Unfolding Properties of Tryptophan-containing \( \alpha \)-Subunits of the Escherichia coli Tryptophan Synthase*  

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The urea-induced unfolding of the Escherichia coli tryptophan synthase \( \alpha \)-subunit is examined via fluorescence measurements with tryptophan-containing \( \alpha \)-subunit mutants, constructed by in vitro mutagenesis. Early unfolding studies with urea and guanidine suggested that the wild type protein unfolded in a two-step process with a stable intermediate composed of a native \( \alpha \)-1 folding unit (residues 1-188) and a completely unfolded \( \alpha \)-2 folding unit (residues 189-268). Recently, more detailed spectroscopic and calorimetric data from the Matthews and Yutani groups indicate that such a structure for the intermediates seems unlikely. Previously, we described the introduction of Trp residues as unfolding reporter groups separately into each of the folding domains and showed that these proteins are wild type enzymatically and in their stability to urea. The unfolding behavior of these \( \alpha \)-subunits, monitored by fluorescence intensity changes at the discrete emission \( \lambda_{\text{max}} \) for each, in both equilibrium and kinetic experiments, suggest that: (a) both folding units commence unfolding simultaneously (near 2 \( \text{M} \) urea); (b) the larger \( \alpha \)-1 unit unfolds in a multistep process, initially yielding a partially unfolded intermediate form which subsequently appears to unfold progressively to completion; and (c) the smaller \( \alpha \)-2 unit unfolds in a single step event. These results are also clearly incompatible with the early proposals on the structure of the intermediate. It is suggested here that the intermediate is heterogeneous, consisting of a stable, partially unfolded form of \( \alpha \)-1 attached to either a completely folded or completely unfolded form of \( \alpha \)-2. These results are consistent with and provide an added dimension to the recent description of the proposed structure of the intermediate.

The unfolding behavior of the \( \alpha \)-subunit of the bacterial tryptophan synthase is characterized by two important features: there appears to be two independent folding units within the protein (1) and there is a stable, populated intermediate in the unfolding pathway (2, 3). The structures of the folding units are defined in terms of the intact native structure. The \( \alpha \)-subunit structure is a variant (4) of the classic \( \alpha/\beta \) barrel structure in that it has several extra \( \alpha \)-helices (5). The amino-terminal (188-residue) \( \alpha \)-1 folding unit consists of an \( \alpha_\beta_6 \) helical form; the carboxyl-terminal (80-residue) \( \alpha \)-2 folding unit is an \( \alpha_\beta_6 \) structure. The unfolding pathway, experimentally inducible by a variety of denaturants (heat, urea, guanidine), has long been known to be a multistep process. However, the structure of the major intermediate in this pathway, detected by calorimetry, circular dichroism, UV absorbance, hydrogen exchange, proton NMR, and tyrosine fluorescence measurements, has not yet been clearly defined.

Interest in this laboratory stems from a calorimetric analysis of nearly 75 mutant \( \alpha \)-subunits of the Escherichia coli enzyme obtained by in vitro mutagenesis and containing single amino acid substitutions (6). The broad spectrum of stability (i.e. unfolding) properties exhibited by these mutant proteins presented the opportunity to explore the unfolding pathway in more detail. The approach initiated here was to introduce a single Trp residue separately into each of the folding units to use as reporter groups for the fluorescence-monitored unfolding of each unit. The wild-type \( \alpha \)-subunit does not contain tryptophan. Three \( \alpha \)-subunits (F139W, F258W, and F139W/F258W) were constructed in which Phe residues were substituted singly at positions 139 (in \( \alpha \)-1) and 258 (in \( \alpha \)-2) and at both sites (7). The positioning of the Trp residues was such that all of the existing mutational alterations can be engineered into either of these tryptophan-containing host \( \alpha \)-subunits. Another critical property of these host proteins is that the Trp substitutions do not affect their enzymatic or stability behavior (7). This report describes the urea-induced unfolding pathway for these wild type-like proteins as monitored by tryptophan-specific fluorescence measurements and provides additional information on the structure of the unfolding intermediate.

MATERIALS AND METHODS  

Chemicals—Ultrapure urea was purchased from Life Technologies, Inc. and was used without further purification. Urea solutions were prepared and filtered freshly for each experiment. Concentrations were determined by refractive index measurements. All other chemicals were reagent grade and were obtained from Sigma.  

Protein Purification—The \( \alpha \)-subunits (F139W, F258W) were purified from E. coli strain RB797 containing pCATtrpF139W, pCAGtrpF258W, respectively. Growth conditions and \( \alpha \)-subunit purification protocols were identical for each and have been described before (14).  

