Identifying the structural boundaries of independent folding domains in the α subunit of tryptophan synthase, a β/α barrel protein

JILL A. ZITZEWITZ, PETER J. GUALFETTI, IEVA A. PERKONS, STACEY A. WASTA,
AND C. ROBERT MATTHEWS

Department of Chemistry, Life Sciences Consortium, and Center for Biomolecular Structure and Function,
The Pennsylvania State University, University Park, Pennsylvania 16802

(RECEIVED November 10, 1998; ACCEPTED February 19, 1999)

Abstract

Two equilibrium intermediates have previously been observed in the urea denaturation of the α subunit of tryptophan synthase (α TS) from *Escherichia coli*, an eight-stranded β/α barrel protein. In the current study, a series of amino-terminal fragments were characterized to probe the elementary folding units that may be in part responsible for this complex behavior. Stop-codon mutagenesis was used to produce eight fragments ranging in size from 105–214 residues and containing incremental elements of secondary structure. Equilibrium studies by circular dichroism indicate that all of these fragments are capable of adopting secondary structure. All except for the shortest fragment fold cooperatively. The addition of the fourth, sixth, and eighth β -strands leads to distinct increases in structure, cooperativity, and/or stability, suggesting that folding involves the modular assembly of $\beta\alpha\beta$ supersecondary structural elements. One-dimensional NMR titrations at high concentrations of urea, probing the environment around His92, were also performed to test for the presence of residual structure in the fragments. All fragments that contained the first four $\beta\alpha$ units of structure exhibited a cooperative unfolding transition at high concentrations of urea with significant but reduced stability relative to the full-length protein. These results suggest that the residual structure in α TS requires the participation of hydrophobic residues in multiple β -strands that span the entire sequence.

Keywords: autonomous folding unit; circular dichroism; equilibrium intermediates; nuclear magnetic resonance; protein fragments; protein folding; residual structure

The equilibrium unfolding reactions of many proteins exhibit two-state behavior in which only the native and unfolded conformations are populated at significant concentrations. This high cooperativity often hinders a detailed analysis of partially folded forms that could provide clues to the relationship between protein sequence and structure. Protein fragmentation offers one approach

toward reducing the cooperativity of the folding reaction and producing stable partially folded forms that can be extensively studied under equilibrium conditions (Kim & Baldwin, 1982; Lecomte & Matthews, 1993).

Protein fragmentation studies, employing proteolysis, chemical cleavage, or protein engineering methods, have provided a variety of insights into protein folding reactions. Fragments corresponding to isolated structural domains in the multidomain proteins phosphoglycerate kinase (Missiakas et al., 1990), streptokinase (Parado et al., 1996), and thermolysin (Corbett & Roche, 1986) have been shown to fold independently, providing evidence for a hierarchical folding mechanism in multidomain proteins (Jaenicke, 1991). A structural subdomain revealed by proteolysis of the dimeric trp repressor (Tasayco & Carey, 1992) has also been demonstrated to fold autonomously. The folding of a 123-residue C-terminal fragment of dihydrofolate reductase to a presumably non-native structure (Gegg et al., 1997) may provide an explanation for the heterogeneity in early folding intermediates of the full-length protein detected by hydrogen-exchange NMR (Jones & Matthews, 1995). The C-terminal fragment of the β_2 subunit of

Reprint requests to: C. Robert Matthews, Department of Chemistry, The Pennsylvania State University, 152 Davey Lab, Box 66, University Park, Pennsylvania 16802; e-mail: crm@psu.edu.

Abbreviations: α TS, full-length α subunit of tryptophan synthase from *Escherichia coli*; α TS(1 – x), amino-terminal residues 1 to x of the α subunit of tryptophan synthase from *E. coli*; Δ ASA, change in solvent accessible surface area; BME, β -mercaptoethanol; CD, circular dichroism; DTE, dithioerythritol; F_{app} , apparent fraction unfolded protein; FL, fluorescence; FPLC, fast protein liquid chromatography; Gdn-HCl, guanidine hydrochloride; I1, equilibrium intermediate of α TS populated at ~ 3 M urea; I2, equilibrium intermediate of α TS populated at ~ 5 M urea; K₂EDTA, ethylenediaminetetraacetic acid, dipotassium salt; MRE, mean residue ellipticity in units of deg cm² dmol^{–1}; PCR, polymerase chain reaction; PDB, Protein Data Bank; SVD, singular value decomposition; TIM, triosephosphate isomerase.

tryptophan synthase, which folds via a nonspecific hydrophobic collapse to a non-native state with significant secondary structure (Chaffotte et al., 1991), has also been proposed to be a model of an early folding intermediate (Chaffotte et al., 1992, 1997). Protein engineering techniques have also been used to generate subdomains by linking noncontiguous sequences, including a molten globule-like helical domain in α -lactalbumin (Peng & Kim, 1994), a helical model for an acid state intermediate in T4 lysozyme (Llinas & Marqusee, 1998), and an organizing scaffold for the heme-binding site in apocytochrome b_5 (Constans et al., 1998). Although fragments of barnase (Kippen et al., 1994) and chymotrypsin inhibitor-2 (de Prat-Gay et al., 1995) show little propensity to fold in solution, the kinetic analysis of complementation studies has offered insights into the folding pathways for the corresponding full-length proteins (de Prat-Gay, 1996).

