereas α1-AT(P3→Cys-IANBD) could only be described using a three-state analysis. The data in Table II and Fig. 4B show that α1-AT(Cys-232-IANBD) has the highest denaturation midpoint (2.8 M Gdn HCl), compared with the tryptophan observed transition (2.5 M Gdn HCl). This indicates that the regions around the tryptophan residues and Cys-232 are significantly more resistant to denaturation than the regions around P3‘Cys and Cys-313. The denaturation midpoint for
midpoint of the second transition is 2.5 M Gdn HCl. This is probe fluorescence emission maximum \((\lambda_{em})\) secondary structure; therefore, deviation in the unfolding profiles are due to local destabilizing effects of the probe. To the probe fluorescence change of "Experimental Procedures." A two-state model was used for all fits except for the two-state and three-state curves fitted as described under "Exper-

A

![Graph A](image)

\(F_{app}\) versus denaturant concentration as described previously (33). A. unfolding was monitored by changes in the Trp residue fluorescence emission maximum \((\lambda_{em} = 350 \text{ nm})\). B. unfolding was monitored by changes in the probe fluorescence emission maximum \((\lambda_{em} = 480 \text{ nm})\). The lines show the two-state and three-state curves fitted as described under "Experimental Procedures." A two-state model was used for all fits except for the probe fluorescence change of \(\alpha_1\)-AT(Cys-232-IANBD) where a three-state model was employed.

\(\alpha_1\)-AT(Cys-313-IANBD) is 1.7 M, over 1 M lower than the value obtained for \(\alpha_1\)-AT(Cys-232-IANBD). The midpoint for the first transition of \(\alpha_1\)-AT(P3-Cys-IANBD) is 0.4 M Gdn HCl, whereas the midpoint of the second transition is 2.5 M Gdn HCl. This is similar to the values obtained from the tryptophan residue and \(\alpha_1\)-AT(Cys-232-IANBD) analysis, suggesting that they may be monitoring the same transition. These data also imply that the regions around both P3 Cys and Cys-313 are relatively unsta-

In contrast, the region around Cys-232 and the tryptophan residues unfold at higher Gdn HCl concentrations, demonstrat-
ing an increased stability compared with the other regions. The sequential spectral changes demonstrate that \(\alpha_1\)-AT unfolding is not a simple two-state process, in which only the fully folded and unfolded states exist. In this case, there appears to be at least one intermediate state, and the protein only becomes fully unfolded at concentrations above 5 M Gdn HCl.

It is possible that the differences in probe monitored unfolding profiles are due to local destabilizing effects of the probe. To assess this, the unfolding curves for the labeled protein were measured using far UV CD (Fig. 5). Far UV CD measures secondary structure; therefore, deviation in the unfolding pro-

B

![Graph B](image)

file from native will indicate that the probes are affecting the unfolding process. However, it can be seen in Fig. 5 that the unfolding profiles for all the labeled proteins are essentially the same as the unlabeled native structure. The far UV CD data show two transitions, with average midpoints of 0.77 and 2.6 M Gdn HCl (Table II). This is similar to the fluorescence changes observed for \(\alpha_1\)-AT(P3-Cys-IANBD); however, the midpoints of the transitions were approximately 0.2 M higher when measured by far UV CD. We cannot discount the possibility that minor local perturbations of the \(\alpha_1\)-AT structure due to labeling may have occurred. However, the coincidence of 1) tryptophan unfolding data (Fig. 4A), 2) far UV CD unfolding data (Fig. 5), 3) tryptophan emission spectra (data not shown), and 4) far UV CD spectra (data not shown) of the labeled proteins compared with native \(\alpha_1\)-AT strongly suggests that the probes do not interfere with the equilibrium unfolding process.

