Long-range side-chain–main-chain interactions play crucial roles in stabilizing the $(\beta\alpha)_8$ barrel motif of the alpha subunit of tryptophan synthase

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Abstract

The role of hither-to-fore unrecognized long-range hydrogen bonds between main-chain amide hydrogens and polar side chains on the stability of a well-studied $(\beta\alpha)_8$ TIM barrel protein, the alpha subunit of tryptophan synthase (α TS), was probed by mutational analysis. The F19–D46 and I97–D124 hydrogen bonds link the N terminus of a β -strand with the C terminus of the succeeding antiparallel α -helix, and the A103–D130 hydrogen bond links the N terminus of an α -helix with the C terminus of the succeeding antiparallel β -strand, forming clamps for the respective $\beta\alpha$ or $\alpha\beta$ hairpins. The individual replacement of these aspartic acid side chains with alanine leads to what appear to be closely related partially folded structures with significantly reduced far-UV CD ellipticity and thermodynamic stability. Comparisons with the effects of eliminating another main-chain–side-chain hydrogen bond, G26–S33, and two electrostatic side-chain–side-chain hydrogen bonds, D38–H92 and D112–H146, all in the same N-terminal folding unit of α TS, demonstrated a unique role for the clamp interactions in stabilizing the native barrel conformation. Because neither the asparagine nor glutamic acid variant at position 46 can completely reproduce the spectroscopic, thermodynamic, or kinetic folding properties of aspartic acid, both size and charge are crucial to its unique role in the clamp hydrogen bond. Kinetic studies suggest that the three clamp hydrogen bonds act in concert to stabilize the transition state leading to the fully folded TIM barrel motif.

Keywords: circular dichroism; protein folding; site-directed mutagenesis; thermodynamic and kinetic mechanisms; $\beta\alpha$ and $\alpha\beta$ hairpins

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Abbreviations: H-bond, hydrogen bond; α TS, α subunit of tryptophan synthase from *Escherichia coli*; HSQC, heteronuclear single quantum coherence; CD, circular dichroism; MRE, mean residue ellipticity; SF, stopped flow; FL, fluorescence; HX, hydrogen exchange; I1, equilibrium intermediate of α TS populated at ~ 3 M urea; I2, equilibrium intermediate of α TS populated at ~ 5 M urea; NMR, nuclear magnetic resonance; N, native state; U, unfolded state; WT, wild type.

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Side chains stabilize the native, functional conformations of proteins by van der Waals interactions between non-polar partners, hydrogen bonds (H-bonds) between polar donors and acceptors, and electrostatic interactions between opposing charged groups. Mutational analysis of buried nonpolar side chains has shown that replacements with alanine can lead to destabilizations of up to 7 kcal mol⁻¹ (Shortle et al. 1990; Eriksson et al. 1992; Serrano et al. 1992). The magnitudes of the effects correlate with the local packing density (Shortle et al. 1990) and the burial of nonpolar surface area (Jackson et al. 1993). With the exception of buried ionic interactions (Anderson et al.

1990; Vaughan et al. 2002), mutational analysis has also shown that the magnitude of an individual side-chain–side-chain or main-chain–side-chain H-bond or an electrostatic interaction is relatively small, $\sim 1\text{--}2\text{ kcal mol}^{-1}$ (Horovitz et al. 1990; Serrano et al. 1992; Myers and Pace 1996; Ibarra-Molero et al. 2004). The small contribution to stability for main-chain–side-chain interactions is underscored by the relatively rapid exchange of their amide hydrogens with solvent through local fluctuations within the native ensemble of conformers (Englander and Kallenbach 1983; Krishna et al. 2004). Thus, the results of a native-state hydrogen exchange (HX) experiment on the alpha subunit of tryptophan synthase (α TS) are quite striking.

α TS is a 29-kDa TIM barrel protein in which the eight parallel β -strands alternate in sequence with amphipathic antiparallel helices that dock on the hydrophobic barrel. H-bonds between $\beta 1$ and $\beta 8$ and van der Waals interactions between nonpolar side chains in $\beta 1$, $\alpha 1$, $\beta 8$, and $\alpha 8'/\alpha 8$ close the barrel (Fig. 1A). Relevant to the present issue, the $^1\text{H}\text{--}^{15}\text{N}$ heteronuclear single quantum coherence (HSQC) NMR spectrum of uniformly ^{15}N -labeled protein (Vadrevu et al. 2003) provided a vehicle for demonstrating that the main-chain amide hydrogens of two residues, F19 and I97, are very resistant to exchange with solvent deuterium (R. Vadrevu, Y. Wu, and C. R. Matthews, in prep.). Inspection of the crystal structure of α TS (Hyde et al. 1988; Nishio et al. 2005) shows that both are involved in long-range main-chain–side-chain H-bonds, F19–D46 and I97–D124 (Fig. 1B). The resistance of the amide hydrogen of F19 to exchange for several days and that of I97 for several months in $^2\text{H}_2\text{O}$ at pH 7.8 and 25°C implies that their amide hydrogens become exposed to solvent in rare high energy states (Bai et al. 1995), not through local fluctuations in the native basin. By implication, the F19–D46 and I97–D124 H-bonds might play unusually important roles in the structure and stability of both native and partially folded states in α TS.

This hypothesis was tested by subjecting the H-bond acceptor side chains, D46 and D124, to mutational analysis. To test the significance of these specific interactions, mutations were also introduced at another long-range main-chain–aspartic acid side-chain H-bond, A103–D130, and a short-range main-chain–serine side-chain H-bond, G26–S33, both of whose amide hydrogens exchange within a few hours. Comparisons were also made with mutations of a pair of surface salt bridges involving aspartic acids in the same N-terminal region, D38–H92 and D112–H146. The results demonstrated that each of the F19–D46, I97–D124, and A103–D130 main-chain–side-chain H-bonds makes a significant contribution to the structure and the stability of α TS that is distinct from the relatively minor perturbations for the other variants.

