Characterization of the Slow Folding Reactions of *trp* Aporepressor from *Escherichia coli* by Mutational Analysis of Prolines and Catalysis by a Peptidyl-prolyl Isomerase†

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ABSTRACT: *Escherichia coli trp* aporepressor (TR) is a highly helical, dimeric protein whose folding has been shown to involve three phases whose relaxation times range from 200 ms to 50 s at 25 °C and pH 7.6 [Gittelman, M. S., & Matthews, C. R. (1990) *Biochemistry 29,* 7011−7021]. All three phases are urea and protein concentration independent below 3 M urea, suggesting that *cis/trans* proline isomerization might limit the folding of TR under these conditions. This hypothesis was tested by measuring the sensitivity of the folding reaction to site-directed mutagenesis and to cyclophilin, a peptidyl-prolyl isomerase. Each of the four proline residues in TR was replaced singly as well as simultaneously, and the effects on the folding mechanism were assessed. All of these mutants, including the version lacking prolines (des-Pro TR), retain three slow, denaturant-independent folding phases similar to those observed for wild-type TR. However, the pattern of catalysis of the two slower folding phases in wild-type and mutant TRs by cyclophilin shows that *cis/trans* isomerization of the Thr44/Pro45 peptide bond can limit folding in proteins containing Pro45. The observation of three urea-independent, slow folding phases in des-Pro TR demonstrates that proline isomerization is not solely responsible for this complex folding behavior. Other types of isomerization or conformational rearrangement reactions appear to limit the folding of this dimeric protein under strongly folding conditions.

The time required for unstructured proteins to fold to their unique, native conformations can range from milliseconds (Alexander *et al.*, 1992; Garel & Baldwin, 1973; Houry *et al.*, 1994; Jackson & Fersht, 1991; Sosnick *et al.*, 1994) to hours (Carrell *et al.*, 1991), depending upon the amino acid sequence and the environmental conditions. It has been argued that, in many instances, reactions requiring more than milliseconds reflect events other than the formation of higher order structure (Sosnick *et al.*, 1994). Examples include the formation of disulfide bonds (Creighton, 1992; Weissman & Kim, 1993), *cis/trans* isomerization at Xaa−Pro peptide bonds (Brandts *et al.*, 1975; Hurle *et al.*, 1991; Kelley & Richards, 1987; Kiefhaber *et al.*, 1990; Kuwajima *et al.*, 1991; Schultz *et al.*, 1992; Wood *et al.*, 1988), and ligand-exchange reactions at heme moieties (Elove *et al.*, 1994; Muthukrishnan & Nall, 1991; Sosnick *et al.*, 1994). Although these reactions may play a significant role in limiting folding *in vitro* and *in vivo*, they are not a fundamental aspect of the mechanism by which the primary structure directs the rapid acquisition of the secondary and tertiary structure. The solution of the folding problem for any protein requires that such reactions, if they occur, be identified and discriminated from actual folding events.

Wild-type *Escherichia coli trp* aporepressor (TR)[1] provides an interesting example of a protein whose slow folding reactions may be candidates for such nonessential constraints on folding. TR is a highly helical, dimeric DNA-binding protein whose subunits are intimately intertwined (Figure 1) (Zhang *et al.*, 1987). Helices A, B, and C in one subunit dock against the same set of helices of the other subunit to form the central core of TR. Helices D and E and the intervening turn form the DNA reading heads. Helix F docks against that part of the core formed by the other subunit. TR contains proline residues at positions 6, 37, 45, and 93, no disulfide bonds, and, in its apo form (*i.e.*, in the absence of the corepressor L-tryptophan), no prosthetic groups. The peptide bonds at Pro37, Pro45, and Pro93 are in the *trans* conformation in the native structure; the isomeric state of Pro6 is not known because it occurs in a region of the sequence which is not well-defined in the X-ray structure.

Previous studies on the folding of TR (Gittelman & Matthews, 1990; Mann & Matthews, 1993) have demonstrated the existence of a burst phase reaction that is complete within 5 ms followed by three slow folding reactions. The burst phase reaction yields a monomeric species which has substantial secondary structure and nonpolar surfaces and displays an estimated stability of ~3 kcal mol^{-1}. For refolding to final urea concentrations between 3 and 5 M, the relaxation times of the three slow folding phases depend upon the protein concentration and the denaturant concentration. Below 3 M urea, these relaxation times become independent of both the protein and denaturant concentration. This behavior has been interpreted in terms of three parallel folding channels which are limited by bimolecular association

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[1] Abbreviations: CD, circular dichroism; des-Pro TR, P6H/P37A/P45G/P93G quadruple mutant of TR; Na_2EDTA, ethylenediaminetetraacetic acid, disodium salt; $NaDodSO_4$, sodium dodecyl sulfate; WT TR, wild-type *trp* aporepressor.

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reactions at moderate urea concentrations and by isomerization reactions, possibly proline isomerization, at low urea concentrations.

To test the role of proline isomerization in these rate-limiting steps in the folding of TR, each of the four prolines residues was replaced individually and collectively by amino acids which do not so constrain the polypeptide backbone. All of the single mutants and the quadruple mutant retain the same number of slow folding phases and the distinctive urea dependences seen in the wild-type protein. The pattern of cyclophilin catalysis of the two slower folding phases in these proteins, however, shows that isomerization of the Thr44/Pro45 peptide bond can limit folding in the proteins which contain Pro45. The retention of three, urea-independent folding phases for des-Pro TR demonstrates that isomerization or rearrangement reactions which are unrelated to proline isomerization can control the folding of TR under strongly folding conditions.

