Conformational Change and Intermediates in the Unfolding of α1-Antichymotrypsin

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Serpins are the prototypical members of the conformational disease family, a group of proteins that undergoes a change in shape that subsequently leads to tissue deposition. One specific example is α1-antichymotrypsin (ACT), which undergoes misfolding and aggregation that has been implicated in emphysema and Alzheimer's disease. In this study we have used guanidine hydrochloride (GdnHCl)-induced denaturation to investigate the conformational changes involved in the folding and unfolding of ACT. When the reaction was followed by circular dichroism spectroscopy, one stable intermediate was observed in 1.5 M GdnHCl. The same experiment monitored by fluorescence revealed a second intermediate formed in 2.5 M GdnHCl. Both these intermediates bound the hydrophobic dye ANS. These data suggest a four-state model for ACT folding N → I1 → I2 → U. I1 and I2 both have a similar loss of secondary structure (20%) compared with the native state. In I2, however, there is a significant loss of tertiary interactions as revealed by changes in fluorescence emission maximum and intensity. Kinetic analysis of the unfolding reaction indicated that the native state is unstable with a fast rate of unfolding in water of 0.4 s⁻¹. The implications of these data for both ACT function and associated diseases are discussed.

Conformational disorders, as defined by Carrell and colleagues (1, 2), arise when a protein undergoes a change in shape that leads to self-association, tissue deposition, and disease. The serpin superfamily includes some of the most studied proteins involved in this group of disorders. The misfolding and aggregation of serpins have been implicated in diseases ranging from emphysema and liver disease, to cancer and dementia (3–5). It is, however, extremely surprising that so little is known about the basic folding pathways of this class of protein. A number of studies have shown that α1-antitrypsin unfolds through one intermediate (6–8), and recent studies with maspin (9) and plasminogen activator inhibitor-1 (10) have shown similar results.

The folding pathway of α1-antichymotrypsin (ACT),² which is involved in regulation of several proteinases, including chymotrypsin, cathespin G, and mast cell chymase, has, so far, received no attention. ACT is an acute phase DNA binding glycoprotein that possesses the characteristic serpin tertiary fold, composed of three β-sheets (A, B, and C) surrounded by nine α-helices (A–I) (11, 12). X-ray crystallographic and biochemical data have shown that ACT is capable of adopting several different conformations (12, 13). Cleavage of the reactive center loop (RCL) by noncognate proteinases causes full insertion of the RCL into the A β-sheet, forming an additional β-strand, which confers additional stability to the protein (12).

The folding pathways of small proteins have received immense attention, primarily due to the ease with which data can be obtained and interpreted (16). These studies, for a small number of proteins, have revealed at high resolution how a linear polypeptide chain folds to its unique three-dimensional structure. Large monomeric proteins are not amenable to many of the high resolution techniques such as NMR applied to small proteins. In this study we have used a combination of spectroscopic approaches, including intrinsic tryptophan fluorescence and circular dichroism spectroscopy, to monitor the structural changes that occur within ACT as it unfolds and folds under equilibrium conditions. We have identified two intermediates on the unfolding pathway; the first shows many similarities to that of α1-AT, whereas the other has not been previously identified for any serpin. Kinetic characterization also demonstrates that the serpins are unstable and designed for dramatic conformational change.

**EXPERIMENTAL PROCEDURES**

Materials—Ultrapure grade guanidine hydrochloride (GdnHCl) was obtained from ICN Biochemicals. 8-Anilinonaphthalene 1-sulfonate (ANS) was purchased from Sigma.

Production of Proteins—ACT and α1-AT were purified as described previously (17–19) and were greater than 95% pure, with no polymeric material found upon native gel analysis. Their concentration and activ-
ity were determined as described previously (20, 21). The stoichiometry of inhibition against chymotrypsin was found to be 1, indicating full activity.

**Spectroscopic Methods**—Fluorescence emission spectra were recorded on a Perkin-Elmer LS50B spectrofluorimeter using a thermostatted cuvette 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 wavelength was monitored in all experiments and remained below 0.05. Circular dichroism spectra were measured on a Jasco 810 spectropolarimeter using a thermostatted cuvette at 25 °C. Far UV spectra from 190 to 250 nm were collected with 5 nm/point signal averaging; spectra were made with the signal averaged over 15 s. The concentration of protein used was 0.1 mg/ml with a 0.1-cm path length.