Figure 1. Long-range main-chain–side-chain interactions in α TS. (A) Ribbon diagram of α TS highlighting the $\beta\alpha$ and $\alpha\beta$ clamps encompassing $\beta 1$ – $\alpha 1$, F19 NH–D46 O δ 2; $\beta 3$ – $\alpha 3$, I97 NH–D124 O δ 2, and $\alpha 3$ – $\beta 4$, A103 NH–D130 O δ 1. The side chains involved in the H-bond clamp interactions are highlighted with the donor and acceptor shown in blue and red, respectively. (B) The three individual $\beta\alpha$ or $\alpha\beta$ clamps, illustrating the respective H-bond distances for each main-chain–side-chain interaction. The H-bonds and the corresponding distances are determined by using the program HBPLUS (McDonald and Thornton 1994). The structures were generated using PyMOL v 0.99 (Delano 2002) and PDB code: 1BKS (Hyde et al. 1988).

Results

The X-ray structure of α TS (Fig. 1A) shows that the F19–D46, I97–D124, and A103–D130 H-bonds each bracket a sequential pair of elements of secondary structure. Each of these three long-range H-bond interactions involves a main-chain amide hydrogen donor and a side-chain oxygen acceptor in aspartic acid. The F19–D46 H-bond links the N terminus of $\beta 1$ with the C terminus of $\alpha 1$, the I97–D124 H-bond links the N terminus of $\beta 3$ with the C terminus of $\alpha 3$, and the A103–D130 H-bond links the N terminus of $\alpha 3$ with the C terminus of $\beta 4$ (Fig. 1B). All three interactions span precisely 27 residues in sequence, and, by analogy, serve as structural clamps for $\beta\alpha$ hairpins, F19–D46 and I97–D124, or an $\alpha\beta$ hairpin, A103–D130, in α TS.

The uniqueness of these structural clamp interactions can be assessed by comparisons with other noncovalent

interactions involving short-range or surface H-bonds. The G26–S33 H-bond braces a tight turn between $\beta 1$ and $\alpha 1$ that contains the only *cis* prolyl peptide bond in α TS between D27 and P28. The D38–H92 and D112–H146 salt bridges on the surface of the barrel link the middle of $\alpha 1$ to the loop between $\alpha 2$ and $\beta 2$ and the N terminus of $\alpha 3$ with the loop between $\alpha 4$ and $\beta 5$, respectively. (In the higher resolution structure of α TS from *Salmonella typhimurium* [Hyde et al. 1988], D112 appears to form a second salt bridge with R145.)

Alanine replacements at D46, D124, or D130 significantly perturb the secondary and tertiary structure of α TS

The effects of replacing D46, D124, or D130 with alanine on the secondary and tertiary structure of the α TS variants were determined by collecting far- and near-

UV CD spectra (Fig. 2). The far-UV CD spectra show a maximum mean residue ellipticity (MRE) at 195 nm and minima at 208 nm and 222 nm, demonstrating that all of the variants retain components of the α -helices and β -strands found in the wild-type (WT) protein. However, the significant decrease in the MRE by $\sim 40\%$ for the variants (Fig. 2A–C) at all three wavelengths indicate that each individual replacement causes a substantial loss of secondary structure.

To probe the effects of the D46A, D124A, and D130A variants on the packing of aromatic side chains, the near-UV CD spectra were collected (Fig. 2E–G). The near-UV CD spectra of the D46A (Fig. 2E) and D124A (Fig. 2F) variants, which are very similar to each other and distinctly different from both D130A (Fig. 2G) and WT α TS, indicate altered chiral environments for one or more of the seven tyrosines and 12 phenylalanines (α TS does

Figure 2. Far-UV (A–D) and near-UV (E–H) CD spectra of the WT α TS (●) and three α TS variants: (A and E) D46A α TS (△), (B,F) D124A α TS (◇), (C, G) D130A α TS (□). Panel D displays all the variant spectra in panels A–C for a direct comparison, and panel H displays all the variant spectra in panels E–G. The protein concentration ranged from 5 μ M to 7 μ M for the far-UV CD spectra and from 50 μ M to 150 μ M for the near-UV CD spectra. The buffer contained 10 mM potassium phosphate, pH 7.8, 0.2 mM K_2EDTA , and 1 mM BME at 25°C.

not contain tryptophan). The nearly featureless spectrum of D130A α TS between 270 nm and 290 nm suggests that the tyrosine side chains are mobile. Small peaks near 260 nm imply that one or more of the phenylalanines experience chiral packing environments.

These data show that the elimination of any one of the F19–D46, I97–D124, or A103–D130 H-bonds and/or the loss of the negative charge when aspartic acid is replaced by alanine have a substantial impact on the secondary and tertiary structure of α TS. The very similar response in the far-UV CD spectrum for all three variants (Fig. 2D) and two of the three variants in the near-UV CD spectrum (Fig. 2H) suggest that the loss of either the F19–D46 or the I97–D124 noncovalent interactions lead to similar partially folded structures. These folds retain a substantial fraction of the secondary structure and maintain tertiary packing around some of the aromatic side chains. The contrasting near-UV CD spectrum for D130A α TS shows that at least some aspect of the tertiary structure of this variant differs from those of the other two clamp variants.