MATERIALS AND METHODS

Chemicals. The synthetic peptide *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide and α-chymotrypsin were purchased from Sigma. Ultrapure urea was purchased from ICN Biomedicals, Inc., and 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 Na_2EDTA at pH 7.6. The final protein concentration in all experiments was 5.3 μM; concentrations are reported in terms of the dimeric form of the protein.

Bacterial Strains and Plasmids. *E. coli* strains and plasmids used for site-directed mutagenesis and protein expression have been previously described (Mann *et al.*, 1993). The plasmid encoding the gene for the P6H mutation was kindly provided by Dr. Barry Hurlburt, University of Arkansas at Little Rock. *E. coli* strain XA90 (Δ*lacpro*XIII, *ara*, *nal*A, *arg*E(am), *thi*, *rif*^{r}, {F'*lac*I^{Q1}ZY, *pro*AB}) was a gift from Dr. Gregory Verdine, Harvard University. Plasmid pHCyPA encoding the wild-type human cyclophilin was a gift from Dr. Christopher T. Walsh, Harvard Medical School.

Enzymes and Nucleotides. Restriction endonucleases, T4 DNA ligase, and T4 polynucleotide kinase were purchased from New England Biolabs. The Sequenase sequencing kit was obtained from United States Biochemical Corp. and [^{35}S]dATPαS from New England Nuclear. A reagent kit for PCR mutagenesis was purchased from Perkin Elmer.

Site-Directed Mutagenesis. The single-proline mutants P6H, P37A, P45G, and P93G were constructed by a previously described procedure (Mann *et al.*, 1993). The quadruple mutant des-Pro was synthesized in a sequential fashion using five rounds of PCR mutagenesis (Dulau *et al.*, 1989; Landt *et al.*, 1990) on a Cetus DNA thermal cycler (Perkin Elmer). For all the mutants produced, the entire *trp* R gene was sequenced to ensure that no undesired mutations had occurred (Sanger *et al.*, 1980).

Protein Purification and DNA-Binding Properties. WT and mutant TR were purified by procedures previously described (Chou & Matthews, 1989; Mann *et al.*, 1993; Paluh & Yanofsky, 1986). Human recombinant cyclophilin was purified according to Liu *et al.* (1990). Protein purity was verified by the presence of a single band on Coomassie blue-stained $NaDodSO_4$−polyacrylamide gels (Schagger & von Jagow, 1987). The DNA-binding properties of each of the mutant proteins were measured by the restriction site protection assay (Joachimiak *et al.*, 1983); the activities of all of the mutants are similar to that of WT TR.

Cyclophilin Activity Assay. The activity of cyclophilin was measured by the method of Schönbrunner *et al.* (1991). Changes in absorbance at 390 nm were monitored with an Aviv/Cary 118 DS spectrophotometer interfaced to an AT&T PC6300 computer. Data were fit with the nonlinear least-squares program NLIN (SAS Institute, Cary, NC).

Circular Dichroism Spectroscopy. CD spectra were collected on an AVIV Associates model 62DS circular dichroism spectrometer. Protein samples were incubated at a given urea concentration for 1 h at 25 °C before recording spectra. Longer incubation times did not lead to further changes in the signal, ensuring complete equilibration under these conditions. The cuvette path length was 2.0 mm.

Stopped-Flow Tryptophan Fluorescence. Stopped-flow tryptophan fluorescence experiments were conducted on a Bio-logic SFM-3 stopped-flow spectrometer. Samples were excited at 295 nm, and emission was detected above 320 nm. The cuvette path length was 2.0 mm, and the dead time of mixing was determined to be 5 ms by the method of Tonomura *et al.* (1978). Three different refolding experiments were performed: (1) Refolding was initiated from 7 M urea to different final urea concentrations at 25 °C. The unfolded protein was incubated at 25 °C for 1 h prior to a 10-fold dilution to induce refolding. (2) Refolding was initiated from 8 to 2 M urea at 10 °C in the presence of cyclophilin at concentrations ranging from 0 to 500 nM. (3) The temperature-dependence of the refolding reaction from 8 to 2 M urea was investigated over the range from 10 to 34 °C.

Data Analysis. (A) Equilibrium Data. Equilibrium data were fit to two-state model (Gittelman & Matthews, 1990):

$$N_2 \overset{K}{\rightleftharpoons} 2U \tag{1}$$

where N_2 is the native dimer, U is the unfolded monomer, and $K = [U]^2/[N_2]$. A detailed description of this procedure can be found elsewhere (Gittelman & Matthews, 1990; Mann & Matthews, 1993).

