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Tryptophan replacements in the *trp* aporepressor from *Escherichia coli*: Probing the equilibrium and kinetic folding models

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Abstract

Mutants of the dimeric *Escherichia coli trp* aporepressor are constructed by replacement of the two tryptophan residues in each subunit in order to assess the effects on equilibrium and kinetic fluorescence properties of the folding reaction. The three kinetic phases detected by intrinsic tryptophan fluorescence in refolding of the wild-type aporepressor are also observed in folding of both Trp 19 to Phe and Trp 99 to Phe single mutants, demonstrating that these phases correspond to global rather than local conformational changes. Comparison of equilibrium fluorescence (Royer, C.A., Mann, C.J., & Matthews, C.R., 1993, *Protein Sci. 2*, 1844-1852) and circular dichroism transition curves induced by urea shows that replacement of either Trp 19 or Trp 99 results in noncoincident behavior. Unlike the wild-type protein (Gittelman, M.S. & Matthews, C.R., 1990, *Biochemistry 29*, 7011-7020), tertiary and/or quaternary structures are disrupted at lower denaturant concentration than is secondary structure. The equilibrium results can be interpreted in terms of enhancement in the population of a monomeric folding intermediate in which the lone tryptophan residue is highly exposed to solvent, but in which substantial secondary structure is retained. The location of both mutations at the interface between the two subunits (Zhang, R.G., et al., 1987, *Nature 327*, 591-597) provides a simple explanation for this phenomenon.

Keywords: cooperativity; folding intermediate; folding mechanism; protein stability; tryptophan fluorescence; tryptophan mutations

Fluorescence spectroscopy is a sensitive and potentially informative method of monitoring both equilibrium and kinetic properties of protein-folding reactions. The hydrophobic character of aromatic residues (i.e., tryptophan, tyrosine, and phenylalanine) often leads to their being sequestered in the interior of the native conformation. Therefore, the intensity, emission maximum, and distribution of various lifetime components of these residues can differ from those exposed to solvent (Beechem & Brand, 1985). Disruption of noncovalent interactions that define the native form and exposure of these aromatic side chains to solvent during a global unfolding reaction can lead to changes that are observed readily in these static and dynamic properties (see, for example, the accompanying paper by Royer et al. [1993]).

A challenge for kinetic studies of folding reactions has been to obtain specific assignments and structural interpretations for observed changes in fluorescence properties. Garvey et al. (1989) solved this problem in dihydrofolate reductase from *Escherichia coli* by using site-directed mutagenesis to replace several of the tryptophan residues individually. These workers found that one of the kinetic phases detected in the refolding of wild-type reductase was eliminated selectively in the mutant, in which Trp 74 was replaced by Phe. This effect was attributed to the burying of Trp 74 in a hydrophobic cluster early in the folding process. Later studies demonstrated that Trp 74 packs against Trp 47 in a native-like fashion in a transient folding intermediate (Kuwajima et al., 1991).

With this precedent, a set of mutations in the tryptophan aporepressor from *E. coli* were constructed with the goal of determining the assignments of the three kinetic phases detected previously by tryptophan fluorescence during refolding of this dimeric protein (Gittelman & Matthews, 1990; Mann & Matthews, 1993). Wild-type

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trp aporepressor (WT TR) is a small, all α-helical, dimeric protein composed of two identical monomers of 107 amino acids (Gunsalus & Yanofsky, 1980). Each monomer is comprised of six α-helices (Zhang et al., 1987; see Kinemage 1), of which three from each monomer (A, B, C, A′, B′, and C′) are intertwined at the dimer interface to form the interior core of the protein. Helices D and E from each monomer make up the "helix-turn-helix" DNA-binding motif common to many prokaryotic and phage DNA binding proteins (for reviews see Steitz [1990] and Freemont et al. [1991]). The last helix, F, packs against the core to form part of the second subunit interface region (see Fig. 1 in the accompanying paper by Royer et al. [1993]). Relevant to the present study is the presence of only two tryptophan residues per monomer: Trp 19 is buried in the interior of the protein core as part of the helix A, and Trp 99 is partially buried and packed against the core as part of helix F (see Fig. 1 in the accompanying paper by Royer et al. [1993]).

Equilibrium and kinetic fluorescence studies of reversible unfolding of the tryptophan 19 → phenylalanine (W19F) and tryptophan 99 → phenylalanine (W99F) mutants reveal that the three kinetic phases observed in WT TR remain in both mutants. Thus, the associated conformational changes must reflect global rather than local phenomena. Curiously, replacement of tryptophan by phenylalanine at either position alters the equilibrium process such that an additional folded form occurs in the transition zone. Fluorescence and circular dichroism properties of this folding intermediate suggest that it is a monomer in which the tryptophan residues are well exposed to solvent, but in which substantial secondary structure is retained. The simplification of spectral properties of proteins by site-directed mutagenesis must, therefore, be used with an appreciation for potential effects on folding reactions.

Results

Spectroscopic analysis

The equilibrium and kinetic properties of urea-induced unfolding reactions were monitored by fluorescence and CD spectroscopy. When exciting into tryptophan absorption at 295 nm, the average emission wavelength provides information on polarity of the local environment of the indole side chains. The average emission wavelength of both tryptophan residues in the native conformation of TR are blue shifted significantly compared to their emissions in the unfolded protein (Royer et al., 1993), making them useful probes of the folding reaction. The far-UV CD is sensitive to secondary structure (i.e., the helical content) of TR and provides an independent measure of the state of folding. Comparisons of unfolding transitions determined by these two techniques offer a means to test equilibrium models. For WT TR, coincident-normalized curves for fluorescence and CD spectroscopy demonstrate that only the native dimer and unfolded monomer are highly populated throughout the urea-induced transition (Gittelman & Matthews, 1990).