Equilibrium folding properties of the clamp-deletion variants

Urea denaturation experiments were performed to test the contribution of the three main-chain amide–aspartic acid side-chain H-bonds to the thermodynamic properties of α TS. The dependence of the MRE at 222 nm on the urea concentration for each variant is compared to WT α TS in Figure 3. The equilibrium unfolding curve of the WT protein displays two overlapping sigmoidal transitions

Figure 3. Urea denaturation equilibrium unfolding curves of D46A α TS (Δ), D124A α TS ($\diamond$), and D130A α TS ($\square$). The data for WT α TS ($\bullet$) are shown for comparison in each panel. The continuous lines represent fits of the data for each α TS variant to a three-state equilibrium folding model as described in the text. The protein concentration was $\sim 5 \mu\text{M}$, and the buffer contained 10 mM potassium phosphate, pH 7.8, 0.2 mM K_2EDTA , and 1 mM βME at 25°C .

that have previously been assigned to interconversions between the native, N, and intermediate, I1, states and between the I1 and the unfolded-like, I2, and unfolded, U, states (Bilsel et al. 1999). The far-UV CD spectra of I2 and U are identical, permitting the application of a three-state model, $\text{N} \rightleftharpoons \text{I1} \rightleftharpoons \text{I2/U}$, to fit the equilibrium data. The I1 state for WT α TS reaches a maximal population of $\sim 60\%$ at 3 M urea (Gualfetti et al. 1999), and its presence is evident in the change in slope of the transition curve at that urea concentration (Fig. 3).

The urea denaturation profile of the D130A clamp-deletion variant could be fit to the same three-state model used for WT α TS. This fitting procedure was justified by the appearance of a limited native baseline below 0.5 M urea and a small shoulder near 2 M urea. As shown in Table 1, the free energy difference between the N and I1 states is greatly reduced, as is the urea-dependence of this free energy difference, that is, the m value, from the values for WT α TS. The $\Delta G_{\text{N} \rightarrow \text{I1}}^{\circ}(\text{H}_2\text{O})$ parameters for D130A α TS and WT protein are 1.17 ± 0.39 and $7.19 \pm 0.58 \text{ kcal mol}^{-1}$, respectively; the respective m values are 1.19 ± 0.21 and $2.85 \pm 0.24 \text{ kcal mol}^{-1} \text{ M}^{-1}$. This three-state fit of the equilibrium unfolding data for D130A was confirmed by a complementary kinetic measurement of the stability of the native state (see below).

Unfortunately, both of the $\beta\alpha$ clamp-deletion variants, D46A and D124A α TS, display broad rather featureless transition curves (Fig. 3) that could not be uniquely fit to a three-state model. In fact, the appearance of the unfolding profiles made it unclear whether the N, I1, and I2/U thermodynamic states were still being sampled in these two variants. As will be shown below, kinetic folding studies can be used to demonstrate the presence of stable folded states in the absence of denaturant and to estimate the stabilities of D46A and D124A α TS.

Kinetic folding properties of the clamp-deletion variants

To obtain insights into the role of the clamp interactions on the kinetic and thermodynamic properties of α TS, a comprehensive set of unfolding and refolding jumps at various final urea concentrations was performed on the clamp-deletion variants. A distinctive feature of the native state of WT α TS is its rate-limiting role in the kinetic unfolding reaction. WT α TS unfolds through two slow phases whose time constants decrease exponentially as the urea concentration is increased (Fig. 4A). It has previously been shown that these two exponential phases correspond to the independent unfolding of a major and a minor native conformer (Bilsel et al. 1999). These conformers reflect the native *cis*, 93%, and nonnative *trans*, 7%, isomers for the D27–P28 peptide bond (Wu and Matthews 2002a). The subsequent unfolding reactions of the corresponding on-pathway I1 intermediates are far

Table 1. Thermodynamic parameters for the urea-induced unfolding of WT α TS and eight variants at 25°C^a

α TS Variants	$\Delta G^\circ_{N \rightleftharpoons I1} (H_2O)$ (kcal mol ⁻¹)	$-m_{N \rightleftharpoons I1}$ (kcal mol ⁻¹ M ⁻¹)	$\Delta G^\circ_{N \rightleftharpoons I2} (H_2O)$ (kcal mol ⁻¹)	$-m_{I1 \rightleftharpoons I2/U}$ (kcal mol ⁻¹ M ⁻¹)	$\Delta \Delta G^\circ_{N \rightleftharpoons I1}$ (kcal mol ⁻¹) ^b
Wild type	7.19 ± 0.58	2.85 ± 0.24	3.04 ± 0.85	0.81 ± 0.17	—
D46A	1.98 ± 0.45 ^c	0.78 ± 0.17 ^c	4.97 ± 1.96 ^d	1.07 ± 0.39 ^d	-5.21 ± 0.73
D124A	2.53 ± 0.40 ^c	1.12 ± 0.19 ^c	3.81 ± 0.64 ^d	0.79 ± 0.16 ^d	-4.66 ± 0.70
D130A	1.17 ± 0.39	1.19 ± 0.21	2.75 ± 0.24	0.80 ± 0.05	-6.02 ± 0.70
D38A	6.11 ± 0.50	2.93 ± 0.24	4.52 ± 0.69	1.14 ± 0.16	-1.08 ± 0.77
D112A	4.90 ± 0.28	2.34 ± 0.14	3.43 ± 0.41	0.89 ± 0.09	-2.29 ± 0.64
S33A	3.73 ± 0.45	2.11 ± 0.24	3.86 ± 0.60	1.00 ± 0.14	-3.46 ± 0.73
D46N	5.04 ± 0.57	3.23 ± 0.38	2.01 ± 0.32	0.67 ± 0.08	-2.15 ± 0.81
D46E	2.15 ± 0.46 ^c	0.93 ± 0.21 ^c	1.91 ± 1.44 ^{d,e}	0.48 ± 0.06 ^{d,e}	-5.04 ± 0.74

^aBuffer conditions: 10 mM potassium phosphate, 0.2 mM K₂EDTA, and 1 mM β ME at pH 7.8.