Fluorescence Measurements—A Perkin-Elmer Luminescence/Fluorescence Spectrometer Model LS50B equipped with a stirrer was used for all fluorescence studies in both equilibrium and kinetic experiments. The excitation wavelength was 295 nm, and fluorescence intensities are presented as arbitrary units in all experiments. All the unfolding experiments were carried out in a 10 mM potassium phosphate buffer (pH 7.8), containing 0.2 mM disodium EDTA and 1 mM \( \beta \)-mercaptoethanol. Corrections were made for buffer and denaturant. The final concentration of protein in both equilibrium and kinetic experiments reported here was 5 \( \mu \)M; similar results were obtained at 10 \( \mu \)M. Equilibrium unfolding measurements were performed after a 12-h incubation at 25 °C in different concentrations of urea. The kinetic experiments, also at 25 °C, involved the rapid addition of 0.5 ml of containing buffer, \( \alpha \)-subunit (dialyzed versus this same buffer), and a specified urea concentration to 1.5 ml of buffer containing urea at concentrations necessary to obtain the final desired concentration. The mixing time is 5-10 s; temperature changes upon mixing were insignificant (±1 °C). Fluorescence intensity measurements were recorded at 0.5-s intervals. The experimental data for the unfolding experiments presented here repre-
sent the average of 3–6 experiments. The standard deviation for each measurement is ±5–10% or less.

Data Analysis—The equilibrium unfolding results were fit by nonlinear regression analyses utilizing the Levenberg-Marquardt method as previously reported (7). The results of urea concentration jumps from 0 M to 6 M were analyzed by single- and biexponential functions using the equation:

$$A(t) - A(\infty) = A_i e^{kt}$$

(Eq. 1)

where $A(t)$ is the fluorescence intensity at a given time $t$, $A(\infty)$ is the intensity when no further change is observed, $k_i$ is the apparent first order rate constant of the $i$th kinetic phase, and $A_i$ is the amplitude of the $i$th phase. The kinetic data were fit to one or more exponentials as appropriate, and the relaxation times and associate amplitudes were extracted with a nonlinear least-squares fitting program of Sigma Plot v.4.1 (Jandel Scientific).

**RESULTS**

Equilibrium Unfolding and Refolding of the Tryptophan-containing $\alpha$-Subunits—Two mutant $\alpha$-subunits, F139W and F258W, are examined here. The substituted Trp residues at the 139 and 258 sites occupy, respectively, the second position in the 8-residue helix-4 and the eleventh position in the carboxy-terminal, 19-residue helix-8. These sites are widely (~30 Å) separated; 10 Å spheres surrounding the side chains at each site are nearly 20 Å apart. Previous results (7) indicated that the UV absorbance and fluorescence spectral properties of equimolar mixtures of the F139W and F258W proteins are virtually identical with those of the double mutant F139W/F258W, suggesting that each substituted Trp residue (and its respective folding unit) is spectroscopically displayed independently. In the absence of urea as shown before (7), the F139W and F258W $\alpha$-subunits have emission $\lambda_{\text{max}}$ values of 328 nm and 341 nm, respectively; at 5 $\mu$M concentrations, the relative fluorescence intensities are 156 and 407, respectively. Fig. 1 presents the fluorescence spectral changes for the F139W (A) and F258W (B) proteins at increasing urea concentrations. For each, the addition of urea results in an intensity decrease at the respective $\lambda_{\text{max}}$ and at 5–6 M urea for F139W and at 4–6 M urea for F258W, fluorescence spectra are obtained that are similar to that for free tryptophan in 6 M urea (dashed line). No further fluorescence intensity changes for either protein are observable at higher (e.g., 8.5 M) urea concentrations. Refolding equilibrium experiments in which each protein was diluted from 6 M urea to intermediate urea concentrations resulted in fluorescence intensity increases (monitored at 12 h after dilution) to >98% of those found in the unfolding experiments. Spectral changes under these conditions are identical with the reverse of those shown in Fig. 1. Thus, by fluorescence intensity criteria, the unfolding of both mutant $\alpha$-subunits F139W and F258W appears completely reversible.