Equilibrium studies

Equilibrium-unfolding CD data at 222 nm and average fluorescence emission wavelength data (Royer et al., 1993) were obtained for WT TR, W19F, and W99F. Plots of average emission wavelength and ellipticity for the wild-type protein and each mutant as a function of urea concentration are compared in Figures 1–3.

As has been reported previously for WT TR (Gittelman & Matthews, 1990), the transition curves obtained from CD and fluorescence studies were coincident within experimental error (Fig. 1). These data support the previous conclusion that urea-induced equilibrium unfolding of WT TR follows a two-state model involving a folded native dimer and an unfolded monomer. In contrast, the transition curves obtained for W19F and W99F were noncoincident (Figs. 2, 3, respectively). The fluorescence transition curve has a lower midpoint than the CD curve for both mutant proteins, indicating that disruption of tertiary and/or quaternary structure precedes the loss of secondary structure. This behavior reflects a breakdown in cooperativity of the folding reaction and indicates that one or more additional, partially folded species must appear in the transition zone. A quantitative assessment of these results is provided in the Analysis section.

Kinetic studies

Of particular interest in this investigation was the possibility that one or more of the three kinetic phases observed previously in the refolding of WT TR might reflect the behavior of an individual tryptophan residue. Thus, the time dependence of total emission above 320 nm was

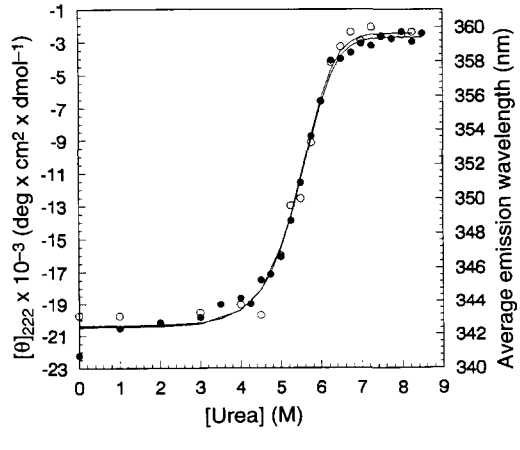

Fig. 1. Dependence of the optical signal for the wild-type *trp* aporepressor (WT TR) on the urea concentration at pH 7.6 and 25 °C, as monitored by CD (●) and fluorescence (○) spectroscopies. Solid lines indicate the global fits for each data set as described in the Materials and methods. Final protein concentration was 5.3 μM.

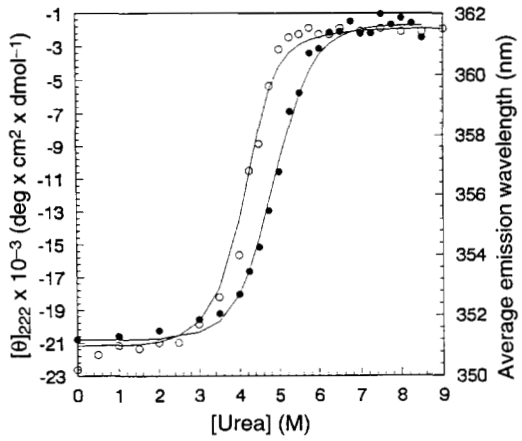

Fig. 2. Dependence of the optical signal for W19F on the urea concentration at pH 7.6 and 25 °C, as monitored by CD (●) and fluorescence (○) spectroscopies. Solid lines indicate the global fits for each data set as described in the Materials and methods. Final protein concentration was 5.3 μM.

monitored on a stopped-flow fluorimeter with a dead time of 4 ms. As described in Gittelman and Matthews (1990), these traces were fit to a sum of three exponentials for jumps ending at or below 3.0 M urea. For jumps above 3.0 M urea, a more complex function was used, in which the three terms also reflect the contribution of the unfolding reaction to the observed relaxation time. In both cases, amplitudes and relaxation times were determined as a function of the final urea concentration.

The refolding of W19F and W99F, similar to WT TR, is described by three kinetic phases, designated τ_1 (slow), τ_2 (intermediate), and τ_3 (fast). The magnitudes of these relaxation times for W19F and their dependencies on the denaturant concentration are very close to those obtained for WT TR (Fig. 4A). The limiting values at 2 M urea are within experimental error, whereas values for the mutant above 3 M urea may be slightly longer for all three phases. The magnitudes and dependence of the amplitudes of each of these phases upon the final denaturant concentration are also very similar to that for WT TR (Fig. 4B). Therefore, Trp 99, the remaining fluorophore in W19F, must be involved in all three of the processes that are detected in WT TR.

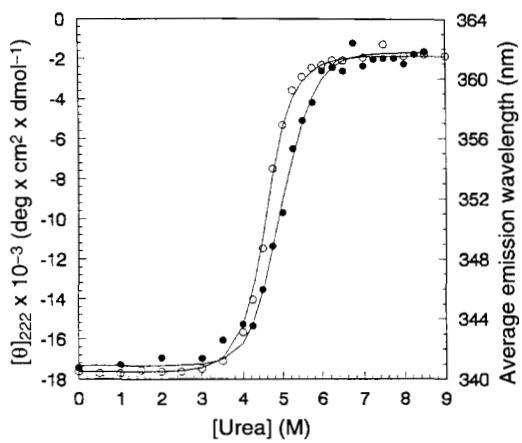

Fig. 3. Dependence of the optical signal for W99F on the urea concentration at pH 7.6 and 25 °C, as monitored by CD (●) and fluorescence (○) spectroscopies. Solid lines indicate the global fits for each data set as described in the Materials and methods. Final protein concentration was 5.3 μM.