^bPerturbation in stability for $N \rightleftharpoons I1$ reaction, calculated by $\Delta \Delta G^\circ_{N \rightleftharpoons I1} = \Delta G^\circ_{N \rightleftharpoons I1}(H_2O)$, variant - $\Delta G^\circ_{N \rightleftharpoons I1}(H_2O, WT)$.

^cDetermined by fitting the urea dependence of the major unfolding kinetic phase to a two-state model.

^dDetermined by fitting the equilibrium unfolding data to a three-state model with parameters for the $N \rightleftharpoons I1$ transition fixed to the values determined as described in footnote c.

^eThe native baseline was allowed to float to an optional value and the unfolded baseline was fixed to the linear dependence on denaturant concentration observed at high urea concentration (Fig. 7B).

faster, rendering the pair of $N \rightleftharpoons I1$ reactions as the rate-limiting steps in unfolding for α TS. If the α TS variants, in the absence of denaturant, occupy a stable thermodynamic state related to the native state of the WT protein, they might be expected to display a vestige of the rate-limiting unfolding reactions.

Similar to the WT protein, the unfolding reactions for the D46A, D124A, and D130A variants each exhibits a major fast and minor slow phase whose relaxation times accelerate exponentially with increasing urea concentration and are nearly coincident with those from the WT protein (Fig. 4A–D). Thus, in the absence of denaturant, D46A, D124A, and D130A α TS all occupy distinct thermodynamic states that retain the major *cis* and minor *trans* isomers at D27–P28 and the rate-limiting barriers to unfolding experienced by WT α TS.

The refolding of WT α TS detected by stopped-flow CD (SF-CD) and stopped-flow fluorescence (SF-FL) proceeds through a submillisecond burst-phase reaction followed by a hundreds of milliseconds reaction that accelerates as the urea concentration is increased (Fig. 4A). This “backtracking” phase reflects the unfolding of a misfolded off-pathway species and controls access to the subsequent on-pathway species, I1. *cis-trans* isomerization at three key prolines, P28, P217, and P261, ultimately limits folding to the major and minor native species evident in the unfolding reaction (Bilsel et al. 1999; Wu and Matthews 2002a,b). These rate-limiting isomerization reactions are the source of the three slow refolding phases in the 10–300 sec time range for the WT protein under strongly folding conditions (Fig. 4A).

By contrast, all three variants are missing two of the three slow urea-independent refolding phases observed in the WT protein (Fig. 4A–D). Only two phases were

detected under strongly folding conditions for D124A (Fig. 4C) and D130A (Fig. 4D) α TS. The relaxation time of the faster phase is weakly dependent on the final urea concentration and coincident with the backtracking phase in the WT protein. The relaxation time of the slower refolding phase was nearly independent of the urea concentration and in the time range of phases previously assigned to *cis/trans* isomerization reactions among a set of four I1 intermediates in parallel folding channels (Wu and Matthews 2002a). In addition to these two refolding phases, D46A also displays a refolding phase in the seconds time range that connects smoothly with an additional unfolding reaction. This additional phase has also been detected for the L48A variant (Wu et al. 2006), and is thought to reflect the interconversion of the I1 and I2 states. Finally, the measurement of the amplitude of the submillisecond CD signal at 222 nm as a function of the final urea concentration confirmed the presence of a burst-phase intermediate for all three variants (Fig. 4F–H).

Taken together, the kinetic refolding data demonstrate that all of the clamp-deletion variants retain the submillisecond off-pathway misfolded intermediate and one of the proline isomerization reactions between two I1-like intermediates. Because the pair of unfolding phases for all three variants so closely agree in relaxation time and amplitude with those previously assigned to isomerization at P28, it is reasonable to suppose that the remaining isomerization reaction in refolding for all three clamp-deletion variants reflects the isomerization at P28. By implication, the P217 and P261 *cis/trans* isomerization reactions no longer limit access to the native conformation in the three clamp-deletion variants. The C terminus of the clamp-deletion variants, including residues P217 and P261, does not appear to be well folded or at least not

Figure 4. Kinetic chevrons for (A) WT αTS, (B) D46A αTS, (C) D124A αTS, and (D) D130A αTS, which show the observed refolding (○◆△□) and unfolding (▼▽■) relaxation times as a function of final urea concentrations monitored by manual-mixing CD (○▼△▽□) at 222 nm and stopped-flow FL (◆■). The solid lines in panels B–D represent the behavior of the WT protein. Refolding burst-phase amplitude at 222 nm (▲) by stopped-flow CD for the (E) WT αTS, (F) D46A αTS, (G) D124A αTS, and (H) D130A αTS. The equilibrium unfolding curves (●) for each protein are shown for reference in panels E–H, and the continuous lines indicate the fit of each data set to a three-state model. The dashed and dotted lines in panels F–H represent the equilibrium unfolding transition and the dependence of the burst-phase amplitude on urea concentration, respectively, for the WT protein.

thermodynamically coupled to the folded N terminus in their respective native states.

Thermodynamic parameters extracted from the kinetic experiments

The kinetic unfolding experiment that verified the presence of stable thermodynamic states for the clamp variants can be modified to determine the stability of their folded states. Because the amplitudes of the unfolding reactions are directly proportional to the populations of the folded states from which these reactions initiate,

the stability of the folded states can be measured by monitoring the amplitudes of the unfolding reactions (to constant urea concentration) as a function of the initial urea concentration. The amplitudes of the slow unfolding phases are expected to decrease in a sigmoidal fashion as the initial urea concentration is increased into the zone where the native states become depopulated.