(B) Kinetic Data. The kinetic refolding traces were analyzed with the nonlinear least-squares statistical program NLIN. Refolding data at or below 3 M urea were fit to the sum of three exponentials and a constant reflecting the steady-state signal amplitude:

$$A(t) = \sum A_i(0) \exp(-t/\tau_i) + A_\infty \tag{2}$$

where $A(t)$ is the total amplitude at time t, A_∞ is the amplitude at infinite time, $A_i(0)$ is the amplitude corresponding to the individual phase, i, at zero time, and τ_i is the associated relaxation time. Refolding data above 3 M urea were fit to the sum of three complex exponentials and a constant:

$$A(t) = \sum A_i(0)\{\exp(-t/\tau_i)/ [1 + q'A_i(0)[1 - \exp(-t/\tau_i)]]\} + A_\infty \tag{3}$$

where the $q'_iA_i(0)[1 - \exp(-t/\tau_i)]$ term reflects the contribution of the unfolding reaction in the unfolding transition zone (Bernasconi, 1976; Gittelman & Matthews, 1990). The

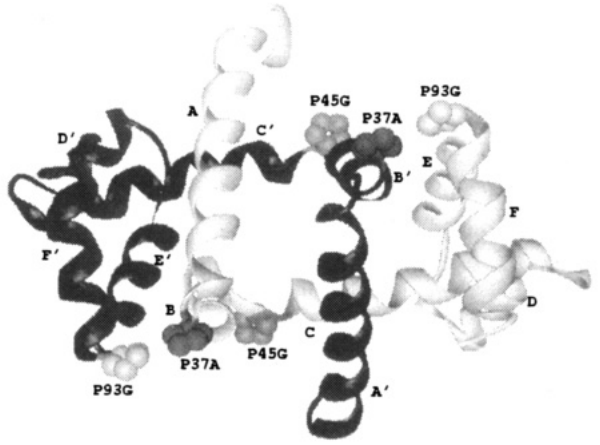

FIGURE 1: Ribbon diagram of dimeric TR (Zhang *et al.*, 1987), in which two subunits are shown in dark and light shading. Pro37, Pro45, and Pro93 are displayed, and the six α-helices are labeled.

relative amplitude of each phase at a given urea concentration, α_i , was calculated from the equation:

$$\alpha_i = A_i(0)/\sum A_i(0) \quad (4)$$

RESULTS

The replacements at Pro37, Pro45, and Pro93 were chosen on the basis of the involvement of these side chains in or between elements of secondary structure (Figure 1). Pro37, in the middle of helix B, was replaced by alanine which has a high helical propensity (Chou & Fasman, 1978; Merutka & Stellwagen, 1990; Lyu *et al.*, 1990; O'Neil & DeGrado, 1990; Padmanabhan *et al.*, 1990). Pro45, at the amino terminus of helix B, was replaced by glycine which is a known helix-breaker (Piela *et al.*, 1987; Fasman, 1989). Pro93, which is located in the turn between helices E and F, was replaced by glycine which is often found in turns (Chou & Fasman, 1978). Pro6, in an unstructured region preceding the core of TR, has been previously replaced by histidine and found to have no effect on function (personal communication, Dr. Barry Hurlburt, University of Arkansas at Little Rock).

Equilibrium Studies. The effects of these amino acid replacements, both individually and collectively, on the stability of TR to urea-induced unfolding are shown in Figure 2A,B. In the absence of denaturant, the mean residue ellipticities of wild-type, P6H, P37A, P45G, P93G, and des-Pro TR at 222 nm are equal within experimental error, $(-20 \pm 2) \times 10^3$ deg cm^2 dmol^{-1}. The data show that these replacements do not perturb the secondary structure of fully folded TR to any significant extent. As the urea concentration is increased to 4 M, the small, linear decreases in ellipticity are consistent with the presence of stable, native conformations. Between 4 and 6−7 M urea (depending upon the protein), the large, sigmoidal decreases in ellipticity reflect cooperative unfolding transitions for all six proteins. Above 7 M urea, the residual ellipticities are very small and indistinguishable, suggesting similar unfolded structures. All of these unfolding reactions are fully reversible (data not shown). Representative examples of this behavior are shown in Figure 2A.

These data were normalized, as described under Materials and Methods, to yield the fraction of unfolded protein, F_{app}, at various urea concentrations (Figure 2B). The midpoints

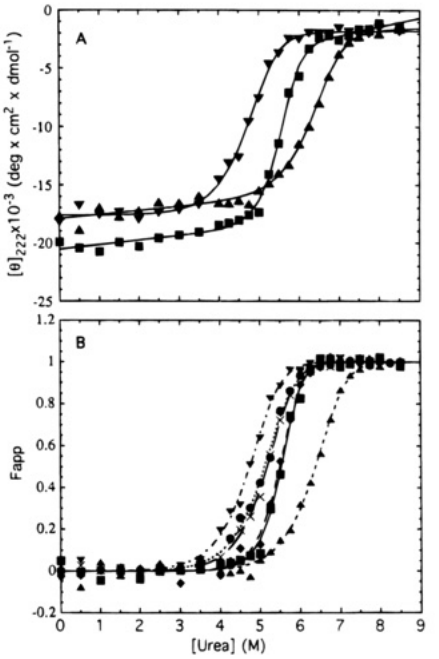

FIGURE 2: (A) Representative plots of the mean residue ellipticity of wild-type TR (■), P37A (▲), and P45G (▼) as a function of the urea concentration at pH 7.6 and 25 °C. (B) Dependence of the apparent fraction of unfolded protein, F_{app}, on urea concentration at pH 7.6 and 25 °C as monitored by CD spectroscopy for wild-type (■), P6H (♦), P37A (▲), P45G (▼), P93G (●), and des-Pro (×) TR. The symbols represent the values calculated from the observed data using the equation: $F_{app} = ([\theta]_O - [\theta]_N)/([\theta]_U - [\theta]_N)$, where $[\theta]_O$ is the observed mean residue ellipticity and $[\theta]_U$ and $[\theta]_N$ are the ellipticities of the native and unfolded forms at the same urea concentration. The lines are the theoretical fits to a two-state model (Mann & Matthews, 1993). The protein concentration was 5.3 μM in all experiments.