The nonsuperimposable transitions observed with different spectroscopic probes and the biphasic nature of the CD unfolding denaturation curve are consistent with the existence of a stable intermediate at 1 M Gdn HCl. The intermediate in 1 M Gdn HCl possesses approximately 20% of the native CD spectral amplitude at 222 nm (Fig. 5), and the fluorescence data suggest that the environment around P3‘ Cys, S313C, and the two tryptophan residues has been altered (Fig. 4). The structure of this equilibrium intermediate state was further char-

**DISCUSSION**

Unfolding Pathway of \(\alpha_1\)-Antitrypsin—Investigations into the pathways by which globular proteins fold are essential to understanding their structural organization. This information is also critical if we are to understand the molecular basis for protein misfolding diseases. In this study, we have characterized the unfolding pathway of \(\alpha_1\)-AT using a combination of spectroscopic techniques. We have placed the fluorescent probe, IANBD, in unique positions within \(\alpha_1\)-AT to obtain information about the movement of specific structural motifs as the protein unfolds. This was achieved by inserting cysteine residues at selected sites and then covalently modifying them with the cysteine reactive probe IANBD. The positions of the fluorescent probes used in this study are shown in Fig. 1. P3‘ Cys is situated on the first strand of the C \(\beta\)-sheet (s1C) (Fig. 1). Chang et al. (11) immobilized s1C using an engineered disulfide bond and found that polymerization did not occur, indicating that mobility of s1C is essential for this process. Cys-232 is the native cysteine residue and is exposed at the rear of the molecule on the B \(\beta\)-sheet (s2B) (Fig. 1). Cys-313 is located at the base of the serpin molecule on a loop connecting strands 5A and 6A of the A \(\beta\)-sheet (Fig. 1). A detailed structural comparison between native and cleaved \(\alpha_1\)-AT showed that this region moves upon reactive center loop insertion into the A \(\beta\)-sheet (36); therefore, this probe will monitor changes in...
Results from Figs. 4 and 5 were fit to either a two-state or three-state unfolding analysis as described in the materials and methods. \( \Delta G_{F \rightarrow U} \), \( \Delta G_{U \rightarrow I} \), and \( \Delta G_{I \rightarrow U} \) represent the free energy change for the unfolding of the protein in water and the free energy change for the unfolding of the protein in water and the free energy change for the \( F \rightarrow I \) and \( I \rightarrow U \) transitions, respectively. \( m_{F \rightarrow I} \) and \( m_{I \rightarrow U} \) represent the m values for the \( F \rightarrow U \), \( F \rightarrow I \), and \( I \rightarrow U \) transitions respectively. Presented are the two- and three-state analyses of the fluorescence probe data (Fig. 4B) and the two-state analysis of the tryptophan fluorescence data (Fig. 4A) and the three-state analysis of the far-UV CD data (Fig. 5).

### Parameters for the two- and three-state fits to the denaturation transition curves

<table>
<thead>
<tr>
<th>Protein</th>
<th>Two-state analysis</th>
<th>Three-state analysis</th>
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<td></td>
<td>( \Delta G_{F \rightarrow U} ) kcal/mol</td>
<td>( m_{F \rightarrow I} ) kcal/mol</td>
</tr>
<tr>
<td>( \alpha_1\text{-AT}_{(P3\text{-Cys-IANBD})} )</td>
<td>3.34 1.19 2.84</td>
<td>1.39 3.44 0.43 3.12</td>
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<tr>
<td>( \alpha_1\text{-AT}_{(P3\text{-Cys-232-IANBD})} )</td>
<td>3.34 1.19 2.84</td>
<td>4.12 5.54 0.74 6.32</td>
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<td>( \alpha_1\text{-AT}_{(P3\text{-Arg})} )</td>
<td>3.27 0.91 2.49</td>
<td>4.41 5.76 0.76 6.60</td>
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<td>( \alpha_1\text{-AT}_{(P3\text{-Cys-313-IANBD})} )</td>
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<td>4.63 5.84 0.79 6.40</td>
</tr>
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<td>( \alpha_1\text{-AT}_{(P3\text{-Cys-337-IANBD})} )</td>
<td>3.27 0.91 2.49</td>
<td>4.14 5.16 0.80 6.20</td>
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Fig. 5. The far UV CD unfolding profile of labeled and unlabeled \( \alpha_1\text{-AT} \). The changes in far UV signal at 222 nm were measured as a function of Gdn HCl concentration for \( \alpha_1\text{-AT}_{(P3\text{-Arg})} \), \( \alpha_1\text{-AT}_{(P3\text{-Cys-232-IANBD})} \), \( \alpha_1\text{-AT}_{(P3\text{-Cys-313-IANBD})} \), and \( \alpha_1\text{-AT}_{(P3\text{-Cys-337-IANBD})} \). A three-state unfolding model was used for the analysis of the data.