**Chemical Denaturation**—Stock solutions of GdnHCl (7 M) in 50 mM Tris, 50 mM NaCl, pH 7.8, were prepared and filtered through 0.2-μm membranes before use. The GdnHCl concentration was determined by refractive index measurements as described previously (22). Equilibrium unfolding and refolding curves were determined by adding a concentrated solution of either native or denatured protein to a series of GdnHCl concentrations. These solutions were incubated for 2 h at 25 °C before analysis by measuring either the fluorescence emission spectra of the protein solution or the change in signal at 222 nm in the far UV spectra, as a function of denaturant concentration. No differences were observed in experiments where spectroscopic measurements were made after more extensive equilibration.

**Equilibrium Unfolding Analysis**—All unfolding data were fit to a three-state unfolding model, using a nonlinear least-squares-fitting algorithm, as described previously (23, 24). This analysis recognizes the presence of one stable intermediate structure, I, populated during the transition from the folded state (N) to the unfolded state (U). The free energy unfolding in 0 M denaturant, ΔG_{N,I}, and m value for each transition were determined from this analysis. The midpoint, D_m, of each transition was calculated by ΔG_{N,I}/m.

**ANS Binding Studies**—Samples of ACT (200 nM) were incubated in increasing concentrations of GdnHCl in the presence of ANS (1 μM) at 25 °C for 30 min. The resulting fluorescence emission spectrum was then scanned (λ_ex = 390 nm) between 450 and 550 nm.

**Unfolding Kinetics**—Unfolding was monitored by changes in fluorescence at wavelengths >320 nm using a cut-off filter and an excitation wavelength of 290 nm. Experiments were performed on an Applied Photophysics SF.18MV stopped-flow apparatus. Unfolding was performed by rapidly mixing one volume of 10 μM protein solution with 10 volumes of concentrated GdnHCl solution at 25 °C. Both solutions contained 50 mM Tris/HCl, 50 mM NaCl, pH 7.8. Data was collected from at least eight experiments, which were averaged and fitted to a single exponential function with a term included for baseline instability using the manufacturer’s software.

**RESULTS**

**Structural and Spectral Properties of ACT**—The three-dimensional structure of ACT conforms with the typical serpin tertiary structure, consisting of three β-sheets surrounded by nine α-helices (Fig. 1). ACT contains three tryptophan residues, which all contribute differently to the overall fluorescence signal observed for the protein. Trp-194 is one of the most highly conserved residues across the serpin family and is located buried between the A and B β-sheets of the molecule, at the top of strand 3 of the A β-sheet (Fig. 1). Trp-276, located on the H helix, is buried against the B β-sheet, whereas Trp-215 is fully solvent-exposed, located at the end of strand 3 of the C β-sheet.

The normalized fluorescence emission spectrum (λ_ex = 290 nm) of native ACT is shown in Fig. 2, and reveals an emission maximum (λ_{max}) of 335 nm. Increasing concentrations of GdnHCl caused a red-shift of λ_{max} along with a concomitant decrease in emission intensity (Fig. 2A). The far-UV CD spectrum for the native state of ACT at 222 nm (Fig. 2B) similar to other serpins, indicating that the serpin family members adopt the same amount of secondary structure (9). The native spectrum was analyzed using the CONTIN algorithm (25) and gave a predicted secondary structure content of 29% α-helix and 37% β-sheet, which agrees closely with the x-ray structure of ACT and other serpins (7, 9, 11).

**Equilibrium Unfolding**—The aim of this study was to investigate the unfolding and refolding pathways of ACT. Throughout these studies we compared the results with those of α1-AT, whose unfolding pathway has been well characterized by many groups, including our own (6–8). The unfolding profiles for ACT and α1-AT monitored by fluorescence emission maximum (λ_{max}) are shown in Fig. 3A and Fig. 4A, respectively. The profiles for ACT were the same over a concentration range of 0.01–0.1 mg/ml protein, and analysis of various samples by native gel electrophoresis and gel filtration indicated that at these low concentrations no polymeric material was present. Similar concentration dependence is observed for α1-AT (6). ACT remained stable below 1 M GdnHCl and was fully unfolded in denaturant concentrations above 4 M. Increasing GdnHCl...
concentrations caused $\lambda_{\text{max}}$ to gradually red-shift to a wavelength of 353 nm, indicative of tryptophan residues fully exposed to solvent. Above 5 M GdnHCl the emission spectra also displayed a distinct peak around 308 nm, indicating the spatial separation of the tyrosine and tryptophan residues (Fig. 2A). The $\lambda_{\text{max}}$ unfolding curve of ACT showed two cooperative transitions (Fig. 3A). The first, associated with a small increase in $\lambda_{\text{max}}$ from 333 to 336 nm, and with a $D_m$ of 1.5 M. The second, larger transition (336–353 nm) occurred in a highly cooperative manner with a $D_m$ of 3.5 M (Fig. 3A). These ACT data indicate the presence of a stable intermediate formed in 2.5 M GdnHCl. The identical experiment with 1-AT resulted in a single cooperative transition. Fluorescence emission and intensity ($I_{330}$) were recorded with a bandpass of 2.5 nm. CD changes were monitored at 222 nm. The lines represent the three-state curves fitted as described under “Experimental Procedures.” Shaded areas represent the conditions that favor formation of the intermediates I₁ and I₂. All experiments were performed at 25 °C.