Table 1: Thermodynamic Parameters for WT TR and Proline Mutants[a]

	$\Delta G°(H_2O)$ (kcal mol^{-1})	$-A$ (kcal mol^{-1} M^{-1})	C_m (M)
WT	25.1 ± 1.7	3.3 ± 0.3	5.54 ± 0.03
P6H	24.0 ± 1.4	3.1 ± 0.3	5.49 ± 0.03
P37A	20.8 ± 1.4	2.2 ± 0.2	6.37 ± 0.08
P45G	16.9 ± 0.9	2.1 ± 0.2	4.74 ± 0.05
P93G	17.7 ± 0.9	2.1 ± 0.2	5.11 ± 0.04
des-Pro	18.8 ± 0.8	2.3 ± 0.2	5.18 ± 0.03

[a] The unfolding transitions were monitored by CD spectroscopy at 222 nm at pH 7.6 and 25 °C. The buffer was 10 mM $NaPO_4$, 0.1 mM Na_2EDTA, and the protein concentration was 5.3 μM in terms of dimer.

of the unfolding transitions vary as do the slopes of the curves in the transition region. Fits of these data to a two-state model involving a native dimer and unfolded monomer (eq 1) yield estimates for the stability in the absence of denaturant, $\Delta G°(H_2O)$, the cooperativity parameter, A, and the midpoint, C_m. The results of these fits are given in Table 1.

Wild-type and P6H TR have virtually identical thermodynamic parameters, as might be expected for a mutation in an unstructured region. P37A, P45G, P93G, and des-Pro TR all have reduced stabilities in the absence of denaturant as compared to WT TR. These mutants also have very similar cooperativity parameters which are significantly less than that of wild-type protein. The decreased cooperativity parameter for P37A is responsible for the lower stability in

water even though its midpoint is higher than for wild-type TR. These equilibrium unfolding curves demonstrate that the proline mutants retain cooperative unfolding transitions and facilitate the design of kinetic experiments to probe their folding mechanisms.

Kinetic Studies. The effects of the individual and collective replacement of the four proline residues in TR on the folding kinetics were examined by stopped-flow fluorescence spectroscopy. The substantially greater signal/noise available with fluorescence spectroscopy studies of the two intrinsic tryptophans and the equality of the relaxation times with those obtained by CD spectroscopy for the wild-type protein (Mann & Matthews, 1993) justify the use of this technique.

The refolding traces of all of the proline mutants, similar to wild-type TR, required three phases for a satisfactory fit. The data between 1.5 and 3 M urea could be fit to a sum of simple exponentials (eq 2) and yielded relaxation times which were independent of the denaturant concentration. At or above 3 M urea, the relaxation times for P6H, P45G, and P93G TR increase at increasing final urea concentration; for des-Pro TR, the increase occurs at 4 M urea. In this range, where subunit association, not isomerization, dominates for all but P37A TR, the traces were fit to a sum of complex exponentials (eq 3), which include a term that reflects the contribution of the unfolding reaction. Because the relaxation times for P37A TR remain independent of the denaturant concentration up to 4.5 M urea, these data were fit to the same sum of simple exponentials used at lower urea concentration. Refolding data could not be collected below 1.5 M urea due to aggregation.

For wild-type TR, the slowest phase, τ_1, has a relaxation time near 10 s, the intermediate phase, τ_2, has a relaxation time near 1 s, and the fastest phase, τ_3, has a relaxation time near 200 ms at 2 M urea and 25 °C (Figure 3). The relaxation times for the P6H mutant are very similar to those of the wild-type protein, consistent with the lack of involvement with the amino-terminal segment in the structure or stability of TR. For P37A TR, the three relaxation times are similar to those for wild-type protein below 3 M urea. Unlike the wild-type protein, all three relaxation times for P37A remain independent of urea between 3 and 4.5 M. This unique behavior may reflect the increase in the midpoint of the equilibrium transition (Figure 2B) and a corresponding increase in the rate of association that would render the isomerization reactions limiting at higher urea concentrations. The τ_2 and τ_3 relaxation times for P45G may be slightly longer than those for wild-type TR below 3 M urea; the τ_1 reaction may be slightly faster. Above 3 M urea, the relaxation times are similar to those for wild-type TR. For P93G, the τ_2 and τ_3 relaxation times are again somewhat longer than those for wild-type TR below 3 M urea but similar at higher urea concentrations; the τ_1 relaxation time is identical to that for wild-type TR under all folding conditions. For des-Pro TR, the relaxation times for the τ_1, τ_2, and τ_3 phases are somewhat shorter and longer, respectively, than wild-type protein; the τ_2 relaxation times are unchanged below 3 M urea. The 10-fold increase in the τ_1 phase between 3.5 and 4 M urea may reflect the inherent error in measurements of a small amplitude phase. The τ_2 and τ_3 phases only display modest increases in relaxation time over this same range. The relative amplitudes for the τ_1, τ_2, and τ_3 phases are also virtually unchanged in these mutant proteins and represent approximately 10%, 40%, and 50%, respectively, of the total change in the fluorescence signal at 2 M urea and 25 °C (Table 2).