The α subunit of tryptophan synthase, an eight-stranded β/α barrel protein, is another excellent candidate for protein fragmentation studies because it contains two equilibrium intermediates, which are populated upon denaturation with urea. One intermediate, I1, is approximately 70% populated at 3.2 M urea when probed by far-UV CD, fluorescence, and absorbance (Matthews & Crisanti, 1981; Gualfetti et al., 1999). A second equilibrium intermediate, I2, has been observed by ^1H -NMR as a peak area shift in the C ϵ proton of His92, with a midpoint of ~ 5 M urea (Saab-Rincon et al., 1993). A four-state equilibrium unfolding model, $\text{N} \rightleftharpoons \text{I1} \rightleftharpoons \text{I2} \rightleftharpoons \text{U}$, was proposed in which the $\text{I2} \rightleftharpoons \text{U}$ transition corresponded to the disruption of residual structure observed by NMR. The I2 species has also been observed more recently by CD, fluorescence intensity, and anisotropy experiments that utilized an automatic titrating system and SVD analysis, confirming the four-state equilibrium folding model for α TS (Gualfetti et al., 1999). I2 attains a maximum population of approximately 80% at 4.6 M urea.

Equilibrium intermediates have also been observed in the folding of other β/α barrel proteins, including phosphoribosylanthranilate isomerase (Eder & Kirschner, 1992; Jasanoff et al., 1994) and indole-3-glycerol-phosphate synthase (Andreotti et al., 1997; Sanchez del Pino & Fersht, 1997), suggesting that multistate unfolding may be a common property of this class of proteins. Fragmentation studies provide a method for probing the structural features responsible for the presence of equilibrium folding intermediates.

Previous studies provide precedence for the ability of fragments of α TS to fold in isolation (Higgins et al., 1979; Miles et al., 1982). In these experiments, trypsin digestion of the trp synthase holoenzyme led to specific cleavage after Arg188 in α TS (Higgins et al., 1979). The amino-terminal fragment α TS(1–188), which comprises the first six strands of the β/α barrel, contained significant secondary structure and unfolded cooperatively when denatured with guanidine-hydrochloride (Miles et al., 1982). By contrast, the carboxy-terminal fragment, α TS(189–268), showed a more modest level of secondary structure, which unfolded in a noncooperative manner. Similar results were obtained for the unfolding of a complementary set of engineered fragments of phosphoribosylanthranilate isomerase, suggesting that these two β/α barrels may be derived from a common ancestor (Eder & Kirschner, 1992).

In this study, a series of amino-terminal fragments of α TS, containing incremental elements of secondary structure, were characterized by CD and NMR. A systematic survey of a series of fragments offers the potential for obtaining important insights into the structural determinants of equilibrium folding intermediates. A comparison of the folding properties of the fragments under both

native and unfolded conditions will yield information on the propensity of protein substructures to fold and maintain residual structure. The insights gained may offer clues to the folding properties of the full-length protein.

Results

Fragment design

The β/α barrel structure of α TS has a relatively simple topology comprised of locally folded and associated elements of secondary structure (Fig. 1A). The eight β -strands align sequentially to form a parallel β -sheet, which is closed by the association of strands 1 and 8. Eleven amphipathic α -helices dock against the β -barrel, providing protection from solvent. The presence of two detectable equilibrium intermediates in the folding of this protein is somewhat surprising given its organization as a single structural domain.

The structural basis for the four-state unfolding process was probed by characterizing a series of fragments that incrementally removed elements of secondary structure from the C-terminus. Figure 1 illustrates the complete series of fragments engineered for this study. The smallest fragment, α TS(1–105), is represented in

Fig. 1. A: Ribbon diagram of α TS generated with MOLSCRIPT, 2.0.2 (Kraulis, 1991). Coordinates for the *Salmonella typhimurium* α TS, which shares 85% sequence identity with the *E. coli* α TS, are from a refined version of PDB file, 1wsy (Hyde et al., 1988; D. Davies, pers. comm.). The dashed line represents missing coordinates for a disordered loop encompassing residues 178–189. **B:** Linear representation for each of the truncated fragments and the full-length α TS, showing the corresponding elements of secondary structure. The approximate position of the seven tyrosine residues are indicated above the full-length representation.

Fig. 2. Far-UV CD spectra of full-length α TS and amino-terminal fragments. Colors represent each fragment as identified in Figure 1B. The full-length α TS spectrum is represented by the black line. Spectra represent the average of 10 traces. Buffer conditions: 10 mM potassium phosphate, pH 7.8, 0.2 mM K_2EDTA , and 1 mM β -mercaptoethanol at 25 °C. Protein concentrations ranged from 5–20 μ M.

red in the ribbon structure (Fig. 1A), and the largest fragment, α TS(1–214), is represented by the full rainbow of colors. Intermediate length fragments are represented by a partial rainbow of colors. All of the fragments are missing the C-terminal residues shown in gray. A linear representation of the complete set of fragments overlaid with the corresponding secondary structural elements observed in the X-ray structure of the full-length α TS is depicted in Figure 1B. Each fragment is color-coded to match the color of its most C-terminal element(s) of secondary structure and contains the secondary structural elements of the full-length protein up to and including that color. The full-length protein contains no tryptophans and seven tyrosines at positions 4, 102, 115, 169, 173, 175, and 204 as indicated in Figure 1B.

Probing secondary structure by CD

Far-UV circular dichroism was used to monitor the secondary structure content of each fragment. CD generally serves as a global probe of the peptide backbone, although tertiary interactions can also contribute to the far-UV CD signal (Kuwajima et al., 1991; Vuilleumier et al., 1993). The CD signal can also be converted to mean residue ellipticity to normalize the data for protein concentration and fragment length, allowing for direct comparisons between fragments. By contrast, a similar comparison of the tertiary structure of the fragments by fluorescence or near-UV CD is hindered by the variable number of chromophores in each fragment.