This procedure was validated on WT αTS (Fig. 5A), where a fit of the sigmoidal decrease in ellipticity at increasing initial urea concentrations to a two-state model yielded a stability of 7.06 ± 0.59 kcal mol⁻¹. This value is in excellent agreement with that for the N₁–I1 transition

Figure 5. The dependence of the amplitude for the major unfolding phase for (A) WT αTS and (B) D46A αTS on the initial urea concentration; the final urea concentration in all cases was 6 M urea. The solid lines represent the fit of the data to a two-state model with $\Delta G^\circ = 7.06 \pm 0.59$ kcal mol⁻¹ and $m = 2.89 \pm 0.24$ kcal mol⁻¹ M⁻¹ for WT αTS and $\Delta G^\circ = 1.98 \pm 0.45$ kcal mol⁻¹, and $m = 0.78 \pm 0.17$ kcal mol⁻¹ M⁻¹ for D46A αTS. The protein concentration ranged from 5 to 6 μM.

from the standard equilibrium titration experiment on the WT protein, 7.19 ± 0.58 kcal mol⁻¹ (Fig. 3; Table 1). The urea dependence of the stability, the m value, is also in excellent agreement, 2.90 ± 0.24 vs. 2.85 ± 0.24 kcal mol⁻¹ M⁻¹ (Table 1). Conformation of the kinetic measurement of stability was provided by the D130a αTS variant. The traditional equilibrium titration data (Fig. 3) yielded a stability of 1.17 ± 0.39 kcal mol⁻¹ and an m value 1.19 ± 0.21 kcal mol⁻¹ M⁻¹ (Table 1). The kinetic measurement yielded a stability of 1.05 ± 0.17 kcal mol⁻¹ and an m value 1.25 ± 0.16 kcal mol⁻¹ M⁻¹ (Table 1).

The stabilities of the folded states of the D46A and D124A αTS clamp-deletion variants were then determined in a similar fashion. Figure 5B displays a representative example of the amplitude of the major unfolding phase for D46A αTS as a function of the initial urea concentration. The stability of the folded state for D46A αTS, 1.98 ± 0.45 kcal mol⁻¹, is 5.21 kcal mol⁻¹ less than that of the N state for the WT protein; the m value is also significantly reduced, 0.78 ± 0.17 vs. 2.85 ± 0.24 kcal mol⁻¹ M⁻¹ (Table 1). The stability of the folded state for D124A αTS is decreased to 2.53 ± 0.40 kcal mol⁻¹. The m value for the N $\rightleftharpoons$ I1 transition of D124A αTS variant is also significantly decreased (Table 1).

Although the absence of a prominent shoulder in the MRE titration data for the D46A (Fig. 4F) and D124A (Fig. 4G) variants leaves the existence of an I1-like state

ambiguous, the folded states for both proteins, as measured by the kinetic amplitude experiment, disappear; for example, at 3–4 M urea for D46A αTS (Fig. 5B), before the unfolded state is completely populated; for example, at 5–6 M urea for D46A αTS (Fig. 3). This discrepancy in the ellipticities implies the presence of a stable intermediate between 3 and 6 M urea. The stabilities of the intermediates for D46A and D124A αTS can be estimated by fitting the unfolding data for these clamp-deletion variants (Fig. 3) to a three-state N $\rightleftharpoons$ I1 $\rightleftharpoons$ I2/U model, for which the parameters for the N $\rightleftharpoons$ I1 reaction are fixed to those obtained from the kinetic unfolding experiments. The thermodynamic parameters obtained for the I1 $\rightleftharpoons$ I2/U transition are shown in Table 1.

The stabilities of the I1 states for all three clamp-deletion variants (relative to their I2/U states) and the m values for the I1 $\rightleftharpoons$ I2/U transition are within error of the values for the I1 state relative to the I2/U state for WT αTS. Because the removal of the clamp interaction by replacing aspartic acid with alanine has no significant effect on the stabilities of the intermediate states, it appears that each of the clamp interactions is broken in the intermediate of the WT protein.

Uniqueness of the clamp interactions

All three clamp interactions are located in the N-terminal half of αTS, which is known to serve as a core of stability in both the N and I1 states (Zitzewitz and Matthews 1999; Rojsajakul et al. 2004). To determine whether the surprisingly large perturbations of structure and stability are related to the participation of these clamp H-bonds in this stable core, the S33A, D38A, and D112A variants of αTS were constructed. S33 forms an H-bond with the main-chain amide nitrogen of G26, D38 forms an electrostatic interaction with H92, and D112 forms an electrostatic interaction with H146. The heavy atom distances for these three interactions are 3.00, 2.75, and 2.61 Å, respectively, comparable to 2.92, 2.61, and 2.85 Å for the F19–D46, I97–D124, and A103–D130 clamp H-bonds (Fig. 1B). The solvent accessible surface areas for the G26–S33, D38–H92, and D112–H146 interactions are 16, 89, and 90 Å², also comparable to those for the F19–D46, I97–D124, and A103–D130 clamp H-bonds, 28, 19, and 80 Å², respectively.

The D38A and D112A variants display a similar reduction in the far-UV CD signal as the clamp-deletion variants (Fig. 6A,B). However, both retain the distinctive near-UV CD spectrum for the WT protein (Fig. 6D,E), which suggests that they remain a similar tertiary structure as WT protein. The reductions in the far-UV CD signal, therefore, likely reflect the local unfolding of the surface helices and loops linked by these salt bridges. The far-UV (Fig. 6C) and near-UV CD (Fig. 6F) spectra of the

Figure 6. Far-UV (A–C) and near-UV (D–F) CD spectra of three clamp-deletion α TS variants: (A,D) D38A α TS (▲), (B,E) D112A α TS (■), and (C,F) S33A α TS (▼). The solid line in each panel represents the behavior of the WT α TS for reference.