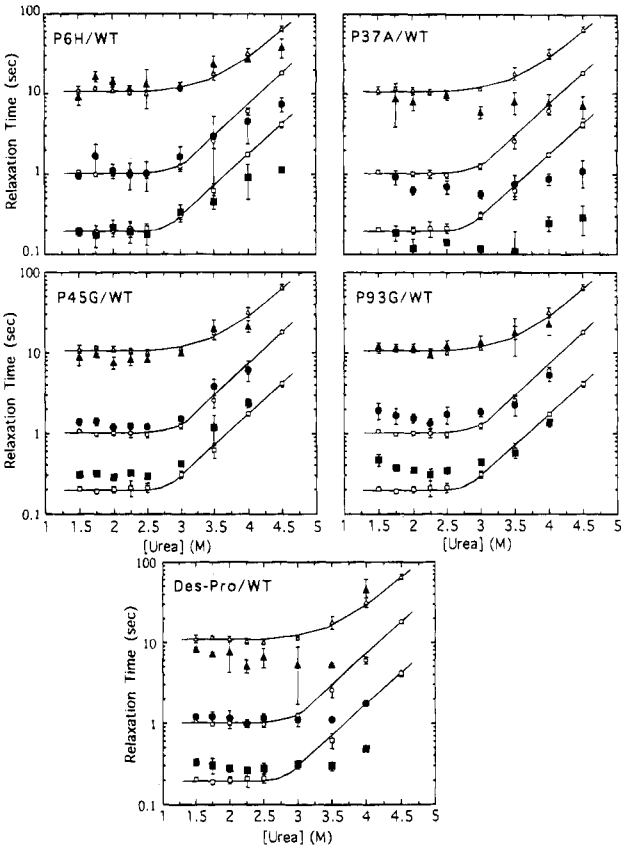

FIGURE 3: Urea dependence of the refolding relaxation times for the τ_1 (△, ▲), τ_2 (○, ●), and τ_3 (□, ■) phases of WT TR (open symbols) and specified proline mutants (closed symbols) at pH 7.6 and 25 °C. All refolding reactions were monitored by stopped-flow fluorescence spectroscopy. The final protein concentration was 5.3 μM. Lines through the WT TR data are drawn to aid the eye. The error bars indicate standard deviations obtained from triplicate runs at each urea concentration.

Catalysis by Peptidy-prolyl Isomerase. The observation of three slow, urea-independent folding phases in all of the proline mutants in TR, including des-Pro TR, at low urea concentrations shows that these reactions must controlled by a phenomenon besides proline isomerization. However, the 2-fold variation in the τ_1 relaxation time for des-Pro at low urea concentrations (Figure 3) suggests that proline isomerization and alternative, rate-limiting reactions may be coupled (Kiefhaber *et al.*, 1992). To test this hypothesis, the slow folding reactions in TR were further investigated for their sensitivity to catalysis by cyclophilin, a peptidyl-prolyl isomerase (Fischer *et al.*, 1989).

The τ_1 phases in wild-type, P6H, P37A, and P93G TR are all sensitive to the concentration of cyclophilin; the observed rate constant increases by a factor of 3−3.5 in the presence of 500 nM cyclophilin (Figure 4). The τ_2 phases for wild-type, P6H, and P93G TR are also accelerated but only by factors of 1.8−2.2. The absence of an effect for the τ_2 phase in P37A may again reflect an enhanced stability of the relevant intermediate in this mutant protein (see Discussion). The τ_3 phases for these four proteins are accelerated by a factor of 1.5 or less at 500 nM cyclophilin, close to the variation observed in des-Pro TR. The apparent acceleration of the τ_3 phase may also reflect the acceleration of the τ_2 phase so that its relaxation time approaches that

Table 2: Relative Amplitudes of the Three Slow Phases for Refolding Jumps of WT and Mutant TRs by Fluorescence Spectroscopy[a]

	WT	P6H	P37A	P45G	P93G	des-pro
			25 °C			
τ_1	13.3 ± 0.6	10.3 ± 0.6	12.2 ± 2.8	12.9 ± 2.2	16.3 ± 1.2	9.0 ± 3.9
τ_2	37.8 ± 2.7	36.0 ± 3.8	36.7 ± 3.6	41.7 ± 2.1	31.7 ± 1.7	38.9 ± 4.3
τ_3	48.9 ± 2.6	53.7 ± 3.4	51.1 ± 2.1	45.4 ± 4.1	52.0 ± 2.2	52.2 ± 8.0
			10 °C			
τ_1	12.9 ± 0.3	12.8 ± 1.1	10.7 ± 0.8	19.8 ± 2.9	16.0 ± 0.5	18.4 ± 2.8
τ_2	43.5 ± 3.1	43.1 ± 3.6	37.3 ± 1.1	53.4 ± 3.9	39.6 ± 5.4	60.1 ± 3.4
τ_3	43.5 ± 3.3	44.1 ± 4.3	52.0 ± 1.8	26.8 ± 6.4	44.4 ± 6.1	21.5 ± 6.1

[a] Data were obtained by refolding TR to 2 M final urea concentration using the same buffer conditions as described in Table 1. The final protein concentration was 5.3 μM in terms of dimer.