The far-UV CD spectra of α TS and the amino-terminal fragments are shown in Figure 2. The spectral shape of each of the fragments resembles the full-length spectrum, exhibiting a maximum near 195 nm, and a minimum near 220 nm, as expected for mixed α/β structures. All of the fragments show evidence of significant levels of secondary structure; however, the decreased intensity at 195 and 220 nm, relative to the full-length α TS, implies less secondary structure on a per residue basis. Closer inspection shows that α TS(1–204) and α TS(1–214) have reduced mean res-

idue ellipticities compared to α TS(1–188), suggesting that residues 189–214 do not fold as well as the 1–188 core. Similarly, α TS(1–147) and α TS(1–157) are not as well folded as α TS(1–133), suggesting the existence of another core involving residues 1–133. The increased MRE for α TS(105), compared to α TS(1–147) and α TS(1–157), probably reflects the greater proportion of helical segments in the smaller fragment. The MRE at 222 nm for each of the fragments is indicated in Table 1. The MRE signal was constant over a range of fragment concentrations (1–30 μ M; data not shown), demonstrating that the small propensity of these fragments to dimerize did not drive the formation of secondary structure under the conditions of these experiments.

The stability of the fragments to urea denaturation was also probed by CD. The far-UV CD spectra for α TS(1–188) at various final urea concentrations decrease in the magnitude of the ellipticity with increasing urea concentrations, indicating that the fragment is unfolding and becoming more random coil-like (data not shown). The MRE values at 222 nm, as a function of urea concentration for α TS(1–188) and full-length α TS are compared in Figure 3. The unfolding of the fragment was >90% reversible as demonstrated by the recovery of ellipticity upon refolding the denatured protein (data not shown). The equilibrium unfolding of the fragment exhibits a smooth sigmoidal transition, indicative of a cooperative unfolding reaction. The break in the cooperativity of the unfolding transition observed for full-length α TS at 3 M urea indicates the presence of the equilibrium intermediate, I2 (Matthews & Crisanti, 1981). By contrast, the unfolding of α TS(1–188) is more two-state-like, showing no direct evidence for the population of an equilibrium intermediate. Similar to previous results on the unfolding of α TS(1–188) in Gdn-HCl (Miles et al., 1982), the apparent midpoint of the unfolding transition for the fragment is closer to that of the second observed transition in the full-length

Fig. 3. MRE at 222 nm for full-length α TS (open squares) and α TS(1–188) (filled circles) as a function of final urea concentration. Lines represent fits of the data to apparent two-state equilibrium unfolding models for the fragment or an apparent three-state model for the full-length α TS. The ellipticity at 222 nm for the I2 species is sufficiently similar to unfolded α TS that titration data at a single wavelength can be fit to a three-state model. Singular value decomposition fits to multiple wavelength data are required to detect I2 and necessitate a four-state model for full-length α TS (Gualfetti et al., 1999). Protein fragment concentration was 13.9 μ M for the fragment and 2.7 μ M for the full-length protein. The buffer conditions are described in the caption to Figure 2.

Table 1. Thermodynamic parameters from CD for unfolding of secondary structure in amino-terminal α TS fragments^{a,b}

Fragment	MRE at 222 nm (deg cm ² dmol ⁻¹) ^c	$\Delta G^\circ(\text{H}_2\text{O})$ (kcal mol ⁻¹)	$-m$ (kcal mol ⁻¹ M ⁻¹)	C_m^d (M)
α TS(1–214)	11,300	3.71 ± 0.78	1.00 ± 0.20	3.71
α TS(1–204)	10,500	3.60 ± 0.51	0.97 ± 0.13	3.72
α TS(1–188)	13,000	4.05 ± 0.22	1.09 ± 0.06	3.73
α TS(1–171)	11,500	2.48 ± 0.41	0.87 ± 0.11	2.87
α TS(1–157)	3,500	ND ^e	ND ^e	ND ^e
α TS(1–147)	6,000	2.41 ± 0.22	0.86 ± 0.11	2.80
α TS(1–133)	10,200	2.31 ± 0.53	0.85 ± 0.15	2.70
α TS(1–105)	8,400	ND ^f	ND ^f	ND ^f
α TS(Full-length)	16,000			
N $\rightleftharpoons$ I1		5.27 ± 0.08	2.01 ± 0.04	2.62
I1 $\rightleftharpoons$ I2		4.80 ± 0.18	1.22 ± 0.04	3.93

^aThe data were fit to the two-state model, F $\rightleftharpoons$ U, with the exception of the full-length data (Bilsel et al., 1999a), which were fit to the three-state model N $\rightleftharpoons$ I1 $\rightleftharpoons$ I2. The I2 $\rightleftharpoons$ U transition is not apparent by CD under the conditions of these experiments, where only the ellipticity at 222 nm is monitored.

^bConditions: 10 mM potassium phosphate, pH 7.8, 0.2 mM K₂EDTA, and 1 mM BME at 25 °C.

^cMean residue ellipticities at 222 nm for each fragment are determined from the far-UV CD spectra shown in Figure 2, and the errors are estimated to be $\pm 10\%$.

^dErrors in C_m are estimated to be ± 0.10 M urea.

^e α TS(1–157) unfolds cooperatively; however, the thermodynamic parameters were not determined because this fragment has a propensity to dimerize.

^fNot determined because the absence of a well-defined native baseline region for α TS(1–105) renders the estimation of the thermodynamic parameters unreliable.

protein (I1 $\rightleftharpoons$ I2). Equilibrium unfolding curves of α TS(1–188) at concentrations ranging from 3–20 μ M were coincident (data not shown), demonstrating that the equilibrium process being monitored was not measurably influenced by the small propensity of this fragment to dimerize. Equilibrium ultracentrifuge experiments with α TS(1–188) were best described by a monomer-to-dimer equilibrium with a dissociation constant of ~ 200 μ M (data not shown), further indicating that the principal species studied under the conditions of the CD experiments is monomeric.