Although $\lambda_{\text{max}}$ reports changes in the solvent exposure of the tryptophan residues, changes in fluorescence intensity ($I_{330}$) can provide further information regarding the solvent exposure and the proximity of neighboring charged residues, which may quench the fluorescence of the Trp residues in the native state. The first transition is followed by a decrease in $I_{330}$ with a midpoint of 2.9 M, similar to the value obtained using $\lambda_{\text{max}}$ as the probe. These data indicate that ACT also unfolds through one intermediate, which exists in low concentrations of denaturant. The unfolding was also monitored by far-UV CD, which follows the changes in overall secondary structure. Both ACT and 1-AT exhibited two transitions that involved a decrease in secondary structural content. The first transition occurred with a midpoint of approximately 0.8 M and was associated with approximately 20% loss of CD signal amplitude at 222 nm (Figs. 3C and 4C). The second, larger transition corresponded to a complete loss of secondary structure, with $D_m$ of 3.5 M. Both CD and fluorescence indicated that the protein was completely unfolded in 4.0 M GdnHCl. It is also interesting to note that, although ACT and 1-AT share only 30% sequence identity, they appear to unfold through similar pathways, as monitored by CD spectroscopy.

Under the low protein concentrations used in these experiments, ACT unfolding was fully reversible. Each data set was therefore analyzed using a three-state model (23, 24). The calculated free energy changes for the $N \leftrightarrow I_1 \leftrightarrow I_2 \leftrightarrow U$ transitions of ACT are 5.3, 7.4, and 11.7 kcal/mol, respectively (Table I). The denaturant dependence of each transition, the $m$ value, decreases from 5.5 to 4.8 to 3.3 kcal/mol/ΔM as the reaction moves from N to U. The $N \leftrightarrow I_1$ transition was monitored by CD spectroscopy, whereas the $I_1 \leftrightarrow I_2$ and $I_2 \leftrightarrow U$ transitions were monitored by the change in $\lambda_{\text{max}}$.

Ptitsyn and colleagues (27) have shown that a specific tool for
detecting intermediate states in protein folding is the binding of the fluorescent probe ANS. ANS often binds weakly to native states, but its binding to equilibrium intermediates is usually much stronger and is accompanied by an increase in fluorescence intensity. Fig. 5 shows that ANS binds weakly to the native state of ACT and that as ACT is denatured, using GdnHCl, there is a dramatic increase in ANS fluorescence. Maximum binding was achieved at 1.7 M GdnHCl which coincides with I1. There was, however, an asymmetry in the binding curve with another maximum at 2.7 M that coincides with I2. Similar asymmetrical binding curves have previously been observed for bovine carbonic anhydrase, which also undergoes a four-state folding pathway (28).

**Unfolding Kinetics**—To thoroughly characterize the folding pathway of ACT, kinetic analysis of both the folding and unfolding reactions was carried out. The increased concentrations of protein required for the stopped-flow experiments, however, resulted in aggregation during refolding, so accurate and reproducible folding data could not be collected. Unfolding was examined beyond the transition region ([GdnHCl] producible folding data could not be collected. Unfolding was of protein required for the stopped-flow experiments, however, folding reactions was carried out. The increased concentrations pathway of ACT, kinetic analysis of both the folding and unfolding transitions, respectively. mN→U, mI→U represent the m values for the N → I and I → U transitions, respectively. DmN→U, DmI→U represent the midpoint of denaturation for the N → I and I → U transitions, respectively. kU→D is the rate of unfolding in the absence of denaturant, mU is the GdnHCl dependence of the unfolding rate constant on GdnHCl concentration.