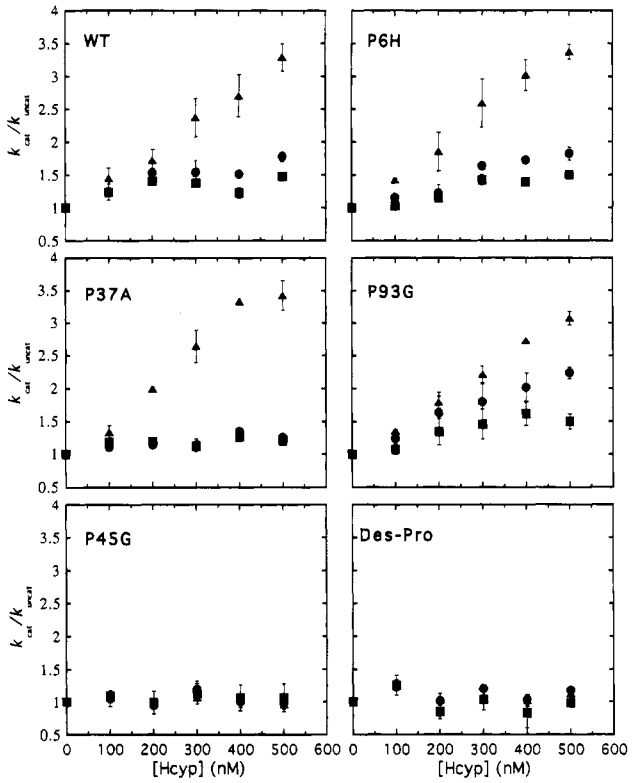

FIGURE 4: Catalytic effect of different concentrations of cyclophilin on the τ_1 (▲), τ_2 (●), and τ_3 (■) phases for WT TR and the proline mutants at pH 7.6 and 10 °C. The final TR protein concentration was 5.3 μM. The refolding rates were obtained from the reciprocal of the corresponding refolding relaxation times (Matthews, 1987). The ratio of k_{cat}/k_{uncat} was calculated from the refolding rate at a given cyclophilin concentration and the rate in the absence of cyclophilin. The error bars are indicated or comparable to the size of the symbols.

for the τ_3 phase. The coalescence of these two phases makes it difficult to resolve them and obtain accurate values for the relaxation times. Considering these complexities, it appears that cyclophilin has little or no effect on the τ_3 phase. The relative amplitudes of all of the refolding phases are independent of the cyclophilin concentration (data not shown), as expected for a folding catalyst. In striking contrast, all three relaxation times for P45G and des-Pro TR are insensitive to the cyclophilin. The simplest explanation for these data is that the τ_1 and τ_2 phases in wild-type TR are coupled to proline isomerization at the Thr44/Pro45 peptide bond.

The dramatic differences in the response of P45G and des-Pro TR to cyclophilin are also observed in the relative amplitudes of the three folding phases at lower temperatures (Table 2). At 10 °C, the relative amplitudes of the τ_1 and τ_2 phases for P45G and des-Pro TR are significantly larger than those for the other proteins in this set. Correspondingly, the amplitude of the τ_3 phase is smaller for P45G and des-Pro TR. Among the proline residues, Pro45 appears to play a unique role in the folding mechanism of TR.

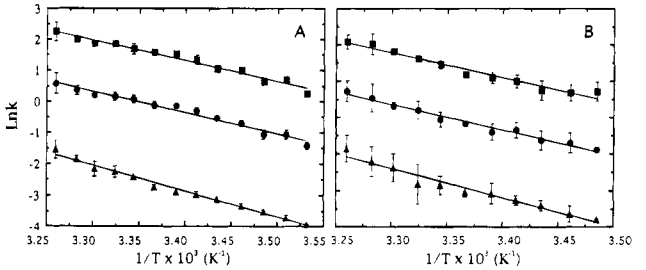

FIGURE 5: Arrhenius plots for the τ_1 (▲), τ_2 (●), and τ_3 (■) phases of (A) WT and (B) des-Pro TR. The error bars are indicated or comparable to the size of the symbols. The lines are the linear fits for each phase.

Activation Energies. Additional information on the nature of the rate-limiting steps in the folding of des-Pro TR was obtained by examining the temperature dependence of the observed rate constants under strongly folding conditions. Arrhenius plots of the data collected over the range from 10 to 34 °C at 2 M urea are shown in Figure 5; similar data for wild-type TR are also provided for comparison. For des-Pro TR, the activation energies for the τ_1, τ_2, and τ_3 phases are 15.7 ± 1.1, 14.0 ± 0.7, and 13.6 ± 0.8 kcal mol^{-1}, respectively; the values for wild-type TR are 16.3 ± 0.6, 13.7 ± 0.7, and 13.3 ± 0.7 kcal mol^{-1}. If one assumes that transition state theory can be applied to these folding reactions (Hammes, 1978; Matthews, 1987), the enthalpic barriers implied by these activation energies ($\Delta H^{\ddagger} = E_a - RT$) are relatively similar for wild-type and des-Pro TR for all three folding phases. These data for wild-type TR supersede those from an earlier, more limited study (Gittelman & Matthews, 1990) which reported a substantially greater activation energy for the τ_1 phase.