S33A variant are both very similar to those for WT α TS. The unfolding transition curves for all three variants reveal native baselines and the biphasic behavior characteristic of a three-state process (Fig. 7A). Fits of these data to a three-state model show that the perturbations on stability for D38A, D112A, and S33A α TS are less than those of their clamp-deletion counterparts for the $N \rightleftharpoons I1$ transition and negligible for the $I1 \rightleftharpoons I2/U$ transition (Table 1). Thus, the effects on the structure and/or stability of the native state accompanying the loss of the $\beta\alpha$ or $\alpha\beta$ clamps in D46A, D124A, and D130A α TS are quantitatively different than those of other main-chain–side-chain or side-chain–side-chain electrostatic interactions in the N-terminal folding unit of α TS.

Uniqueness of aspartic acid as an H-bond donor in the clamp interaction

The significance of the formal negative charge and the size of the aspartic acid side chain for the clamp interaction was examined by replacing the aspartic acid at position 46 with asparagine or glutamic acid. The asparagine side chain has a very similar size but lacks the formal charge, and glutamic acid retains the charge but

has an additional methylene in the side chain. The D46N α TS variant experiences a 20% reduction in the far-UV CD signal and retains the three-state equilibrium unfolding profile (Fig. 7B). The free energy change for the $N \rightleftharpoons I1$ reaction is 5.04 ± 0.57 kcal mol⁻¹, only 2.15 kcal mol⁻¹ less than that of WT α TS (Table 1). The absence of a significant perturbation in the free energy change for the $I1 \rightleftharpoons I2/U$ reaction is consistent with the results for the D46A variant and the disruption of the clamp H-bond in the I1 state for WT α TS. Kinetic analysis showed that D46N α TS retains the two unfolding phases observed for the WT protein; however, two of the three proline isomerization-limited refolding reactions are absent (data not shown). Thus, the isosteric replacement of aspartic acid with asparagine at position 46 in the N-terminal domain appears to preclude the proper folding and/or docking of the C-terminal region containing P217 and P261.

The ellipticity at 222 nm for the D46E α TS variant is smaller than that for any other variant examined, including that for D46A α TS (Fig. 7B). D46E α TS retains the pair of slow unfolding reactions characteristic of the native conformation of α TS (data not shown), and the kinetic measurement of the free energy change for the $N \rightleftharpoons I1$

Figure 7. Urea denaturation equilibrium unfolding curves of (A) D38A α TS (▲), D112A α TS (■), and S33A α TS (▼), and (B) D46A α TS (△), D46N α TS (◆), and D46E α TS (○). The data for WT α TS (●) are shown for comparison in each panel. The continuous lines represent fits of the data for each α TS variant to a three-state equilibrium folding model as described in the text. The protein concentration was $\sim 5 \mu\text{M}$, and the buffer contained 10 mM potassium phosphate, pH 7.8, 0.2 mM K_2EDTA , and 1 mM βME at 25°C .

transition is also greatly destabilized, $2.15 \pm 0.46 \text{ kcal mol}^{-1}$ (Table 1). Similar to D46A and D46N, the free energy difference for the $\text{I1} \rightleftharpoons \text{I2/U}$ reaction is within error of that for WT α TS. Neither asparagine nor glutamic acid can completely recapitulate the spectroscopic, thermodynamic, and kinetic folding properties of aspartic acid at position 46.

Discussion

The role of clamp interactions in the structure and stability of α TS

Elimination of any one of the three $\alpha\beta$ or $\beta\alpha$ clamp interactions in α TS, F19–D46, I97–D124, or A103–D130, results in surprisingly large decreases in the stability of the native state relative to the equilibrium intermediate, I1. Equally surprising was the appearance of what appear to be closely related partially folded states with substantially diminished secondary structure and altered tertiary structure for all three clamp-deletion variants. This tentative conclusion is based on (1) virtually identical far-UV CD spectra for all three clamp-deletion variants, and identical near-UV CD spectra for the D46A and D124A

α TS variants; (2) similar reductions in the stability of the N state, $4.66\text{--}6.02 \text{ kcal mol}^{-1}$; and (3) a pair of unfolding reactions with identical relaxation times and relative amplitudes for all three proteins. The contrasting near-UV CD spectrum for D130A α TS (Fig. 2G) may reflect the peculiar and localized behavior of Y102, adjacent to the peptide bond containing the amide hydrogen of A103 with which D130 forms an H-bond. The observation of a common urea-independent slow refolding reaction, in the absence of the prolyl isomerization reactions for P217 and P261, implies that the C-terminal region of α TS cannot properly fold and/or dock on the N-terminal domain in the native states of all three clamp-deletion variants.

Other variants in which either aspartic acid, which serves as the anionic partner in surface electrostatic interactions, D38–H92 and D112–H146, or serine, which serves as an H-bond acceptor for a nearby main-chain amide nitrogen donor, G26–S33, is replaced with alanine show smaller decreases in stability and lesser effects on the secondary and/or tertiary structure. The comparable range of H-bond lengths and solvent-accessible surface areas with the clamp-deletion variants suggest that other factors, possibly the concerted disruption of all three clamp H-bonds (see below), are responsible for the dramatically different effects. The observation that neither asparagine nor glutamic acid can completely recover the structural, thermodynamic, or kinetic folding properties of WT α TS implies, at least for this position, that both the formal charge and the precise positioning of that charge are crucial for full acquisition of the TIM barrel structure and stability.