Similar kinetic studies of W99F show that relaxation times for the τ_2 and τ_3 phases are virtually identical to those for WT TR (Fig. 5A). The τ_1 phase may be somewhat retarded in the mutant, but the small amplitude of this phase makes this conclusion tenuous. The amplitude of the fastest phase is somewhat enhanced at the expense of the intermediate phase (Fig. 5B); however, the overall behavior is again similar to WT TR. The fluorophore in W99F, Trp 19, is also sensitive to the same three events that occur in WT TR. Because Trp 19 and Trp 99 are components of different hydrophobic cores in the native conformation (see Fig. 1 in the accompanying paper by Royer et al. [1993]), structural changes corresponding to these phases must involve global rather than local events.

Analysis

The above kinetic data show that no substantial changes in the kinetic folding mechanism are introduced by replacing either tryptophan in WT TR with phenylalanine. However, the noncoincidence of the equilibrium fluorescence and CD transition curves suggests either that what was once an unstable, transient intermediate may now have become sufficiently stable to be populated at equilibrium, or that the dimer has become sufficiently unstable at equilibrium to enhance the population of an intermediate. A key observation in proposing a candidate for this intermediate is that the tryptophan residues become exposed to solvent *before* the secondary structure dissolves. A Lee and Richards (1971) analysis of the solvent-exposed surface area of TR shows that 133 Å^2 of Trp 19 and 121 Å^2 of Trp 99 are protected from solvent exposure by the other subunit in the dimer (Zhang et al., 1987; see Kinemage 1). These two tryptophan residues are two of only three residues that show more than 100 Å^2 change in their surface area when the partner subunit is removed. In effect, both tryptophans are at different interfaces between the subunits.

A simple model to explain how replacement of either residue could result in the same optical effects assumes that the intermediate is a partially folded, monomeric species:

$$D \overset{K_{12}}{\longleftrightarrow} 2M \overset{K_{23}}{\longleftrightarrow} 2U. \quad (1)$$

In this model, D represents the folded dimer, M represents a monomer with substantial secondary structure and

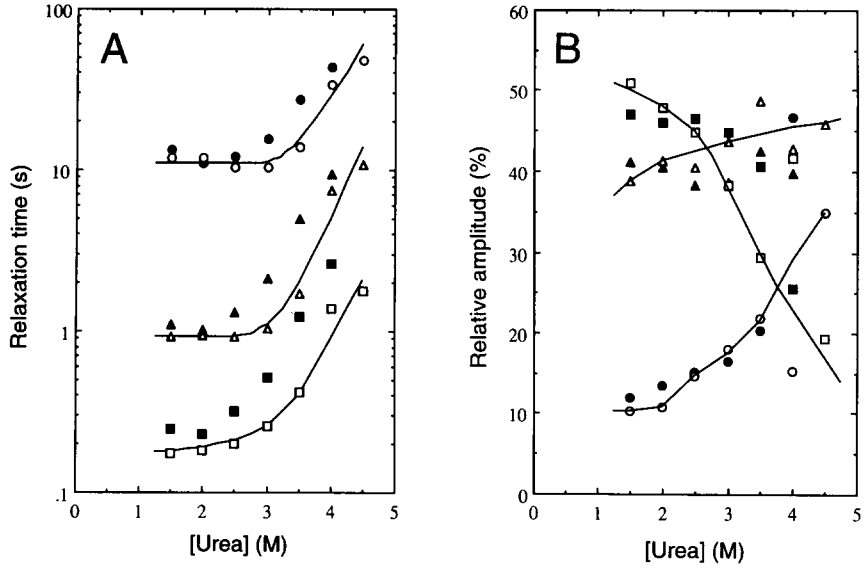

Fig. 4. A: Urea dependence of the W19F (closed symbols) and WT TR (open symbols) relaxation times τ_1 (●, ○), τ_2 (▲, △), and τ_3 (■, □) at pH 7.6 and 25 °C as monitored by stopped-flow intrinsic tryptophan fluorescence. Final protein concentration was 5.3 μM. Lines through WT TR data are drawn to aid the eye. **B:** Urea dependence of W19F (closed symbols) and WT TR (open symbols) relative amplitudes of the τ_1 (●, ○), τ_2 (▲, △), and τ_3 (■, □) refolding reactions. Final protein concentration was 5.3 μM. Lines through WT TR data are drawn to aid the eye.

high exposure of both Trp 19 and Trp 99 to solvent, and U represents the unfolded monomer. The appropriate equilibrium constants are defined as $K_{12} = [M]^2/[D]$ and $K_{23} = [U]^2/[M]^2$.

The equilibrium unfolding data from both spectroscopic techniques for each mutation (Figs. 1-3) were fit simultaneously to the model above using the global-fitting procedure described in the Materials and methods. Fluorescence and CD properties of the intermediate were allowed to vary independently. The free energies of formation of the dimer and intermediate monomer were assumed to depend linearly on denaturant concentration (Schellman, 1978; Pace, 1986). The results of these fits for W19F and W99F are compared to the data in Figures 2 and 3. The good agreement between predicted and observed behavior in both cases shows that this model is a plausible description of the equilibrium unfolding processes.