Given the fact that no further intensity changes are observed between 6 M and 8.5 M urea, it is assumed that the fluorescence intensity changes achieved at 6 M urea represent those for a total unfolding of the respective folding units. Utilizing the intensity decreases at the $\lambda_{\text{max}}$ for the native form of each $\alpha$-subunit, the fractional unfolding, $F_{\text{app}}$, $F_{\text{app}} = (1 - f_i)I_{\text{u}} - f_i I_{\text{u}}$ where $I$ is the observed intensity at some urea concentration, and $I_{\text{u}}$ and $I_{\text{f}}$ are the intensities of native and unfolded species (closed circles); open circles represent the $F_{\text{app}}$ observed upon dilution from 6 M urea to the indicated urea concentration. B, fraction of native ($f_{\text{n}}$), intermediate ($f_{\text{i}}$), and unfolded ($f_{\text{u}}$) forms (the dashed lines are the values obtained from UV absorbance measurements, Ref. 7). C, the dashed and solid lines are the calculated $F_{\text{app}}$ values for a one- or two-step transition, respectively.

Previous UV absorbance data obtained with the F139W...
α-subunit (7) suggested a three-state, two-step unfolding model, namely

\[ \begin{align*}
N & \xrightarrow{k_1} I \xrightarrow{k_2} U \\
N \equiv I \equiv U
\end{align*} \]  

(Eq. 2)

The F139W α-subunit fluorescence-based denaturation curve (Fig. 2A) is relatively broad over the transition range, and deviations from linearity for the ΔG versus urea concentration plot (8) are also observed. In addition, an examination of the intersecting fluorescence spectra found during the N → U transition (Fig. 1A) reveals no clear isosbestic point. These observations also suggest a multistep unfolding process for this protein when monitored by fluorescence measurements. A two-step model can be described as before (7) by

\[ F_{\text{apo}} = \frac{K_i(Z + K_o)}{1 + K_i/K_o} \]  

(Eq. 3)

where \( K_1 \) and \( K_2 \) are the equilibrium constants for the N → I and I → U transition, respectively, and \( Z \), the fractional change in intensity for the intermediate form = \((I_I - I_N)/(I_U - I_N)\) where \( I_I, I_U, \) and \( I_N \) are, respectively, the intensities of the intermediate, unfolded, and native forms. The fluorescence equilibrium data were fit statistically to a one-step (Fig. 2C, dashed line) or a two-step (Fig. 2C, solid line) model over the transition range. From these results, a two-step transition seems more likely. Assuming a linear dependence of free energy on urea concentration, the free energies of unfolding in the absence of urea for each step, \( \Delta G_{1,0}^{\text{apo}} \) and \( \Delta G_{2,0}^{\text{apo}} \), were estimated according to Pace (8): \( \Delta G_1 = \Delta G_{1,00}^{\text{apo}} + m_1 C \) and \( \Delta G_2 = \Delta G_{2,00}^{\text{apo}} + m_2 C \) where \( m_1 \) and \( m_2 \) are the cooperativity factors and \( C \) is the urea concentration. The parameters, \( Z, \Delta G_{1,00}^{\text{apo}}, \Delta G_{2,00}^{\text{apo}}, m_1, \) and \( m_2 \), are, respectively, 0.33, 5.6 ± 0.8 kcal/mol, 4.9 ± 0.9 kcal/mol, -2.3 ± 0.3 kcal/mol/m, and -1.3 ± 0.2 kcal/mol/m. The fractional distribution of native, intermediate, and unfolded forms for the F139W α-subunit are given in Fig. 2B. The concentration of intermediate form reaches a maximum at ~3 M urea and is subsequently converted to more unfolded forms. This pattern is quite similar to that obtained for this protein when more global UV absorbance differences were employed (7) to monitor unfolding (Fig. 2B, dashed line).

A similar treatment of the equilibrium unfolding data for the F258W α-subunit is given in Fig. 3. The unfolding transition initiates at ~2 M urea, and apparent complete unfolding in the region around Trp-258 is observed at 4–4.5 M (Fig. 3A). The transition range for denaturation is more narrow (Fig. 3A) than that for the F139W α-subunit, and unfolding is fully reversible. Although a two-step unfolding transition was observed for the F258W α-subunit when UV absorbance measurements were employed (7), attempts to fit the fluorescence data to a one-step (Fig. 3B, dashed line) or two-step (Fig. 3B, solid line) process do not provide a mutually exclusive case for either. A plot of ΔG versus urea concentration can best be judged as linear and indicative of a simple one-step unfolding process. This conclusion also appears consistent with subsequent kinetic data. Based on this interpretation, estimated \( \Delta G_{1,0}^{\text{apo}} \) and \( m \) values are 7.0 ± 0.7 kcal/mol and -2.5 ± 0.4 kcal/mol/m, respectively.