DISCUSSION

Rate-Limiting Folding Reactions. The results presented in this and previous studies (Gittelman & Matthews, 1990; Mann & Matthews, 1993) demonstrate that the rate-limiting steps in the folding of dimeric TR can involve either of two classes of reactions. The relaxation time of the first class depends upon both the protein and the denaturant concentration and clearly reflects the subunit association reaction. The second class does not depend upon either the protein or the denaturant concentration. These properties are characteristic of isomerization reactions which could involve either the

backbone, *e.g.*, *cis/trans* prolyl isomers (Brandts *et al.*, 1975), or rearrangements of folded domains which result in little or no net change in the exposure of side chains or backbone to solvent. The hypothesis that the second class of reactions could reflect domain rearrangements is based upon the proposal that the denaturant dependence of unfolding/refolding rate constants is directly related to differences in the solvent exposure of side chains or backbone in kinetic intermediates and subsequent transition states (Chen *et al.*, 1989; Kuwajima *et al.*, 1983).

Which class of reactions dominates the folding of TR depends upon both the protein and denaturant concentrations. At urea concentrations above 3 M and at TR concentrations of 5−20 μM, three distinct protein concentration-dependent reactions limit folding at 25 °C (Gittelman & Matthews, 1990). Below 3 M urea at similar protein concentrations, three protein and denaturant concentration-independent reactions control folding. Catalysis by cyclophilin of the τ_1 and τ_2 phases in wild-type TR and all but the P45G and des-Pro mutants at low urea concentration demonstrates that isomerization at the Thr44/Pro45 peptide bond is part of the rate-limiting process in TR variants which contain Pro45. However, at low urea concentrations, the folding of des-Pro TR is also limited by three denaturant and protein concentration-independent, high activation energy reactions. This observation shows that another type(s) of conformational rearrangement or isomerization reaction must underlie the proline isomerization reaction.

The assignment of these phases for des-Pro TR to alternative conformational rearrangements or isomerization reactions is also consistent with the linearity of the Arrhenius plots for the $\tau_1-\tau_3$ refolding phases (Figure 5). The linearity implies that small differences exist between the heat capacities of a set of transient intermediates and the rate-limiting transitions states in each channel. The correlation between the heat capacity change and the exposure of nonpolar and polar groups to solvent (Privalov & Makhatadze, 1990) suggests that little net change in the surface area of the protein occurs as the set of intermediates accesses their respective transition states. This behavior contrasts with that for folding reactions in other proteins which have nonlinear Arrhenius plots and are known to reflect substantial development of higher-order structure and the burial of nonpolar surfaces (Jackson & Fersht, 1991; Segawa & Sugihara, 1984).

Development of a Kinetic Folding Model. A kinetic folding model for TR must incorporate the following observations:

(1) TR folds within 5 ms to a monomeric intermediate which is stable, has substantial secondary structure, and can bind ANS to nonpolar surfaces (Mann & Matthews, 1993).

(2) Under marginally folding conditions (3−5 M urea), the folding of wild-type TR involves three urea and protein concentration-dependent reactions (Gittelman & Matthews, 1990; Mann & Matthews, 1993).

(3) Under strongly folding conditions (<3 M urea), the folding of both wild-type and des-Pro TR involves three urea and protein concentration-independent reactions. These reactions have activation energies between 13 and 16 kcal mol^{-1} and involve small changes in the solvent exposure of side chain and backbone atoms.

(4) *cis/trans* isomerization of the Thr44/Pro45 peptide bond appears to limit folding in two of the three folding channels for proteins containing Pro45. Catalysis by cyclophilin is significantly greater for the slowest folding phase than for the intermediate folding phase; the fastest folding phase is apparently not affected by cyclophilin.

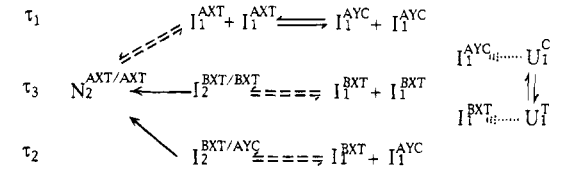

FIGURE 6: Proposed kinetic folding mechanism for wild-type TR. The symbols are described in the text. The burst phase reactions are indicated by dotted lines, the association reactions are indicated by dashed lines, and the isomerization reactions are indicated by solid lines. The relaxation times associated with the rate-limiting steps in each of the folding channels are indicated in the left margin.

(5) The unfolding of TR is governed by one kinetic phase which is consistent with a single monomer/dimer association/dissociation reaction in and above the transition region (Gittelman & Matthews, 1990).

A model which incorporates all of these observations is shown in Figure 6. U and N_2 represent unfolded monomeric TR and folded dimeric TR, respectively. I designates partially-folded forms, with the subscript denoting the number of chains in the species and the superscript indicating the state of three different isomeric pairs, A/B, X/Y, and T/C. T/C reflects the *cis/trans* isomers at Pro45 while A/B and X/Y represent unspecified, alternative conformational states which are not due to proline isomerization. Unfolded, monomeric WT TR is proposed to consist of two slowly-interconverting populations, U^T and U^C, which are distinguished by the isomeric state of the Thr44/Pro45 peptide bond. When refolding is initiated, the two populations of unfolded TR collapse to either of two monomeric forms, I_1^{BXT} (the major species) or I_1^{AYC} (the minor species) within 5 ms. The specific coupling of the BX conformers with T and AY with C is required to explain the existence of only three folding channels. Other possible couplings would lead to additional channels, a prediction which contradicts the experimental observations.