Quantitative parameters derived from these fits and the fit of the WT TR data to a two-state model are shown in Tables 1 and 2. The relatively large number of parameters and their high degree of correlation lead to considerable uncertainty in the recovered free energies. For example, the recovered free energy of WT TR is almost identical to that of W99F. However, from the data in Figures 1 and 3, it can be seen that the WT TR transition midpoint occurs at significantly higher urea concentrations (5.2 M) than either the fluorescence (4.6 M) or CD (4.9 M) profiles of W99F, implying that this mutant native dimer is slightly less stable than WT TR. The recovered values for the stability of W19F are significantly smaller than those

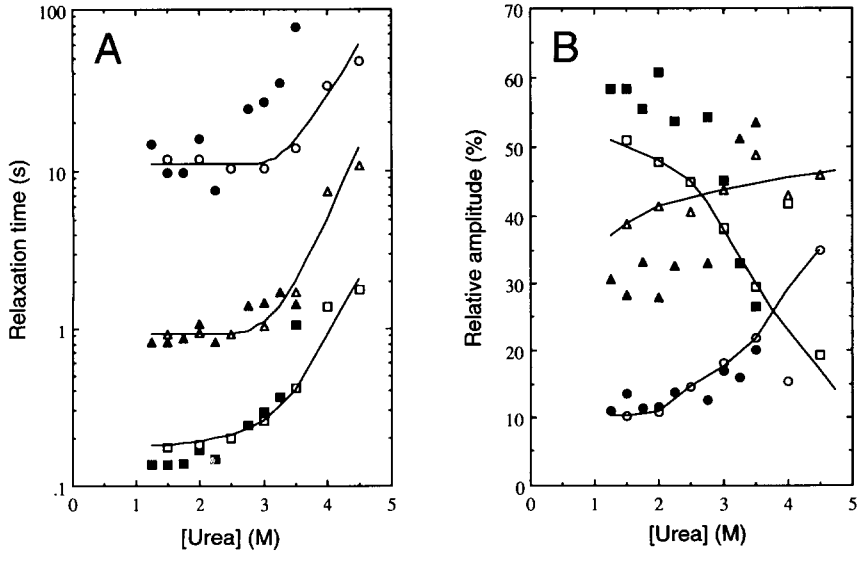

Fig. 5. A: Urea dependence of W99F (closed symbols) and WT TR (open symbols) relaxation times τ_1 (●, ○), τ_2 (▲, △), and τ_3 (■, □) at pH 7.6 and 25 °C as monitored by stopped-flow intrinsic tryptophan fluorescence. Final protein concentration was 5.3 μM. Lines through WT TR data are drawn to aid the eye. **B:** Urea dependence of W99F (closed symbols) and WT TR (open symbols) relative amplitudes of the τ_1 (●, ○), τ_2 (▲, △), and τ_3 (■, □) refolding reactions. Final protein concentration was 5.3 μM. Lines through WT TR data are drawn to aid the eye.

Table 1. *Thermodynamic parameters for the urea-induced unfolding of WT TR and mutants W19F and W99F*

Protein	$D \leftrightarrow 2U$ $\Delta G^{0_{H_2O}}_{app}$ [c]	$2M \leftrightarrow 2U$ $\Delta G^{0_{H_2O}}_{app}$ [d]	$D \leftrightarrow 2M$ $\Delta G^{0_{H_2O}}_{app}$ [e]	χ^2 [f]
WT TR[a]	18.2 ± 2.0	–	–	1.2
W19F[b]	15.5 ± 2.0	8.6 ± 4.0	6.9	0.74
W99F[b]	18.6 ± 0.5	11.2 ± 3.0	7.4	0.34

[a] WT TR data were fit to a two-state equilibrium model.
[b] The data for W19F and W99F were fit to a three-state equilibrium model.
[c] Apparent free energy of folding (in kcal mol·dimer^{-1}) at pH 7.6 and 25 °C based on the global fit analysis of both spectroscopic data sets as described in the Materials and methods and Royer (1993). Errors in the free energy values are derived from rigorous confidence limit testing at the 67% level.
[d] The apparent free energy of folding (in kcal mol^{-1}) for a pair of monomers.
[e] Apparent free energy of folding values derived from free energy values in columns 2 and 4 (in kcal mol·dimer^{-1}).
[f] Values are global χ^2 weighted for the error in the measurements and the degrees of freedom of the fit and as such are relative values. They are presented for comparison of goodness of fit for the three proteins indicated.

of W99F and WT TR, and both CD and fluorescence unfolding profiles for W19F are shifted to much lower urea concentrations than WT TR or W99F (4.1 and 4.7 M urea, respectively, for fluorescence and CD).

Because the fluorescence profiles for both mutants undergo a larger shift to lower urea concentrations with respect to WT TR than the CD profiles, and because the fluorescence profiles are indicative of exposure of these interfacial tryptophans to water, it is not unreasonable to suggest that the destabilization introduced by mutation occurs predominantly in disruption of the dimer interface, not in perturbation of the intermediate monomer. The monomeric intermediates for the single tryptophan mutant proteins were predicted to be at least 4 kcal/(mol dimer) more stable than the unfolded form, and possibly as much as 8.6 and 11.2 kcal/(mol dimer) for W19F and W99F, respectively (Table 1, column 3). The lower limit was obtained from rigorous confidence limit testing. Unfortunately, stability of the monomer is not well-defined due to correlation with the values for its optical properties. Parameter correlation plots demonstrated that a higher estimated stability results in a higher population and a smaller ellipticity or redder average emission wavelength for the intermediate.

Although the molar ellipticity of the native form of W19F is within experimental uncertainty of that of WT TR, the value for W99F is reproducibly less negative (Table 2). The implied loss in helicity may reflect a direct effect on helix F (which contains this residue), an indirect effect on helices D and E (which are tethered to the core by helix F), or both. Whatever alternative native form is adopted by W99F, it is sufficiently stable to display a nearly urea-independent baseline region up to 3 M urea (Fig. 3).

The optical properties of the intermediate for W19F indicate an average emission wavelength of 360.3 nm and a molar ellipticity of -15.4×10^3 deg-cm^2/(dmol dimer), whereas those for W99F are 361.8 nm and -7.7×10^3 deg-cm^2/(dmol dimer) (Table 2). Given that the average emission wavelength for the completely unfolded W19F and W99F mutants occurs at 361.5 nm, the fluorescence results suggest that each tryptophan has 100% exposure to solvent in the intermediate ($Z = 1.00$ for both W19F and W99F). The molar ellipticities indicate that this intermediate for W19F retains about 80% ($Z = 0.22$) and the intermediate for W99F retains about 50% ($Z = 0.51$) of the native secondary structure (Table 2).