The equilibrium unfolding curves were converted to plots of apparent fraction unfolded protein (F_{app}) to allow for a direct comparison of the disruption of secondary structure by urea in the various fragments. A subset of these F_{app} curves is shown in Figure 4. The coincidence of the curves for fragments α TS(1–188), α TS(1–204), and α TS(1–214) implies that the addition of $\alpha 6$ or $\beta 7$ to the C-terminus of α TS(1–188) provides no increased resistance to urea denaturation. By contrast, the removal of $\beta 6$ to produce α TS(1–171) has a noticeable effect on the stability, as indicated by the substantial shift in the midpoint to lower urea concentrations. The incremental removal of additional secondary structural elements from the C-terminus to produce α TS(1–147) and α TS(1–133) results in a slight but systematic decrease in the m value and midpoint of the unfolding transition. Fragment α TS(1–105) is predominantly folded although it does not exhibit a well-defined native baseline region (data not shown), suggesting that it is further destabilized. These data suggest that $\beta 4$ and $\beta 6$ define structural boundaries for autonomous folding units.

Quantitative comparisons of the apparent stabilities of the fragments were performed by fitting the equilibrium unfolding curves to an apparent two-state transition between folded (F) and unfolded (U) monomers. The results are shown in Table 1. The two-state approximation for the fragments is suggested by the

smooth sigmoidal transition curves, which lack the obvious break in cooperativity observed for the full-length α TS. Fluorescence titrations were performed on fragments α TS(1–133), α TS(1–188), α TS(1–204), and α TS(1–214) to further confirm the two-state approximation (data not shown). The coincidence of the midpoints obtained by both CD and FL for these fragments is consistent with two-state unfolding although the steep native baselines observed

Fig. 4. Normalized fraction unfolded plot for amino-terminal fragments: α TS(1–133) (∇), α TS(1–147) (Δ), α TS(1–171) ($\circ$), α TS(1–188) ($\bullet$), α TS(1–204) ($\blacktriangle$), and α TS(1–214) ($\blacklozenge$). Solid lines represent fits of the data to an apparent two-state equilibrium unfolding model. The dashed line represents the apparent fraction unfolded plot for the full-length α TS fit to a three-state model. Conditions are described in the caption to Figure 2.

by FL results in a high uncertainty in the estimation of the thermodynamic parameters.

All of the fragments that contain at least $\alpha_0(\beta/\alpha)_{1-4}$ exhibit measurable stability. Addition of the $\alpha_4(\beta/\alpha)_5\beta_6$ segment results in an increase in stability of $1.5 \text{ kcal mol}^{-1}$ with a corresponding shift of 0.85 M in the midpoint to higher urea. Inclusion of the $\alpha_6(\beta/\alpha)_{7-8}$ carboxyl-terminal segment leads to the three-state behavior observed for the full-length α TS.

Probing residual structure by NMR

One-dimensional ^1H -NMR was used to probe for the presence of residual structure in the unfolded fragments at high concentrations of urea where the secondary structure is predominantly disrupted. The experiment monitors the chemical environment around His92, which resides in a loop region just before β_3 and is present in all of the fragment sequences. ^1H -NMR spectra were taken after equilibration with urea concentrations that varied in 0.5 M increments between 4 and 9 M urea. Figure 5 shows representative NMR spectra of the histidine C ϵ proton region for α TS(1–157), α TS(1–147), and α TS(1–133) at three different urea concentrations. All of the peaks in Figure 5 exhibit a linear downfield chemical shift with increasing urea concentration. This linear dependence on urea concentration does not reflect a conformational change, but rather is a consequence of the urea effect on the frequency of the water resonance that was used to reference the spectra. The NMR spectra of all of the fragments are simplified relative to the full-length spectrum by the removal of one or more of the histidine residues at positions 146, 195, and 244 (Saab-Rincon et al., 1996).

For α TS(1–157), both of the histidine residues experience similar environments at 4 M urea (Fig. 5A). At higher urea concentrations, a new downfield peak appears that increases in area with increasing urea concentrations. This new peak represents the slow exchange process involving His92 and indicates the presence of

residual structure in α TS(1–157). All of the fragments larger than α TS(1–157) also exhibit a similar slow exchange process which spans a similar urea concentration range to that of α TS(1–157), indicating residual structure in these fragments as well (data not shown). The fragment α TS(1–147) also shows evidence for residual structure; however, in this case, the structure is more destabilized as indicated by the presence of the slow exchange His92 resonance at the lowest urea concentration monitored, 4 M (Fig. 5B). Fragments α TS(1–133) and smaller, which all contain only a single histidine residue at position 92, showed neither a peak area change nor a chemical shift change beyond a solvent effect, providing no evidence for stabilized residual structure (Fig. 5C).

For all of the fragments, the His92 C ϵ proton experiences a second environment at all urea concentrations, as indicated by the small upfield resonance at 8 M urea observed in Figure 5. This peak represents the small population (5–10%) of the *cis* conformation around the peptide bond between His92 and Pro93 (Saab-Rincon et al., 1996).