The role of the clamp interactions in the folding mechanism of α TS

The retention of the burst-phase ellipticity and the hundreds of milliseconds unfolding reaction under refolding conditions, that is, the backtracking of the off-pathway burst-phase intermediate to the productive folding pathway, for all three clamp-deletion variants (Fig. 4F–H) demonstrate that none of the clamps are essential for the misfolding reaction. The observation of a single urea-independent subsequent refolding reaction attributed to the *trans* $\rightarrow$ *cis* isomerization of the peptide main chain at D27–P28 shows that the prolyl isomerizations at P217 and P261 are no longer rate limiting in folding for any of the clamp-deletion variants. By contrast, the retention of the *cis* isomer for P28 in the native state of all three variants implies that the tight turn between $\beta 1$ and $\alpha 1$, responsible for stabilizing this higher energy form, is preserved in the absence of the clamps.

These results can be explained by examination of the properties of a stable C-terminal truncated version of α TS, 1–188 α TS (Zitzewitz and Matthews 1999). 1–188

α TS retains the burst-phase reaction, the early back-tracking reaction, a single slow folding reaction, and two slow unfolding reactions. All but the unmeasured burst-phase reaction have relaxation times similar to full-length α TS. Although the secondary and tertiary structures of the clamp-deletion variants are somewhat disrupted compared to the WT 1–188 fragment (with the clamp H-bonds), the similarities of the kinetic folding properties of 1–188 α TS (Zitzewitz and Matthews 1999) and the clamp-deletion variants suggest that the C-terminal region of each of the clamp-deletion variants is not well folded under native-favoring conditions. Apparently, the loss of any of the clamp interactions in the N terminus alters the structure of the N terminus so as to preclude the folding and docking of the C terminus. An earlier fragmentation analysis of α TS (Higgins et al. 1979) showed that the C-terminal region, residues 189–268, is poorly folded in isolation. The spontaneous recovery of structure and function for α TS when these two fragments are complemented showed that the N-terminal region serves as the template for the folding and docking of the C-terminal region. If the template structure is altered, as appears to be the case for all three clamp-deletion variants, the C-terminal region cannot fold and dock on the N-terminal region.

It is also interesting to note that the two slow unfolding relaxation times exhibited by all three variants are within error of those for WT α TS at high denaturant concentration. Given the substantial loss in the stability of their respective native states, the similar destabilization of the unfolding transition states for all three variants means that all three clamp interactions in the WT α TS must be maintained in both unfolding transition states. Because the $N \rightleftharpoons I1$ reaction is reversible, these clamps must therefore also be very native-like in the transition states for the refolding reactions. The absence of the clamps in the I1 state, as implied by the lack of perturbation in the nature of $\Delta G^\circ_{I1 \rightleftharpoons I2/U}$ (H_2O) when aspartic acid is replaced by alanine (Table 1), implies that the clamps first appear in the final, rate-limiting transition state for folding, after the $U \rightarrow I2$ and $I2 \rightarrow I1$ reactions are complete. By acting in concert to stabilize the transition state of WT α TS, the clamps direct the folding to the fully formed TIM barrel structure rather than to the partially folded states observed for the clamp variants. The stabilization of the N terminus of the native state for WT α TS by the clamps presumably enhances the folding of the C terminus by providing a properly structured template on which the C terminus can fold.

The closely related partially folded structures implied by the nearly identical far-UV CD spectra (Fig. 2D) and unfolding rates (Fig. 4B–D) for the clamp-deletion variants suggest that the loss of a single clamp results in the rupture of the two remaining clamps. Thus, the

unusually large decrease in stability observed for each clamp-deletion variant (Table 1) does not reflect the strength of a single side-chain–main-chain H-bond. This conjecture is supported by the substantial perturbations in the far- and near-UV CD spectra that imply the loss of numerous noncovalent interactions in these variants.

Structural implication of strong protection against HX for F19 NH and I97 NH

The absence of the F19–D46 and I97–D124 clamp interactions in the stable I1 folding intermediate for α TS (Table 1) begs the question as to the source of the unusually strong protection of these amide hydrogens against HX with solvent. If I1 were that source, one would have expected exchange at both of these positions in a few hours at 25°C and pH 7.8 via an EX1 mechanism (Englander and Kallenbach 1983; Bilsel et al. 1999). The implication of protection sufficient to retard exchange for days and months is either that these amide hydrogens have an alternative H-bond acceptor partner and/or are buried in nonnative nonpolar environments that inhibit exchange with solvent in the I1 state. Exchange must occur through other, higher energy states on the folding free energy surface, for example, the I2 state that lacks discernable secondary structure. Either implied explanation means that the N termini of at least two β -strands, $\beta 1$ and $\beta 3$, are involved in a very stable but nonnative structure(s) that differentiate the I1 and the N state. It is interesting to speculate that both aspartic acid H-bond donors are replaced by carbonyl oxygens in adjacent strands, that is, $\beta 2$ and $\beta 4$, whose lengths are extended to enable these additional stabilizing interactions. Whatever the source of these nonnative stabilizing interactions, they must be disrupted to enable formation if the clamp H-bonds are required to access the transition state leading to the fully folded TIM barrel.

Clamp interactions define the boundaries of $\beta\alpha$ and $\alpha\beta$ hairpins

The clamp interactions probed in this analysis are analogous to helix-capping interactions previously identified by Rose (Presta and Rose 1988). H-bond acceptor side chains near the N termini of helices can form H-bonds with unsatisfied main-chain amide hydrogen donors within but near the N termini of the helices. These H-bonds are typically sheltered from solvent by a pair of nonpolar side chains that presumably serve to increase the strength of the H-bond. Inspection of the local environments of each of the $\beta\alpha$ and $\alpha\beta$ clamp interactions in α TS shows hydrophobic clusters in those regions: A103 has van der Waals contacts with V128 and V131, I97 has contacts with L85 and I88, and F19 has contacts with

V259 and M262. As for the helix–capping interactions, screening of the solvent by hydrophobic clusters would be expected to strengthen the clamp H-bond.