As noted above, the high correlation between stability and optical properties of the intermediate make estimates of these parameters somewhat imprecise. However, the lower midpoint for fluorescence transition compared to CD transition observed for both mutant proteins

Table 2. *Optical parameters for the urea-induced unfolding of WT TR and mutants W19F and W99F*

Protein	Technique	Native dimer: Optical maximum[a]	Intermediate monomer: Optical maximum[a]	Intermediate monomer: Z value[b]	Unfolded monomer: Optical maximum[a]
WT TR	CD (at 222 nm)	−22.2	–	–	−2.5
	Fl. av. em.[c]	343.0	–	–	359.5
W19F	CD (at 222 nm)	−20.8	−15.4	0.22	−1.7
	Fl. av. em.	351.0	360.3	1.00	361.5
W99F	CD (at 222 nm)	−17.3	−7.7	0.51	−1.7
	Fl. av. em.	340.5	361.8	1.00	361.5

[a] In units of 10^{-3} deg × cm^2 × dmol·dimer^{-1} for CD data and nm for the fluorescence average emission wavelength data.
[b] The Z value is the fractional change in the total optical signal in the native dimer to intermediate monomer transition and is defined as $Z = (Y_i - Y_n)/(Y_u - Y_n)$, where Y_i is the observed optical maximum for the intermediate monomer form of the protein, Y_n is the observed optical maximum for the native dimer form of the protein, and Y_u is the observed optical maximum for the unfolded monomer form of the protein.
[c] Fluorescence average emission.

(Figs. 2, 3) definitively shows that solvent exposure of the tryptophan residues must be proportionally greater than disruption of secondary structure.

An alternative equilibrium unfolding model for these mutant proteins would involve a dimeric intermediate with altered spectral properties. Although it is possible to discriminate between these two models by varying the protein concentration, simulations indicate that the concentration would have to be decreased more than 10-fold to observe reliable differences. Unfortunately, insufficient signal intensity under these conditions precludes such a test. It is, however, difficult to imagine how tryptophan exposure to solvent could increase in such dramatic fashion in a dimeric species. Another argument in favor of a monomeric intermediate is that both mutations have a similar effect on fluorescence and CD transition curves. The only obvious common property of Trp 19 and Trp 99 is that they are both located at interfaces between the subunits. Disruption of the subunit association energy by replacements at the interface could enhance the dissociation reaction and favor the formation of monomers. A similar phenomenon has been observed previously in urea-induced unfolding of the α subunit of tryptophan synthase. Mutations at the interface between two folding units in this monomeric protein enhance the population of a stable folding intermediate (Chen et al., 1992).

Discussion

Simplification of the fluorescence properties of the TR dimer by replacing either of the two tryptophans with a nonfluorescent analog demonstrates that the three kinetic phases detected in refolding of the wild-type protein reflect common rather than unique structural events. This conclusion is based on the observation that, although Trp 19 and Trp 99 (in the W99F and W19F mutants, respectively) are involved in different hydrophobic cores, both display the same three refolding phases as WT TR. In fact, the slight changes in amplitudes obtained for the two faster refolding phases of these mutants support previous work (Gittelman & Matthews, 1990) that suggested the involvement of transient intermediates. By contrast, the amplitude for the slowest refolding phase remained unchanged for these mutants because this reaction does not involve transient intermediates.

The current kinetic folding model proposes that these phases correspond to the rate-limiting step in each of three channels leading from the unfolded monomer to the native dimer (Gittelman & Matthews, 1990; Mann & Matthews, 1993). In each channel, the association of prefolded, monomeric intermediates is limiting between 3 and 5 M urea. Below 3 M urea, isomerization reactions that are independent of both urea and protein concentration become limiting (Figs. 4A, 5A). The isomerization reaction for the slowest, τ_1, channel is proposed to occur in an unfolded monomer and to precede the association step. The isomerization reactions for the intermediate, τ_2, and fast, τ_3, channels are thought to reflect rearrangements after the association step.

The present mutational analysis shows that both the association reactions and, in the cases of the τ_2 and τ_3 channels, the isomerization reactions, involve structural rearrangements in the vicinity of both Trp 19 and Trp 99. The location of these two tryptophans at the interfaces between subunits provides a ready explanation for the change in solvent exposure during the association reaction. If the assignment of the τ_2 and τ_3 isomerization reactions to postassociation events is correct, then the core (which contains Trp 19) and helix F (which contains Trp 99 and tethers the DNA reading heads to the core) must repack in the final stages of folding in these channels.

A very surprising consequence of replacing either tryptophan residue in TR with a phenylalanine is that equilibrium unfolding behavior becomes more complex. The noncoincidence of the fluorescence and CD transition curves shows clearly that disruption of the secondary and tertiary structure can be uncoupled, much as it is in α-lactalbumin for either a guanidine HCl-induced intermediate (Kuwajima et al., 1976) or for the acid form (Kuwajima et al., 1976; Kuwajima, 1977). The thermodynamic analysis of the TR data suggests that this intermediate species appears at substantial concentrations in the unfolding transition zone (as much as 50% of the population, based upon the data in Table 1).