The relative area of the His92 C ϵ proton resonance as a function of urea is shown in Figure 6 for α TS and a subset of the fragments. For all of the fragments, the unfolding transitions occur over a wider urea range with a midpoint that is equal to or lower than that of the full-length protein, suggesting a destabilization of the residual structure as a consequence of the missing C-terminal residues. α TS(1–147) exhibits only a partial transition curve, suggesting a further destabilization of the residual structure. The urea dependence of the relative area of the His92 resonance was reasonably well described by a two-state model in all cases. Table 2 shows the apparent thermodynamic parameters extracted from fits of the data to the two-state model, $\text{I2} \rightleftharpoons \text{U}$. All of the fragments exhibit free energies and m values that are less than those for the full-length protein, suggesting that the removal of $\alpha_7(\beta/\alpha)_8$ results in a significant destabilization of the residual structure. The partial tran-

Fig. 5. A 500 MHz FT-NMR spectra taken at 4, 6, and 8 M urea for (A) α TS(1–157), (B) α TS(1–147), and (C) α TS(1–133). The histidine residue corresponding to each C ϵ proton resonance is identified, and the peaks corresponding to the *cis* and *trans* isomers of His92 are designated H92c and H92t, respectively. Protein concentrations were ~ 0.5 mM, and the buffer conditions were 58 mM potassium phosphate, 0.2 mM K_2EDTA , and 2.0 mM DTE at pH 7.8 and 25°C .

Fig. 6. Normalized relative areas for the His92 C ϵ proton as a function of urea for full-length α TS ($\square$) and amino-terminal fragments: α TS(1–147) ($\triangle$), α TS(1–157) ($\diamond$), α TS(1–171) ($\circ$), and α TS(1–204) ($\blacktriangle$). Lines represent fits of the data to a two-state model, with the full-length fit represented by the thicker line. Conditions are described in the caption to Figure 5.

sition observed for α TS(1–147) suggests a further destabilization upon removal of β 5. Removal of β 4 results in the absence of stable residual structure in the smallest fragments.

Discussion

Structure in the folded fragments

Amino-terminal fragments containing as little as 40% of the α TS sequence have been demonstrated to fold cooperatively and have significant stability. Although somewhat surprising for a single structural domain protein, these results may be consequences of

the relatively large size of these amino-terminal fragments, 105–214 residues, and the local disposition of the elements of secondary structure. Fragments of less than 100 amino acids for other single structural domain proteins do not fold significantly, including barnase (Kippen et al., 1994) and chymotrypsin inhibitor-2 (de Prat-Gay et al., 1995). In a study of fragments of dihydrofolate reductase, only fragments containing 107 or more residues showed evidence for significant structure and stability (Gegg et al., 1997). These results suggest that a minimum sequence length may be necessary to bury enough hydrophobic surfaces and release solvent to account for the entropic loss resulting from ordering the backbone and side chains. In the case of α TS, the conformational entropy penalty for folding will also be mitigated by the simple topology of the β/α barrel, in which the neighboring secondary structural elements in the three-dimensional structure are also adjacent in the linear sequence.

Insights into the folded conformations of these fragments can be inferred from the thermodynamic parameters in Table 1. The near constant values for the free energies and m values of fragments α TS(1–188), α TS(1–204), and α TS(1–214) suggest that the segments from 189–214 do not fold well, largely consistent with the MRE data shown in Figure 2. Similarly, fragments α TS(1–133), α TS(1–147), and α TS(1–171) have comparable but reduced stabilities and m values, suggesting that residues from 134–171 also do not fold well. A further decrease in stability is suggested for α TS(1–105) by the absence of a well-defined native baseline region for this fragment. When taken together, these data suggest individual $\beta\alpha\beta$ modules exhibit a strong preference for structure. The previous demonstration of partial folded structure in a C-terminal fragment comprising residues 189–268, containing $\alpha_6(\beta/\alpha)_{7-8}$ is consistent with this observation (Miles et al., 1982).

The structural requirement for a pair of strands and an intervening helix may reflect both a minimal size necessary to bury sufficient hydrophobic surface, ~ 50 residues, as well as the stabilizing effect of hydrogen-bonding interactions between neighboring strands. The docking of these individual $\beta\alpha\beta$ modules onto each other appears to lead to enhanced stabilization in the larger fragments. Presumably, helix 0 (Yee et al., 1996) and the other helices that link these modules also provide hydrogen bonding and hydrophobic contacts that contribute to stability in all of the fragments.

It was originally proposed by Schellman (1978), and later demonstrated by Myers et al. (1995), that the m value is proportional to the change in solvent exposed surface area upon unfolding. The m value estimates for the largest fragments, α TS(1–188) to α TS(1–214), suggest Δ ASA of $\sim 6,500$ Å², which corresponds to the exposure of ~ 80 residues in a globular protein. Similarly, Δ ASA is estimated to be $\sim 4,300$ Å² for fragments α TS(1–133) to α TS(1–171), corresponding to the exposure of ~ 55 residues. In all cases, the estimated number of buried residues is less than half of the total residues in the fragment, suggesting that the side chains are more loosely packed and exposed to solvent in the folded conformation of the fragments than in the native full-length protein.