Fig. 6. Stern-Volmer plot for iodide quenching of the tryptophan fluorescence of \( \alpha_1\text{-AT}_{(P3\text{-Cys-IANBD})} \), \( \alpha_1\text{-AT}_{(P3\text{-Cys-232-IANBD})} \) (200 nm) was titrated with KI, and the fluorescence emission intensity (\( \lambda_{ex} = 290 \text{ nm} \); \( \lambda_{em} = 337 \text{ nm} \)) was recorded after each addition. In the presence of 1 M Gdn HCl, KI was added to individual samples so that the Gdn HCl was not diluted. \( \Delta \) represents the states of native \( \alpha_1\text{-AT}_{(P3\text{-Cys-IANBD})} \) and \( \bigcirc \) represents \( \alpha_1\text{-AT}_{(P3\text{-Cys-232-IANBD})} \) in the presence of 1 M Gdn HCl. The lines represent a least-squares fit of the experimental data as described previously (34).

the A \( \beta \)-sheet and in the surrounding regions. The fluorescent properties of the native tryptophan residues (194 and 238) were also used in this study. Trp-194 is at the top of s3A and is sandwiched between the A and B \( \beta \)-sheets, whereas Trp-238 on s3B is partially exposed to the solvent. Previous studies have shown that Trp-194 is the major contributor to the fluorescence properties of \( \alpha_1\text{-AT} \) (3, 37).

Two-state unfolding has been observed in only a few small monomeric proteins (38). It is often characterized by the coincidence of unfolding curves monitored using different spectral probes. Fig. 4B shows that the fluorescence changes are non-superimposable when the different fluorescent probes are used to follow the unfolding pathway. This implies that the probes placed in different positions within \( \alpha_1\text{-AT} \) are reporting local structural changes. Therefore, \( \alpha_1\text{-AT} \) unfolds via a series of structures. It can be argued that the presence of the fluorescence probes themselves affects the \( \alpha_1\text{-AT} \) unfolding pathway. We cannot entirely discount this; however, we have shown that the tryptophan residue and far UV CD spectral changes for the labeled proteins are indistinguishable from the native protein during unfolding. Furthermore, the labeled proteins are fully active as inhibitors. Although small perturbations due to the presence of the probes cannot be ruled out, we believe that the probes are reporting genuine structural changes.

Examination of the x-ray crystal structure of native \( \alpha_1\text{-AT} \) (17) in conjunction with the fluorescence and far UV CD data allows insight into the structural changes as \( \alpha_1\text{-AT} \) unfolds. It is evident that the probes placed at P3\text{-Cys} and Cys-313 are in regions of structure that are significantly less resistant to denaturation than the structural areas around Cys-232 and the tryptophan residues (Fig. 4, A and B). The midpoint of unfolding for both \( \alpha_1\text{-AT}_{(P3\text{-Cys-IANBD})} \) (first transition) and \( \alpha_1\text{-AT}_{(P3\text{-Cys-337-IANBD})} \) (second transition) occurs at significantly lower Gdn HCl concentrations than the corresponding values for \( \alpha_1\text{-AT}_{(P3\text{-Arg})} \) and \( \alpha_1\text{-AT}_{(P3\text{-Cys-232-IANBD})} \). It has previously been shown that conformational changes in the reactive center loop and s1C cause a movement at the base of the serpin (36). Therefore, the initial unfolding event, which occurs at low Gdn HCl concentrations, may represent a movement in the reactive center loop and an opening of the A \( \beta \)-sheet. This would alter the spectral properties of the fluorescence probes at positions P3\text{-Cys} and Cys-313. Conformational changes in these areas would be predicted not to affect the region around Cys-232, and indeed the emission maximum of Cys-232 was unaffected. The circular dichroism of the structure at 1 M Gdn HCl in the far UV (222 nm) is decreased approximately 20% (Fig. 5), suggesting partial unfolding and disruption of some secondary structure. This decrease may represent a local loss of secondary structure as indicated by the changes in the fluorescence of the probes at
positions P3’Cys and Cys-313. There is a small change (< 8%) in the environment of the tryptophan residues at 1 M Gdn HCl. Kwon et al. (37) predicted that the opening of the A β-sheet would make Trp-194 more accessible to solvent and hence alter its fluorescence properties. This was confirmed by the 2-fold increase in the Stern-Volmer constant of the tryptophan residues (Table I). Complete disruption of the A β-sheet has been shown to account for the change in tryptophan fluorescence (37); therefore, the small change we observed at low denaturant concentrations may represent a prelude to this conformational change.