**Unfolding Antichymotrypsin**

**DISCUSSION**

Understanding the conformational changes involved in protein folding is essential if we are to one day solve the so called “protein folding problem.” This information is also fundamental to understanding many disease states. The ability to undergo conformational change is crucial for the physiological function of serpins and is the basis for their metastability (29, 30). This conformational property, however, makes the serpins exceptionally susceptible to mutation and predisposes carriers to disease (2, 15). Many different mutations may cause a serpin to self-associate during folding, which leads to tissue deposition. The aim of this study was to characterize the folding pathway of ACT, which has been implicated in numerous disorders, to provide the essential background for understanding how mutations can have such deleterious effects.

| Protein | ΔGO(N→I) kcal/mol | mN→I kcal/mol | DmN→U μ | ΔGO(I→U) kcal/mol | mI→U kcal/mol | DmI→U μ | kU→D s⁻¹ | mU |
|---------|------------------|--------------|-------|------------------|--------------|-------|---------|-----|
| ACT    | 7.43             | 4.84         | 1.54  | 11.71            | 3.33         | 3.52  | 0.35    | 0.80|
| α₁-AT  | 2.18             | 1.46         | 1.49  | 8.17             | 2.21         | 3.70  | 0.36    | 0.76|
| I330   | 2.53             | 3.00         | 0.84  | 3.62             | 1.23         | 2.94  |         |     |
| Far-UV CD | 5.34             | 5.49         | 0.97  | 4.46             | 1.30         | 3.43  |         |     |
| ACT    | 4.01             | 4.92         | 0.81  | 5.10             | 1.66         | 3.07  |         |     |

**Fig. 5.** ANS binding profile of ACT. ACT was incubated in various concentrations of GdnHCl, then the fluorescent probe ANS was added to each condition, and fluorescence intensity at 480 nm (λex = 390 nm) was recorded as a function of denaturant concentration. Bandpass was 2.5 nm, and all records were made at a temperature of 25 °C.

**Fig. 6.** Determination of the first order rate constants of the unfolding of ACT and α₁-AT. A, a representative stopped-flow trace for ACT at 25 °C. B, the observed rate constant was plotted as a function of GdnHCl concentration for ACT (○) and α₁-AT (□). Unfolding was initiated by a 1:10 dilution of the protein (native state) into varying concentrations of GdnHCl (>4 M) and was monitored by the emitted fluorescence at >320 nm at 25 °C. The lines represent the best fit of data, fit according to a single exponential equation.

**TABLE I**

Analysis of the equilibrium and kinetic unfolding data

Results from Figs. 3 and 4 were fit to three-state unfolding analysis as described under “Experimental Procedures”; the results presented represent the average of five individual curves ΔGO(N→I), ΔGO(I→U), represent the free energy change for the unfolding for the N → I and I → U transitions, respectively. mN→I, mI→U represent the m values for the N → I and I → U transitions, respectively. DmN→U, DmI→U represent the midpoint of denaturation for the N → I and I → U transitions, respectively. kU→D is the rate of unfolding in the absence of denaturant, mU is the GdnHCl dependence of the unfolding rate constant on GdnHCl concentration.
Data presented here showed that the equilibrium unfolding reaction of ACT as monitored by fluorescence and CD did not coincide, indicating a multistep process. The unfolding transitions observed by both techniques are compatible with a four-state model, involving at least two stable, partially unfolded intermediates in addition to the native and unfolded states that can best be described by the pathway $N \leftrightarrow I_1 \leftrightarrow I_2 \leftrightarrow U$.

The presence of two intermediates in a serpin folding pathway is, so far, unique to ACT, because $\alpha_1$-AT and other serpins that have been studied to date possess a single equilibrium intermediate (6, 9, 10, 31). The native state structure of ACT has been solved by crystallography and is well characterized (11). The spectral properties of the unfolded state strongly indicate that it lacks any secondary structure and the tryptophan residues are fully exposed to solvent. From the data presented here we can begin to comment on the structure of both $I_1$ and $I_2$, and compare them to previously reported intermediates in the serpin folding pathway.