This supposition is supported by the measurement of the rotational correlation time for α TS(1–188) by time-resolved fluorescence anisotropy, which yields a hydrodynamic radius for this fragment that is $\sim 15\%$ larger than expected for a globular protein of this size (Bilsel et al., 1999b). An eight-stranded β/α barrel structure provides optimal side chain packing in the interior of the barrel (Lesk et al., 1989). It is reasonable to suppose that the removal of β 8 and further deletions would lead to an open, sheet-like structure. If so, then side chains that are buried inside the

Table 2. Thermodynamic parameters from NMR for unfolding of residual structure in α TS and amino-terminal fragments^{a,b}

Fragment	$\Delta G^\circ(\text{H}_2\text{O})$ (kcal mol ⁻¹)	$-m$ (kcal mol ⁻¹ M ⁻¹)	C_m^c (M)
α TS(Full-length)	7.7 $\pm$ 0.9	1.3 $\pm$ 0.1	5.9
α TS(1–214)	4.3 $\pm$ 1.0	0.7 $\pm$ 0.2	6.1
α TS(1–204)	4.8 $\pm$ 0.4	0.9 $\pm$ 0.1	5.2
α TS(1–188)	4.2 $\pm$ 0.5	0.8 $\pm$ 0.2	5.1
α TS(1–171)	4.2 $\pm$ 0.3	0.8 $\pm$ 0.1	5.4
α TS(1–157)	3.5 $\pm$ 0.5	0.6 $\pm$ 0.1	5.5
α TS(1–147) ^d	2.1 $\pm$ 0.2	0.5 $\pm$ 0.1	~ 4.5
α TS(1–133)	ND ^e	ND ^e	ND ^e
α TS(1–105)	ND ^e	ND ^e	ND ^e

^aThe data were fit to the two-state model, $I_2 \rightleftharpoons U$.

^bConditions: 58 mM potassium phosphate, pH 7.8, 0.2 mM K₂EDTA, and 1 mM DTE at 25 °C. The pH is uncorrected for the effect of ²H₂O.

^cErrors in C_m are estimated to be ± 0.2 M urea.

^dAlthough only a partial transition was detected, the data could be accurately fit because the folded baseline has a peak area value of zero.

^eND = not detected.

barrel in the full-length protein would be more mobile and exposed to solvent in the folded forms of these fragments. The steep native baselines observed in the urea-induced unfolding of the fragments monitored by fluorescence and the similar spectral shapes of the near-UV CD spectra of folded α TS(1–188) and unfolded full-length α TS (Higgins et al., 1979) are consistent with this view. Thus, while the folded forms of these fragments maintain a β/α backbone pattern, the three-dimensional structures are likely to be different than those for the same segment in the native conformation of the full-length protein.

The addition of $\alpha_4(\beta/\alpha)_5\beta_6$ to α TS(1–133) to produce α TS(1–188) provides a discrete and significant increase in the cooperativity and stability of the fragments that is not further enhanced by the addition of the next two secondary structural elements, α_6 and β_7 . Monte Carlo simulations on the folding of α TS also suggest that the six-stranded amino-terminal fragment should have unique stability (Godzik et al., 1992). Fragments containing 2 β -strands and 1–3 α -helices had some intrinsic stability, but they formed and dissolved spontaneously over the course of the simulation. When three such elements combined, they formed a stable six-stranded β -barrel whose hydrophobic core is partially covered by helices making non-native contacts. The rate-limiting step in the simulation was the breaking of these non-native contacts and the opening of the six-stranded barrel to incorporate the last two strands. Interestingly, the six-stranded barrel intermediate exclusively comprised the amino-terminal residues in α TS. A simulation of the folding of another β/α barrel protein, triosephosphate isomerase (TIM), showed that it also folded through a six-stranded barrel intermediate; however, TIM showed no preference for amino- or carboxy-terminal residues in the intermediate. Fragmentation analysis of TIM would be an interesting test of this simulation, as would an examination of longer carboxy-terminal fragments of α TS.

The supposition that α TS(1–188) corresponds to the equilibrium intermediate I1 in full-length α TS was originally based on the similarity of the midpoints for the Gdn-HCl denaturation of α TS(1–188) and the second transition in the full-length protein (Miles et al., 1982). Tritium exchange studies supported this conclusion (Beasty & Matthews, 1985). The urea unfolding data presented in Table 1, however, does suggest a modest decrease in stability and midpoint of α TS(1–188) relative to the I1 $\rightleftharpoons$ I2 transition in the full-length protein (Table 1), implying that the C-terminal residues may play a role in I1. Previous mutagenesis studies have demonstrated that β_6 and β_7 interact in I1 while strands β_1 and β_8 only interact in the native conformation (Tsuji et al., 1993). The similar stabilities observed for α TS(1–204) and α TS(1–214) suggest that β_8 and/or helices α_7 – α_8 may also contribute to the stability of I1.

Residual structure in the urea-unfolded fragments

The carboxy-terminal residues of α TS must also play a role in the formation of the high urea intermediate I2, as indicated by the decreased stability of the residual structure in the fragments relative to the full-length protein (Table 2). Previous mutagenic studies have shown that residues in β_3 , the strand adjacent to His92, participate in I2. However, neither β_3 nor α_2/β_3 is sufficient to stabilize the residual structure as demonstrated by the lack of residual structure in the corresponding 15- or 25-residue peptides spanning these regions (Saab-Rincon et al., 1996). Residual structure in some, but not all, of the fragments at high concentrations of urea further confirms that interactions between side chains distant

in sequence are required to stabilize I2. The similarity of the I2 $\rightleftharpoons$ U transition for fragments ranging from 147–214 residues (Table 2) suggests that interactions between a minimum of four strands are necessary to stabilize this residual structure. The abrupt increase in stability and m value for full-length α TS suggests either that the $(\beta/\alpha)_{5-8}$ module stabilizes $\alpha_0(\beta/\alpha)_{1-4}$ or that β_8 itself plays a unique role in stabilizing the amino terminal region. The previous observation that β_8 does not interact with β_1 until the native conformation of full-length α TS (Tsuji et al., 1993) favors the former explanation.