Noncoincident fluorescence and CD transition curves for an oligomeric protein have been observed previously for the dimeric β_2 subunit of tryptophan synthase (Zetina & Goldberg, 1980). The multistate behavior was attributed to weak intersubunit interactions that give rise to monomeric folding intermediates (Carrey & Pain, 1978; Zetina & Goldberg, 1980). In other cases, greater separation between the native–intermediate and the intermediate–unfolded transitions leads to an inflection in the transition curve, e.g., λ repressor (Banik et al., 1992) and aspartate aminotransferase (Herold & Kirschner, 1990). The higher concentration of intermediate in these latter instances is an advantage for solution-phase structural studies. Additional mutations at the domain interface in TR might be useful in this regard.

Summary

The ability to manipulate the amino acid sequence of a protein to probe its folding reaction provides unique opportunities to test the role of specific side chains in this complex conformational transition (Matthews, 1987, 1991; Goldenberg, 1988; Matouschek & Fersht, 1991). In the case of TR, selective replacement of the two tryptophan residues reveals that the three kinetic refolding phases detected previously by fluorescence spectroscopy (Gittelman & Matthews, 1990) reflect global rather than local conformational changes. The present study also shows that

even these limited modifications in the sequence can significantly alter the equilibrium properties of a folding reaction. The breakdown in cooperativity detected for TR demonstrates that mutagenesis can also be used to enhance the population of a partially folded form, potentially simplifying the study of the folding mechanism.

Materials and methods

Chemicals

Ultrapure urea was purchased from Schwarz/Mann and was used without further purification. All other chemicals were reagent grade. The buffer used in all experiments was 10 mM sodium phosphate and 0.1 mM disodium ethylenediaminetetraacetate, pH 7.6, at 25 °C. The final protein concentration was 0.13 mg/mL (5.3 μM) for all experiments. All aporepressor concentrations are reported for the dimer.

Bacterial strains and plasmids

The *E. coli* strain DH5αF′ (φ80δ*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(r_K^-, m_K^+) *sup*E44 λ^- *thi*-1 *gyr*A *rel*A1) was obtained from Bethesda Research Laboratories. Strain CJ236 (*dut*1 *ung*1 *thi*1 *rel*A1/pCJ105[Cm^r]) was obtained from International Biotechnologies, Inc. Strain CY15070 (W3110 (*tna*A2 *trp*R2 *lac*I^q)) and the plasmid pJPR2, used for expression of WT TR, were provided by Dr. Charles Yanofsky, Stanford University. Bacteriophage M13mp18 and M13mp19 were obtained from Bethesda Research Laboratories.

Enzymes and nucleotides

Restriction endonucleases, T4 DNA ligase, and T4 polynucleotide kinase were purchased from New England Biolabs. Sequenase® DNA polymerase was purchased from United States Biochemical Corp. Deoxyribonucleotides were purchased from Pharmacia LKB Biotechnology. The [^{35}S]dATPαS was purchased from New England Nuclear.

Oligonucleotides

Oligonucleotides were purchased from the Protein and DNA Chemistry Facility, The Pennsylvania State University Biotechnology Center. The following oligonucleotides, with altered base(s) underlined, were synthesized: (1) removal of the BamHI restriction site mutagenic oligonucleotide, dGCCGGCCGGTTCCGTCGACC; (2) W19F mutagenic oligonucleotide, dCACCAGGAGTTTTTAC GTTTTGTGGACCTG; (3) W99F mutagenic oligonucleotide, dCTGCGCCAGTTTCTGGAGGAGGTGTTG; (4, 5) sequencing primers, dGAGAATAACCATGGCC CAAC and dTGGGGACTCGAGTGCGTATTG.

Site-directed mutagenesis

Mutagenesis procedures were initiated by isolation of the EcoRI/HindIII fragment from pJPR2 (Paluh & Yanofsky, 1986) containing the gene for WT TR. This fragment was ligated into the EcoRI/HindIII-digested M13mp19 DNA to produce mp19-*trp*R. Uracil-containing single-strand DNA was produced (Kunkel et al., 1987) and hybridized with mutagenic oligonucleotide 1. Double-stranded DNA was used to transform competent DH5αF′ cells (Hanahan, 1985), and the resulting viral plaques were used to prepare single-strand DNA for sequencing and identification of mutants (Sanger et al., 1980) using oligonucleotides 4 and 5. This first round of mutagenesis was used to remove the BamHI restriction site approximately 60 base pairs from the 3′ end of the *trp*R gene to aid in subsequent mutagenesis reactions. The mutated *trp*R DNA was removed from the mp19-*trp*R construct by digestion with BamHI/HindIII and recloned back into pJPR2 to test wild-type protein expression. As expected, expression of WT TR appeared to be normal from this new DNA construct, referred to as pJPR2-2B.

In the second round of mutagenesis, the BamHI/HindIII fragment from pJPR2-2B containing the gene for WT TR was ligated into BamHI/HindIII-digested M13mp19 DNA to produce mp19-*trp*R(2B). Uracil-containing single-strand DNA was produced from this construct as described above and hybridized with the W19F or W99F mutagenic oligonucleotides 2 and 3, respectively. Double-stranded DNA was used for transformation of competent cells and subsequent sequencing and identification of mutants as described above. For both single tryptophan mutants, the entire *trp*R gene was sequenced. The mutated *trp*R DNA was removed from the mp19-*trp*R(2B)-W19F or W99F constructs by digestion with BamHI/HindIII and recloned into BamHI/HindIII-digested pJPR2-2B to form the mutant constructs, pW19F and pW99F, respectively. These plasmids were used for protein expression and purification.

Protein purification

Wild-type TR and the mutants were purified by procedures described previously (Paluh & Yanofsky, 1986; Chou et al., 1989). Protein purity was verified by the presence of a single band on Coomassie blue-stained sodium dodecyl sulfate-polyacrylamide gels (Schagger & von Jagow, 1987).