Kinetics of Unfolding of the Tryptophan-containing α-Subunit—The unfolding kinetics for each of the tryptophan-containing α-subunits were determined by monitoring the loss of native structure (i.e. the intensity decreases at 328 nm for F139W and at 341 nm for F258W) following rapid changes in urea concentration. Fig. 4 presents some of the data for the F139W α-subunit. Only the first 6 min of the intensity changes are shown; in all cases, continued monitoring until ≥98% of total change are observed revealed no additional slope change.

This is the case for all of the kinetics presented here for both proteins.

Fig. 4, A and B, shows that the kinetics of typical urea concentration jumps from 0 M to ≥4 M urea. A simple linear relationship suggests a single kinetic event. In contrast, typical results for urea concentration jump from 0 M to ≥4 M (Fig. 4, C–E) indicate two kinetic events, a fast and slow step. The amplitudes of the changes for the fast phases are ≈90%, and
those of the slow phase are ~10%. The rate constants for these changes are given in Fig. 5A (solid and open circles are for the fast and slow phases, respectively). Similar results are seen in urea concentration jumps from x M to 6 M urea (Fig. 4, F–J). In these experiments, the protein was allowed to reach equilibrium at a specified urea concentration (usually for 12 h) and subsequently rapidly adjusted to a final concentration of 6 M urea. Fig. 4, F–H, gives typical results of urea concentration jumps from 0–2.75 M to 6 M. Two phases are again observed. Fig. 5B gives the apparent rate constants for the fast (closed circles) and slow (open circles) phases. As shown in Fig. 5C, the amplitude of the fast phase is ~90% (closed circles) and ~10% for the slow phase (open circles). In contrast to these data, the results of urea concentration jumps from ~3 M to 6 M indicate an abrupt loss of the fast phase (Fig. 4, I–J). The amplitude of the fast phase decreases to a point where the slow phase only accounts for essentially all of the observed intensity change (Fig. 5C). The apparent rate constants shown for the fast phase also appear to exhibit a decrease (Fig. 5B) in these experiments, but the estimation of these rate constants is problematic because of the decreased amplitudes. The apparent rate constants for the slow phase do not appear to change.

Both sets of experiments agree well and are consistent with the equilibrium data which indicate that the N → L transition occurs over the urea concentration range of 0–3 M and the L → U transition proceeds over the 3–6 M urea concentration range. The kinetics indicate that urea concentration jumps within the 0 M to 3–4 M range (Fig. 4, A and B) and within 3–4 M to 6 M range (Fig. 4J) each exhibit essentially single kinetic events. Urea concentration jumps that span the 3–4 M range (Fig. 4, C–E, F–I) exhibit two events, a fast phase with an amplitude of ~90% followed by a slower phase with an approximate amplitude of 10%.

Comparable kinetic experiments with the F258W α-subunit employing urea concentration jumps either from 0 M to x M urea (Fig. 6, A–D) or from x M to 6 M urea (Fig. 6, E–H) indicate that a single kinetic event is sufficient to achieve ~98% unfolding. The rate constants obtained for both sets of experiments are given in Fig. 7, A and B.

**DISCUSSION**

The unfolding properties reported here involving the regions surrounding Trp-139 and Trp-258 are clearly different and are consistent with the notion of two independent folding units in the α-subunit. Neither here nor in many other folding studies with this protein is there any unequivocal indication that such folding units cannot function as such although the initial experiments defining them remain the strongest evidence that they exist. We have interpreted our results in these terms, namely, that the fluorescence-monitored unfolding properties of the F139W and F258W α-subunits reflect some of the unfolding properties of the α-1 and α-2 folding units, respectively.

Regardless of whether or not there are independent folding units, there is little doubt concerning the presence of the relatively stable unfolding intermediate for this protein. Initial conclusions that it consisted of an intact α-1 unit and an unfolded α-2 unit have been largely discarded by recent evidence from both the Matthews and Yutani groups (9–13). Evidence here also support this conclusion. Unfolding of the region surrounding both Trp-139 (in α-1) and Trp-258 (in α-2) commence unfolding nearly simultaneously at urea concentrations near 2 M. Thus, no completely intact structure in either folding unit can exist during the unfolding transitions.