Mutagenic and temperature-dependence studies have previously demonstrated that the hydrophobic effect is responsible for stabilizing I2 (Saab-Rincon et al., 1996). Surprisingly, the prediction of hydrophobic folding units in the native α TS structure by the method of Tsai and Nussinov (1997) parallels the results from NMR at high urea concentrations. By using a cutting procedure that considers the compactness, isolatedness, hydrophobicity, and connectedness of subdomains, two hydrophobic folding units, corresponding to residues 216–257/19–145 and residues 146–215, are suggested for α TS (PDB file: 1wsya). The cooperative I2 $\rightleftharpoons$ U transitions observed for fragments α TS(1–147) to α TS(1–214) and absence of such a transition in the smaller fragments suggest that the 1–147 hydrophobic core is uniquely stable and primarily responsible for this behavior. Because these hydrophobic folding units are defined by consideration of the native structure, this correlation suggests that the interactions defining the residual structure in the unfolded protein are fundamental in the protein sequence. Specifically, the patterning of hydrophobic and hydrophilic residues may be the critical factor in determining the fold and stability of the residual structure in α TS. Kamtekar et al. (1993) have shown an appropriate bipolar pattern can result in highly helical polypeptides.

General implications for protein folding

The results on amino-terminal fragments of α TS demonstrate that large fragments (>100 residues) can behave as autonomous folding units. The data further suggest a strong preference for structure in even smaller $\beta\alpha\beta$ modules. The role of these local folding modules may be to serve as initial scaffolds, which enhance the assembly process for full-length proteins. Segmental folding could accelerate folding by simplifying the search of conformational space, but also might be responsible for creating stable intermediates that are separated from the native conformation by energy barriers. One of the rate-limiting steps in the folding of α TS is the conversion of the I1 intermediate to the native form (Bilsel et al., 1999a). The assembly of supersecondary structure modules can be considered as an extension of the framework model (Kim & Baldwin, 1982) or an example of the subdomain model (Oas & Kim, 1988).

The current study also demonstrates that residual structure can involve long-range interactions, not just local interactions, as observed in 434 repressor (Neri et al., 1992). This residual structure could play a key role in early folding events by limiting the conformational search process prior to the formation of secondary structure. The recent report of residual structure in Gdn-HCl unfolded cytochrome *c* suggests that other motifs, e.g., all helix, may also form by a similar mechanism (Segel et al., 1998). Understanding the transition from the hydrophobic clusters that stabilize the residual structure to the higher order secondary and tertiary structure observed in native-like intermediates (Bilsel et al., 1999a) is critical to solving the folding problem for β/α barrel proteins and

may potentially offer insights into the folding of other protein architectures as well.

Materials and methods

Reagents

Oligonucleotides used for mutagenesis were obtained from the Midland Certified Reagent Company (Midland, Texas). Ultrapure urea was purchased from ICN Biomedicals, Inc. (Costa Mesa, California) and used without further purification. $^2\text{H}_2\text{O}$ (100% pure) and ^2HCl (99.0% pure) were purchased from MSD Isotopes (Montreal, Canada). NaO^2H (99.0% pure) was purchased from ICN Biomedicals. All other chemicals were reagent grade.

Site-directed mutagenesis

The fragments were engineered by introducing a double stop-codon, TAATAG, into the αTS gene in regions coding for loops between incremental elements of secondary structure. For fragments $\alpha\text{TS}(1-188)$, $\alpha\text{TS}(1-204)$, and $\alpha\text{TS}(1-214)$, the "megaprimer" PCR method (Sarkar & Sommer, 1990) was used to incorporate the double stop codon into the αTS gene after residues 188, 204, or 214, respectively. The plasmid pXH (Chen & Matthews, 1994), a derivative of pTZ18 (Pharmacia Biotech, Inc., Piscataway, New Jersey) that contains the gene for the αTS from *Escherichia coli*, was used as the DNA template. For the first round of PCR, a 27 base-pair primer containing the double stop-codon, TAATAG, and a primer that contained the SalI restriction site and flanked the 3' end of the gene were used as primers. The resulting amplified PCR product was used as the "megaprimer" in the second round of PCR along with a primer that contained the SacI site and flanked the 5' end of the gene. The resulting PCR product was digested with SacI and SalI, ligated into the pXH plasmid, and transformed into the *E. coli* strain CB149 (a generous gift from E. Miles, National Institutes of Health, Bethesda, Maryland) for expression of the truncated gene product.

For fragments $\alpha\text{TS}(1-171)$ and shorter, a single round of PCR was used to incorporate the double stop-codon, TAATAG, and the restriction site, HindIII, into the αTS gene at the desired locations. In these cases, the plasmid containing the $\alpha\text{TS}(1-214)$ gene, pXH(1-214), was used as the DNA template. The 5' primer used in all cases was before the start of the gene and included the unique EcoRI site. For each fragment, a different 40 base-pair 3' primer was used that contained 21 nucleotides that base-paired to the plasmid and 19 non-base-pairing nucleotides, which coded for the double stop-codon and the HindIII restriction site. The resulting PCR product, which did not contain the undesired C-terminal codons, was digested with EcoRI and HindIII and ligated into the plasmid, pXH(1-214), for expression of the truncated gene products. For all of the fragments, DNA sequences were verified by sequencing the gene product using the Sequenase sequencing kit (U.S. Biomedicals).

Protein purification

The amino-terminal fragments were expressed as inclusion bodies, and a modified version of the protocol of Tsuji et al. (1993) was used to recover the insoluble fragments. The fragments were purified by anion exchange on a DEAE-52 column, using a 0–300 mM KCl

exponential gradient in 10 mM potassium phosphate, pH 7.8, 2 mM K_2EDTA and 1 mM DTE. The fragments were further purified by sizing chromatography under native conditions in a buffer containing 10 mM potassium phosphate, pH 7.8, 2 mM K_2EDTA and 1 mM DTE. To avoid solubility problems, no more than 50 mg of ion-exchange purified fragment was loaded onto the 100×2.5 cm G-100 superose sizing column at a flow rate of 0.5 mL min^{-1} . Approximately 10–20 mg of purified protein was recovered per sizing column to give a total yield of 50–100 mg of each fragment after multiple purification steps. All purification steps were carried out at 4°C . Full-length αTS was expressed and purified using the method of Kirschner et al. (1975) with previously described modifications (Bilsel et al., 1999a).