The extinction coefficients of WT TR and the three mutant proteins at 280 nm were determined in the presence and absence of 6 M guanidine hydrochloride by the method of Gill and von Hippel (1989); the values for each protein in the absence of denaturant are shown in Table 3. The extinction coefficient for WT TR is about 7% lower than that reported by Joachimiak et al. (1983), 29,700 M^{-1} cm^{-1}, but within the estimated error of the method

Table 3. *Extinction coefficient and DNA binding activity measurements of WT TR and mutants W19F and W99F*

Protein	E_{280} (M^{-1} cm^{-1})[a]	K_d (nM)[b]
WT TR	27,500	0.8–0.9
W 19F	16,400	0.3–0.5
W 99F	16,400	0.6–1.0

[a] Extinction coefficients determined in triplicate by the method of Gill and Von Hippel (1989). The standard errors of the extinction coefficient values are ±300 (M^{-1} cm^{-1}).
[b] DNA binding measurements performed by Dr. Jannette Carey.

(10%). The dominant contribution of the tryptophan residues to the absorbance spectrum is obvious.

The effect of tryptophan replacements on the function of TR was determined by a DNA mobility-shift assay (Carey, 1988). As shown in Table 3, the binding affinities of the mutants are very similar to those of WT TR, indicating functional equivalence between WT TR and the two tryptophan mutants.

CD spectroscopy

Protein samples were equilibrated with denaturant for 1 h at 25 °C before recording spectra. Equilibrium CD spectra were determined on an AVIV Associates model 62DS circular dichroism spectrometer. The cuvette pathlength was 2.0 mm. More than 95% of the native ellipticity was observed when fully unfolded protein was diluted with buffer to return TR to the native conformation. Thus, the unfolding reaction is fully reversible for all proteins examined in this study.

Fluorescence spectroscopy

Data were collected using the procedures and instrumentation described in Royer et al. (1993).

Stopped-flow tryptophan fluorescence

Fluorescence experiments were conducted using a Bio-Logic SFM-3 stopped-flow module equipped with a Bio-Logic modular light source and monochromator. Samples were excited at 295 nm and emission above 320 nm was detected using a cut-off filter supplied by Bio-Logic. The cuvette pathlength was 2.0 mm, and the dead time was calculated to be 4 ms, using the procedure of Tonomura et al. (1978).

Data fitting

Equilibrium CD and fluorescence data were analyzed according to a three-state model that includes folded dimer, partially folded monomer, and unfolded monomer, using the numerically based program Bioeqs (Royer et al., 1990) with recent modifications (Royer, 1993). These modifications allow for consideration of multiple species with identical stoichiometries (i.e., folded and unfolded monomer), as well as chemical denaturant perturbations. The ellipticities and average fluorescence emission wavelengths of the partially folded monomeric intermediate species were variable parameters in the fits, as were the free energies of formation for the folded dimer and the intermediate. Also allowed to float were the values of the slopes, i.e., the cooperativity parameter, of the urea-induced unfolding reaction. The free energies and slopes were linked between the two data sets (fluorescence and CD) for each mutant or wild-type protein.

Using the nonlinear least-squares program NLIN (SAS Institute Inc., Cary, North Carolina), kinetic refolding data at or below 3.0 M urea were fit to a sum of three exponential terms and a constant term reflecting the steady-state signal amplitude:

$$Y(t) = \sum Y_i(0)\,[\exp(-t/\tau_i)] + Y_\infty, \qquad (2)$$

where $Y(t)$ is the total amplitude at time t; Y_∞ is the amplitude at infinite time; $Y_i(0)$ is the amplitude corresponding to the individual phase, i, at zero time; and τ_i is the associated relaxation time. Refolding data above 3.0 M urea were fit to a sum of three exponential terms of the type:

$$Y(t) = \sum Y_i(0)\left\{\frac{\exp(-t/\tau_i)}{1 + q_i' Y_i(0)\,[1 - \exp(-t/\tau_i)]}\right\} + Y_\infty, \qquad (3)$$

where the $1 + q_i' Y_i(0)\,[1 - \exp(-t/\tau_i)]$ term reflects the contribution of the unfolding reaction to the relaxation time in the unfolding transition zone (Bernasconi, 1976; Gittelman & Matthews, 1990).

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References

Banik, U., Saha, R., Mandal, N.C., Bhattacharyya, B., & Roy, S. (1992). Multiphasic denaturation of the λ repressor by urea and its implications for the repressor structure. *Eur. J. Biochem. 206*, 15–21.

Beechem, J.M. & Brand, L. (1985). Time-resolved fluorescence of proteins. *Annu. Rev. Biochem. 54*, 43–71.

Bernasconi, C.F. (1976). *Relaxation Kinetics*. Academic Press, New York.

Carey, J. (1988). Gel retardation at low pH resolves *trp* repressor–DNA complexes for quantitative study. *Proc. Natl. Acad. Sci. USA 85*, 975–979.

Carrey, E.A. & Pain, R.H. (1978). Conformations of a stable intermediate on the folding pathway of *Staphylococcus aureus* penicillinase. *Biochim. Biophys. Acta 533*, 12–22.

Chen, X.C., Rambo, R., & Matthews, C.R. (1992). Amino acid replacements can selectively affect the interaction energy of autonomous folding units in the α subunit of tryptophan synthase. *Biochemistry 31*, 2219–2223.

Chou, W.-Y., Bieber, C., & Matthews, K.S. (1989). Tryptophan and 8-anilino-1-naphthalenesulfonate compete for binding to *trp* repressor. *J. Biol. Chem. 264*, 18309–18313.

Chou, W.-Y. & Matthews, K.S. (1989). Serine to cysteine mutations in *trp* repressor protein alter tryptophan and operator binding. *J. Biol. Chem. 264*, 18314–18319.

Freemont, P., Lane, A.N., & Sanderson, M. (1991). Structural aspects of protein-DNA recognition. *Biochem. J. 278*, 1–24.