$I_1$ was observed by CD spectroscopy and possesses only 80% of the far-UV signal of native ACT. From the fluorescence data it is clear that this loss of structure does not involve regions around the three Trp residues. Because their fluorescence properties are unchanged. Our previous study with $\alpha_1$-AT indicated that this loss of structure involved movement around the A and C $\beta$-sheets (6). It has been hypothesized that the recent structure of an inactive variant of ACT (termed $\delta$ Leu55-Pro ACT), in which the RCL is partially inserted into the A $\beta$-sheet, and the F-helix partially unwound, may approximate to the intermediate state in polymerization (14). The conformational change required to form this structure from the native state would cause no significant change in the environment of the tryptophan residues, and the change in secondary structure from $\alpha$-helix to $\beta$-sheet would lead to a decrease in ellipticity at 222 nm. This suggests that $I_1$ may indeed have some of the structural characteristics of $\delta$ ACT, although further structural studies will be required to confirm this.

The transition from the $I_1$ to $I_2$ state, perhaps surprisingly, does not involve any loss of secondary structure. The conformational changes are more subtle, involving a combination of increased solvent exposure and alteration of side chain conformations around the Trp residues. Although the $I_1$ state involves a loss of secondary structure, $I_2$ results from a loss of tertiary interactions suggesting that $I_2$ has some of the properties of a molten globule state as defined by Pritsyn (28), i.e. it possesses substantial secondary structure but lacks rigid tertiary structure. From the position of the Trp residues we propose that these changes are around Trp-194 and Trp-276. Trp-215 is fully solvent-exposed on the C $\beta$-sheet (Fig. 1), and it is unlikely that it would contribute to fluorescence changes as the protein unfolds. Trp-194 and Trp-276 are buried against different faces of the B $\beta$-sheet. In our previous study with $\alpha_1$-AT, we proposed that the B $\beta$-sheet was the last secondary structural element to unfold (6). These data support this hypothesis, suggesting that the structural transition from $I_1$ to $I_2$ involves loss of tertiary interactions around this critical $\beta$-sheet, which precede total unfolding.

One important observation in our study came from the examination of the rate constants for unfolding. We examined the unfolding rate at high denaturant concentration (GdnHCl > 4 M) so that the folding reaction played no part. In doing so we were able to estimate $K_{U}^{\mathrm{eq}}$, the rate of unfolding in the absence of denaturant. For both ACT and $\alpha_1$-AT, this value was around $0.4 \text{ s}^{-1}$, which is a relatively fast rate even more so considering their relatively large size (400 amino acid residues). The unfolding rate of most small proteins (>150 amino acid residues) in the absence of denaturant is very slow, generally around $10^{-2}$ to $10^{-6} \text{ s}^{-1}$ (16). Exceptions to this at 25 °C are the cold shock family of proteins (12 $\text{s}^{-1}$; approximately 66 amino acid residues) and tumor suppressor protein p16 (0.8 $\text{s}^{-1}$; 156 amino acid residues) (32, 33). It is proposed that these fast rates are indicative of flexibility within the protein that is required for normal function (32). The data presented here agree with this model. Serpins undergo dramatic conformational change for inhibition in which the A $\beta$-sheet opens and the RCL inserts, resulting in a translocation of the proteinase from one pole of the molecule to another (30, 34). Possessing a high $K_m^\text{I2}$ implies conformational flexibility that would facilitate such conformational changes. The structure and folding of serpins are well known to be highly sensitive to point mutations (2, 15, 35), which may be due to their inherently low barrier to unfolding. There is some evidence (36) that in the case of $\alpha_1$-AT the mutation causes a preferential stabilization of the intermediate, $I_1$. This may be a consequence of the conformational flexibility of the wild type protein being reduced.

Previous work on serpin folding has identified one intermediate and indicated that the serpin folding pathway is conserved (6, 9, 10). The fluorescence and CD data presented here for $\alpha_1$-AT only identified $I_1$, which may indicate that ACT and $\alpha_1$-AT are unfolding via different pathways or that $I_2$ was only detected because of the different tryptophan location within ACT. This latter option appears probable given the almost identical far-UV CD unfolding traces for ACT and $\alpha_1$-AT. A substantial body of work, however, has been performed on the unfolding pathways of $\alpha_1$-AT mutants, using probes placed at various sites around the molecule, and no second intermediate has been detected by these methods (6). It is possible that the unique optical properties of the stable intermediates of ACT have allowed the identification of an additional intermediate, $I_3$. Although possessing a similar amount of secondary structure, lacks crucial tertiary interactions that presumably maintain the serpin fold. It would be very interesting to introduce the same optical probes into other serpins to determine whether $I_3$ exists in their pathways. If so, it would suggest that the folding pathway of the serpin superfamily is conserved.

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