The structural description of this intermediate here is based on the combined fluorescence equilibrium and kinetic data from both F139W and F258W α-subunits. Maximum urea denaturation (5–6 M urea for F139W and 4–5 M for F258W) leads to similar spectra for both of these proteins and free L-tryptophan. Small intensity differences remain, however, and there is a question of whether or not there is complete unfolding for the
respective folding units. NMR data (10) indicate that pockets of local native structure may remain at 6 M urea. His-92, for example, becomes totally exposed only at 6.5–7 M urea. Although our data alone cannot prove complete unfolding of each unit, it is clear that no further fluorescence spectral changes are seen at urea concentrations as high as 8.5 M. Thus, the final fluorescence intensity levels for each tryptophan-containing α-subunit are considered to represent those for complete unfolding. With these caveats in mind, together with the basic assumption that the regions surrounding Trp-139 and Trp-258 represent, respectively, the α-1 and α-2 folding units, a relatively simple unfolding scheme for the α-subunit is suggested (Fig. 8). Although previous data (7) have indicated a strong similarity between the wild-type and the Trp-containing α-subunits, it must be noted that the relaxation times shown here apply specifically to the F139W and F258W α-subunits. These time constants, however, might be expected to be at least qualitatively similar to those for the wild-type protein. It can be seen that, although incompatible with the initial ideas of the structure for the major unfolding intermediate as noted above, this pathway is consistent with other more recent data and illustrates the potential value in introducing specifically positioned reporter residues.

The α-1 folding unit (as exemplified with the F139W α-subunit) appears to unfold in multiple steps starting at −2 M urea. The equilibrium unfolding data are most consistent with a simple two-step (N → I = U) unfolding process in which the intermediate form appears maximally at −3 M urea. The kinetic data also indicate at least two kinetic events (fast and slow) for the N → U transition. The assignment of the fast and slow steps to the N → I and I → U transitions, respectively, is based on the two types of urea concentration jump experiments. Urea concentration jumps from 0 to ≤4 M and from ≥4 M → 6 M exhibit a single fast and a single slow rate, respectively. Maximum values for the fast and slow rates correspond to relaxation times of 12–16 s and 125–130 s, respectively, with the fast step accounting for ~90% of the total observed change. From a statistical analysis of the equilibrium experiments, the predominant unfolding reaction below 3 M is the N → I transition and above 3 M is the I → U transition.

The α-2 folding unit (i.e. as exemplified with the F258W α-subunit) also initiates unfolding at −2 M urea. The equilibrium data can be fit to a one-step (N = U) process, and, consistent with this, the kinetics of urea concentration jumps over any concentration range can be described by a single, uniform rate with maximum value corresponding to a relaxation time of 54–58 s. By these criteria, it appears that at any denaturing urea concentration, the α-2 folding unit is either completely native or completely unfolded.

Thus, these fluorescence studies also suggest that there is a major α-subunit unfolding intermediate at about 3 M urea as others have proposed. At 3 M urea, the α-1 folding unit appears to exist primarily in a stable, partially unfolded state. The α-2 unit is either native or completely unfolded at all denaturant concentrations, and, at 3 M urea, it is ~50% native and ~50% unfolded. This leads to our suggestion that the intermediate is heterogeneous, consisting of a stable partially unfolded α-1 folding unit joined to either a totally unfolded or totally folded α-2 folding unit. These conclusions are not inconsistent with Yutani’s (12, 13) structural description of the intermediate as a molten globule form nor with the more detailed picture by Matthews group (9–11). In the latter description, for example, the α-2 folding unit is thought to remain partially organized. We have concluded that the α-2 unit exists as both completely folded and completely unfolded in the intermediate structure. Secondly, the intermediate is proposed to be an “opened” form of the barrel structure in which ~40% of the secondary and tertiary structure is retained but the packing is loosened. We suggest that the region surrounding Trp-139 has reached a stable, partially exposed state in which α-1 is attached to either a native or completely unfolded α-2. Thirdly, it is concluded that there is partial contact between β-strand 6 (in α-1) and β-strand 7 (in α-2). Our conclusion is that a fraction of a completely intact α-2 remains associated with a partially unfolded α-1 unit.

The structure of an additional, stable refolding intermediate proposed from UV and CD spectroscopy by the Matthews group (10) is thought to contain little secondary structure with much of its tyrosyl residues exposed but yet retaining some local (near His-92) tertiary structure. Our fluorescence data do not clearly indicate any additional intermediates although the slow step for α-1 unfolding may be related to the unfolding of the second intermediate proposed by Matthews’ group. The structural identity of an additional potential additional folding unit within α-1 may come from a closer examination of an hypersensitive trypsin site at Arg-70 (15), similar to, although apparently less hypersensitive than, the Arg-188 site. Whether or not this represents a site between two smaller folding units within α-1 is, at best, conjecture. This possibility is being explored by the approach used here.

Given the sensitivity of fluorescence to environmental factors, a further exploration of the unfolding/refolding characteristics of these wild-type-like proteins together with an examination of their behavior when our destabilizing/stabilizing...
amino acid substitutions are introduced should prove useful in resolving these events.

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