I_1^{BXT} can either associate with itself to form the dimeric intermediate $I_2^{BXT/BXT}$ or combine with I_1^{AYC} to form dimeric $I_2^{BXT/AYC}$. I_1^{AYC} can also slowly isomerize at Pro45 to I_1^{AXT} which can then self-associate to form the native dimer, $N_2^{AXT/AXT}$. The $I_2^{BXT/BXT}$ and $I_2^{BXT/AYC}$ dimers must undergo unimolecular isomerization or rearrangement reactions which convert them to the native dimer. The observed relaxation times associated with the rate-limiting steps in each of these channels are also indicated in Figure 6. The relative amplitudes of the τ_1, τ_2, and τ_3 phases, 10%, 40%, and 50%, are consistent with a 70:30 distribution between the I_1^{BXT} and I_1^{AYC} intermediates ($I_1^{AYC} + I_1^{AYC}$ reflects 30% × 30% = 9% of the signal, $I_1^{AYC} + I_1^{BXT}$ reflects 30% × 70% × 2 = 42%, and $I_1^{BXT} + I_1^{BXT}$ reflects 70% × 70% = 49%). The failure to detect a contribution from the $I_2^{AYC/AYC}$ species might reflect the inherent instability of a dimer whose subunits both contain the nonnative Y and C isomers.

The relative amplitudes of these phases for P45G and des-Pro TR display a significantly different dependence on temperature than do those for the remaining members of this set of proteins (Table 2). The redistribution of material flowing through the three channels is consistent with a 50:50 ratio for I_1^{BX} to I_1^{AY} at 10 °C, implying that Pro45 plays

an integral role in defining the structure and stability of this pair of early intermediates.

Cyclophilin specifically catalyzes *cis/trans* isomerization at Pro45, thereby accelerating the $I_1^{AYC} \rightarrow I_1^{AXT}$ and $I_2^{BXT/AYC} \rightarrow N_2^{AXT/AXT}$ reactions in TR variants containing Pro45. The effect on the former reaction, *i.e.*, the τ_1 phase, would be greater than the latter, τ_2 phase, because the target of the enzyme would be a monomeric, less stable form of TR. The lack of effect of cyclophilin on the τ_2 phase for P37A (Figure 4) may reflect an increased rate of folding and stability of the $I_2^{BXT/AYC}$ species in P37A. Cyclophilin would not affect the rate of the $I_2^{BXT/BXT} \rightarrow N_2^{AXT/AXT}$ reaction, *i.e.*, the τ_3 phase, because Pro45 is already in the *trans* conformation.

The postulate that there are two pairs of non-proline isomers or conformers reflects the observation that, under strongly folding conditions, des-Pro TR displays three denaturant and protein concentration-independent phases. One pair of conformers is required to retain the three folding channels in the quadruple-mutant protein. The other is required to explain the fact that each of these channels is limited by a step that is independent of the denaturant concentration.

Although is it not possible to prove that the model depicted in Figure 6 is *the* folding mechanism, an alternative mechanism which places the Pro45 isomerization reaction before a trio of association steps and three dimer rearrangement reactions after association can be eliminated. Catalysis of *cis/trans* proline isomerization by cyclophilin would accelerate all three folding phases, with the greatest effect on the fastest channel. This prediction is contrary to the observed behavior (Figure 4). Also, this and other models that involve dimeric intermediates in all three channels would not explain the unfolding kinetics which suggest that the native dimer is in direct exchange with a pair of monomers (Gittelman & Matthews, 1990). Although the model shown in Figure 6 is consistent with all available data, further experiments are required to test its validity.

Kinetic folding models which invoke parallel channels that are not due to proline isomerization have also been proposed for lysozyme (Radford *et al.*, 1992) and dihydrofolate reductase (Jennings *et al.*, 1993). In both cases, it appears that the development of secondary and/or tertiary structure can proceed along more than one pathway in the early stages of folding. Because the energy barriers between these pathways are greater than those separating the partially folded forms from the final, native conformation, the structural differences can persist until the final stages of folding. Bryngelson *et al.* (1995) have developed a statistical analysis of the energetics of a protein folding landscape, based on spin glass theory, and concluded that the ruggedness required to define multiple folding channels reflects the early formation of a collapsed conformation(s). The postulated monomeric intermediates, I_1^{AYC} and I_1^{BXT}, could fulfill such a role for TR.

CONCLUSIONS

A mutational analysis of the nature of the rate-limiting steps in the folding of dimeric tryptophan repressor has shown that proline isomerization is not responsible for the formation of three parallel folding channels nor for the urea and protein concentration-independent reactions at low denaturant concentration. The two pairs of isomers or other conformational states postulated to explain the transient response of des-Pro TR during folding may be due to alternative packings of helices in the monomeric early intermediates. The highly intertwined nature of the two subunits in native TR makes it very unlikely that the isolated monomer would adopt precisely the same conformation as found in the dimer. The hydrophobic effect would probably dictate that the helices would collapse upon themselves to minimize the exposure of nonpolar residues to solvent. Thus, it is possible that the monomer might fold to more than one stable conformation in an effort to minimize its free energy under strongly folding conditions. Structural studies of stable, monomeric forms of TR would provide a test of this hypothesis; amino acid replacements at the interface between the subunits might provide a source of such material (Mann *et al.*, 1993).

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