Garvey, E.P., Swank, J., & Matthews, C.R. (1989). A hydrophobic cluster forms early in the folding of dihydrofolate reductase. *Proteins Struct. Funct. Genet. 6*, 259–266.

Gill, S.C. & von Hippel, P.H. (1989). Calculation of protein extinction coefficients from amino acid sequence data. *Anal. Biochem. 182*, 319–326.

Gittelman, M.S. & Matthews, C.R. (1990). Folding and stability of *trp* aporepressor from *Escherichia coli*. *Biochemistry 29*, 7011–7020.

Goldenberg, D.P. (1988). Genetic studies of protein stability and mechanisms of folding. *Annu. Rev. Biophys. Biophys. Chem. 17*, 481–507.

Gunsalus, R.P. & Yanofsky, C. (1980). Nucleotide sequence and expression of *Escherichia coli trp*R, the structural gene for the *trp* aporepressor. *Proc. Natl. Acad. Sci. USA 77*, 7717–7121.

Hanahan, D. (1985). Techniques for transformation of *Escherichia coli*. In *DNA Cloning: A Practical Approach*, Vol. 1 (Glover, D.M., Ed.), pp. 109–135. Oxford University Press, Oxford, UK.

Herold, M. & Kirschner, K. (1990). Reversible dissociation and unfolding of aspartate aminotransferase from *Escherichia coli*: Characterization of a monomeric intermediate. *Biochemistry 29*, 1907–1913.

Joachimiak, A., Kelley, R.L., Gunsalus, R.P., Yanofsky, C., & Sigler, P.B. (1983). Purification and characterization of *trp* aporepressor. *Proc. Natl. Acad. Sci. USA 80*, 668–672.

Kunkel, T.A., Roberts, J.D., & Zakour, R.A. (1987). Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol. 154*, 367–383.

Kuwajima, K. (1977). A folding model of α-lactalbumin deduced from the three-state denaturation mechanism. *J. Mol. Biol. 114*, 241–258.

Kuwajima, K., Garvey, E.P., Finn, B.E., Matthews, C.R., & Sugai, S. (1991). Transient intermediates in the folding of dihydrofolate reductase as detected by far-ultraviolet circular dichroism spectroscopy. *Biochemistry 30*, 7693–7703.

Kuwajima, K., Nitta, K., Yoneyama, M., & Sugai, S. (1976). Three-state denaturation of α-lactalbumin by guanidine hydrochloride. *J. Mol. Biol. 106*, 359–373.

Lee, B. & Richards, F.M. (1971). The interpretation of protein structures: Estimation of static accessibility. *J. Mol. Biol. 55*, 379–400.

Mann, C.J. & Matthews, C.R. (1993). Structure and stability of an early folding intermediate of *Escherichia coli trp* aporepressor measured by far-UV stopped-flow circular dichroism and 8-anilino-1-naphthalene sulfonate binding. *Biochemistry 32*, 5282–5290.

Matouschek, A. & Fersht, A.R. (1991). Protein engineering in analysis of protein folding pathways and stability. *Methods Enzymol. 202*, 82–112.

Matthews, C.R. (1987). Effect of point mutations on the folding of globular proteins. *Methods Enzymol. 154*, 498–511.

Matthews, C.R. (1991). The mechanism of protein folding. *Curr. Opin. Struct. Biol. 1*, 28–35.

Pace, C.N. (1986). Determination and analysis of urea and guanidine hydrochloride denaturation curves. *Methods Enzymol. 131*, 266–280.

Paluh, J.L. & Yanofsky, C. (1986). High level production and rapid purification of the *E. coli trp* repressor. *Nucleic Acids Res. 14*, 7851–7860.

Royer, C.A. (1993). Improvements in the numerical analysis of thermodynamic data from biological complexes. *Anal. Biochem. 210*, 91–97.

Royer, C.A., Mann, C.J., & Matthews, C.R. (1993). Resolution of the fluorescence equilibrium unfolding profile of *trp* aporepressor using single tryptophan mutants. *Protein Sci. 2*, 1844–1852.

Royer, C.A., Smith, W.M., & Beechem, J.M. (1990). Analysis of binding in macromolecular complexes: A generalized numerical approach. *Anal. Biochem. 191*, 287–299.

Sanger, F., Coulson, A.R., Barrell, B.G., Smith, A.J., & Roe, B.A. (1980). Cloning in single-stranded bacteriophage as an aid to rapid DNA sequencing. *J. Mol. Biol. 143*, 161–178.

Schagger, H. & von Jagow, G. (1987). Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem. 166*, 368–379.

Schellman, J.A. (1978). Solvent denaturation. *Biopolymers 17*, 1305–1322.

Steitz, T.A. (1990). Structural studies of protein-nucleic acid interaction: The sources of sequence-specific binding. *Q. Rev. Biophys. 23*, 205–280.

Tonomura, B., Nakatani, H., Ohnishi, M., Yamaguchi-Ito, J., & Hiromi, K. (1978). Test reactions for a stopped-flow apparatus. Reduction of 2,6-dichlorophenolindophenol and potassium ferricyanide by L-ascorbic acid. *Anal. Biochem. 84*, 370–383.

Zetina, C.R. & Goldberg, M.E. (1980). Reversible unfolding of the β2 subunit of *Escherichia coli* tryptophan synthetase and its proteolytic fragments. *J. Mol. Biol. 137*, 401–414.

Zhang, R.-G., Joachimiak, A., Lawson, C.L., Schevitz, R.W., Otwinowski, Z., & Sigler, P.B. (1987). The crystal structure of *trp* aporepressor at 1.8 Å shows how binding tryptophan enhances DNA affinity. *Nature 327*, 591–597.