In addition to enhancing the stability of the native conformation, helix–capping interactions are thought to limit the length of the helices and, thereby, promote the formation of loops and turns that link the helices to neighboring elements of secondary structure. The clamp interactions may serve a similar role for $\beta\alpha$ and $\alpha\beta$ hairpins by delineating and reinforcing the boundaries of its constituent β strand and α helix. The specificity conferred by the formation of a clamp interaction would ensure the proper register of the strand and helix forming the hairpin. The identification of these long-range clamp interactions in the amino acid sequence of TIM barrel proteins of unknown structure could, therefore, assist in the prediction of their structures from the sequence. Nonpolar side chains at the N termini of predicted β strands or α helices that precede polar H-bond acceptor side chains at the C termini of subsequent predicted helices or strands would be candidates for the clamp H-bonds.

Broader involvement of clamp interactions

Similar $\beta\alpha$ and $\alpha\beta$ hairpin clamps exist in other TIM barrel proteins, including indole-3-glycerol phosphate synthase (IGPS) from *Sulfolobus solfataricus* (I107–D128, T129–E155), the iolI protein from *Bacillus subtilis* (L106–N72), the cyclase moiety of imidazole-3-glycerol phosphate synthase (HisF) from *Thermotoga maritima* (F77–D98), N-(5'-phosphoribosyl)anthranilate isomerase (PRAI) from *Escherichia coli* (I355–D374), and the founding member of the motif, triosephosphate isomerase (TIM) from chicken (I206–D227). Expansion of the search to other types of $(\beta\alpha)_n$ structures, for example, the $\alpha/\beta/\alpha$ sandwich flavodoxin-fold, the Rossmann-fold, and the leucine-rich repeat-fold revealed the presence of one or more clamps in members of these motifs as well (S. Kathuria, unpubl.). Further experimentation is required to determine if the enhancement of stability gained by linking consecutive elements of secondary structure in α TS through these types of long-range non-covalent interactions is a general property of the TIM barrel and other $(\beta\alpha)_n$ classes of proteins.

Materials and Methods

Site-directed mutagenesis

Oligonucleotides for mutagenesis were purchased from Operon, and the Quickchange site-directed mutagenesis kit was obtained from Stratagene. The method involved one round of PCR amplification using two complementary primers, and the variant

was amplified by PCR in the pXH plasmid (Wu and Matthews 2002b). The endonuclease, DpnI, was used to selectively digest the methylated parent plasmid, and the product was transformed into XL1-Blue competent cells from Stratagene. The site-directed mutations were confirmed by DNA sequence analysis (UC Davis Sequencing Facility).

Protein expression and purification

α TS variant proteins were expressed in CB149 cells and purified as described previously (Wu and Matthews 2002b). Purity was demonstrated by the appearance of a single band by Coomassie blue-stained PAGE.

Circular dichroism

CD spectroscopy was employed to monitor the secondary structure and the tertiary structure near aromatic side chains. Spectra were obtained on a Jasco Model J-810 spectropolarimeter equipped with a thermoelectric cell holder. Far-UV CD data were collected from 280 nm to 185 nm at a scan rate of 50 nm/min and at 1-nm intervals using a 0.1-cm pathlength cell, a bandwidth of 2.5 nm, and an averaging time of 8 sec. Three replicate spectra were collected and averaged. The protein concentration was ~ 5 μ M. Near-UV CD data were collected from 320 nm to 250 nm at 5 nm/min using a 0.5-cm path length cell, and the protein concentration was 50–150 μ M. The temperature was maintained at 25°C with a computer-controlled Peltier system.

Equilibrium experiments

The stability of the α TS variants was measured by urea denaturation as described previously (Wu and Matthews 2002b) in a buffer containing 10 mM potassium phosphate, pH 7.8, 0.2 mM K_2EDTA , and 1 mM β ME. A Hamilton 540B automatic titrator was used to prepare the samples to enhance the precision of the measurements. The samples were incubated overnight at 25°C to ensure equilibration.

Kinetic experiments

CD manual-mixing kinetic experiments were performed on a Jasco Model J-810 spectropolarimeter equipped with a thermoelectric cell holder using a 1-cm pathlength cell, a bandwidth of 2.5 nm, and an averaging time of 1 sec. The dead time of the experiments was 3 sec and the instrument response time was about 5 sec. The change in ellipticity as a function of time was monitored at 222 nm. Stopped-flow CD experiments were performed with an Aviv model 202SF stopped-flow CD spectrometer. The cell path length was 1.0 mm, and the dead time for mixing was 5 msec. Stopped-flow fluorescence experiments were performed on an Applied Photophysics SX-18MV stopped-flow fluorometer. The excitation wavelength was 280 nm, and the emission above 300 nm was detected through a cutoff filter.

Kinetic unfolding experiments to determine the stability of the folded states of the clamp-deletion variants were performed by jumping from different initial urea concentration (0–5.6 M) to a final concentration 6 M urea. Protein samples were first equilibrated in the initial urea concentration overnight and

jumped to corresponding urea buffer solutions by a 1:10 dilution. The final protein concentration was around 5 μ M.

Data analysis

Equilibrium data were fit to a three-state model (Bilsel et al. 1999), and the kinetic data were fit to a sum of exponentials and a constant, as described previously (Bilsel et al. 1999). All thermodynamic and kinetic folding data were fit using Savuka version 6.2, an in-house, nonlinear, least-squares program.

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