An increase in the denaturant concentration causes further structural changes in the protein that leads to complete unfolding. As a result, the probes at P3’Cys and Cys-313 become fully exposed to solvent, and the major portion of the tryptophan residue fluorescence transition occurs. The final conformational change observed using these probes is the disruption of the area around the B β-sheet (Cys-232). These data demonstrate that the B β-sheet region is the most resistant to denaturant. The pathway of conformational change suggested by these fluorescence data begins with an opening at the top of the A β-sheet and a movement in s1C to form a stable intermediate. This is then followed by the unfolding of the A and C β-sheet regions, which in turn leads to loss of the B β-sheet and formation of the unfolded structure.

α1-AT and other serpins can undergo a conformational switch termed the stressed (native) to relaxed (latent) transition. This transition is crucial to the inhibitory properties of the serpin family (5, 39). The area that holds the molecule in this trapped, stressed state has been located to a region within the hydrophobic core of the molecule (37, 39). Through side chain locking, these core residues prevent the rearrangement of native α1-AT into a more stable but inactive state (39). Our study suggests that the residues that make up this core around the B β-sheet are unaffected by low denaturant concentrations. Therefore, this arrangement is intact in the intermediate structure formed in 1 M Gdn HCl. Once the denaturant concentration is increased, the residues within the core region become disordered and the molecule unfolds completely. These data suggest that regions within the serpin molecule have evolved that are significantly more resistant to denaturant than others; this has implications for both the inhibitory mechanism and polymerization process.

Implications for Serpin Mechanism—Specific parts of the structure of a serpin, such as the reactive center loop and hinge region, are extremely flexible; this mobility is required for their inhibitory function (16, 20, 28). In recent years, the conformational changes that occur within the serpin structure when bound to a proteinase have been intensively studied (40, 41). Recent studies suggest that the proteinase, once docked to the exposed reactive center loop, attempts to cleave the scissile bond, which causes the reactive center loop to insert into the A β-sheet. The extent of this insertion is unknown; however, biophysical studies have shown that the effect is to move the proteinase from the top of the molecule to a position probably on the face of the A β-sheet (40, 41). The unfolding results presented above explain how α1-AT is designed to undergoing these conformational changes. We show here that the regions around the top and bottom of the A β-sheet are less stable than the other parts of the molecule, unfolding at lower Gdn HCl concentrations. Comparison of x-ray crystallographic structures of native and cleaved serpins show that the area around Ser-313 moves upon either full (complete A β-sheet opening) or partial (partial A β-sheet opening) reactive center loop insertion (36). Furthermore, the x-ray crystal structures of latent antithrombin (10) and plasminogen activator inhibitor-1(42) demonstrate the stability of this β-sheet for these conformational changes, and our study shows that this is a far more stable region in the protein (unfolding with a midpoint at 2.8 M Gdn HCl). The data presented herein show that the region around the top of the A β-sheet of α1-AT is more flexible and facilitates movement of the reactive center loop into the A β-sheet once the proteinase is bound.

Implications for Serpin Polymerization—The fluorescence data in this paper showed that the top of the A β-sheet is more flexible than the rest of the molecule, and this is required for correct serpin function. However, if this instability is enhanced due to mutations, this can induce the unwanted effect of polymerization. α1-AT polymerization in vivo leads to a plasma deficiency of the inhibitor and eventually to lung and liver damage (6, 43). A number of mutations have been identified that cause the A β-sheet movement to be disturbed (9, 44, 45). An alternative explanation may be that these mutations alter further the conformational instability of this region, which would lead to open A β-sheet, and therefore increased chances of polymerization and disease.

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