The purity and length of the fragments were confirmed by the presence of a single band of the appropriate molecular weight on 15% Laemmli polyacrylamide gels. The molecular weights of fragment $\alpha\text{TS}(1-188)$ were also confirmed by mass spectrometry. The fragments were stored in 80–90% saturated ammonium sulfate solutions containing the sizing column purification buffer. The fragment concentrations were determined by measuring the tyrosine absorbance at 278 nm, using the method of Gill and von Hippel (1989) to measure the extinction coefficients of the folded forms. The extinction coefficients under native conditions were typically $\sim 10\%$ higher than expected for fully solvent-exposed tyrosines. Errors in protein concentration measurements are estimated to be $\pm 10\%$. All of the fragments had a modest propensity to dimerize as demonstrated by FPLC (5–25% dimer at $\sim 100 \mu\text{M}$, data not shown), presumably as a consequence of removing the carboxy-terminal strand(s) and exposing the interior of the barrel to solvent. $\alpha\text{TS}(1-157)$ in particular tended to aggregate in the μM concentration range and, to a lesser extent, $\alpha\text{TS}(1-147)$.

CD spectroscopy

All experiments were carried out at a temperature of 25°C in buffer solutions containing 10 mM potassium phosphate, 0.2 mM K_2EDTA , and 1 mM BME at pH 7.8. Far-UV CD spectra were collected on an AVIV model 62DS spectrometer. Spectra were collected from 320 to 185 nm using a 0.1 or 0.2 cm pathlength cell and a 2 s averaging time. The CD signal was converted to MRE using the following equation:

$$\text{MRE} = \theta / (10 \times l \times c \times N_A) \quad (1)$$

where θ is the ellipticity in mdeg, l is the pathlength in centimeters, c is the molar concentration, and N_A is the number of residues. The equilibrium unfolding of the fragments was monitored by collecting CD spectra from 300 to 200 nm as a function of urea. To compare unfolding curves for the different fragments, the signal at 222 nm was normalized by calculating the apparent F_{app} , as a function of urea:

$$F_{app} = (y_F - y_o) / (y_F - y_U) \quad (2)$$

where y_o is the observed CD signal at 222 nm at a given urea concentration, and y_F and y_U are the values for the presumed linear folded and unfolded baselines, respectively, at that same urea concentration (Pace et al., 1990).

NMR spectroscopy

The samples used in the NMR studies were prepared in $^2\text{H}_2\text{O}$ following previously reported procedures (Saab-Rincon et al., 1993).

For each fragment, two samples were prepared in 4.0 and 9.0 M urea that represented the end points of the titration. The protein concentrations were ~ 0.5 mM, and the fragments did not dimerize under these conditions because of the presence of high concentrations of urea. The buffer conditions were 58 mM potassium phosphate, 0.2 mM K₂EDTA, and 1 mM DTE, pH 7.8 (pH meter reading was uncorrected for the effect of ²H₂O). After collecting NMR spectra on the two titration end point samples, appropriate volumes were successively mixed to obtain samples at 0.5 M urea intervals. All samples were equilibrated for ~ 1 h prior to collecting spectra. NMR spectra were obtained at 500 MHz on a Bruker AM 500 spectrometer in the Fourier transform mode using previously described instrumental settings (Saab-Rincon et al., 1993). The areas for each histidine C ϵ peak in the region from 7.6–7.9 were determined by fitting to a sum of Lorentzians, using the program FELIX 95.0. The sum of the areas was normalized to the total number of histidine residues in each fragment. The relative area for the His92 C ϵ proton was plotted as a function of urea and fit as described below to extract thermodynamic parameters.

Data analysis

Data were fit to two-state models, assuming a linear dependence of the free energy of unfolding on the denaturant concentration, $\Delta G^\circ = \Delta G^\circ(\text{H}_2\text{O}) + m[\text{urea}]$ (Schellman, 1978). Thermodynamic parameters were extracted from the equilibrium unfolding curves according to previously described procedures (Finn et al., 1992). Individual data sets were fit using either NLIN (SAS Institutes, Inc., Cary, North Carolina) or the in-house nonlinear least-squares program SAVUKA 5.1 (Zitzewitz et al., 1995) to yield the free energy change in the absence of denaturant, $\Delta G^\circ(\text{H}_2\text{O})$, the transition midpoint, C_m , and the sensitivity of the transition to denaturant, m . For the CD data, the baseline slopes and intercepts were adjustable parameters. For the NMR data, the y-intercept of the native baseline at 0 M urea and the slopes of both the native and unfolded baselines were all fixed to zero.

Acknowledgments

The authors would like to thank Drs. Osman Bilsel, Katherine Bowers, Colin Gegg, and Virginia Smith for insightful discussions. This work was supported by NIH grant GM 23303 to CRM. Partial support for JAZ was provided by NIH postdoctoral fellowship, GM 14954.

References

- Andreotti G, Cubellis MV, Di Palo M, Fessas D, Sannia G, Marino G. 1997. Stability of a thermophilic TIM-barrel enzyme: Indole-3-glycerol phosphate synthase from the thermophilic archaeon *Sulfolobus solfataricus*. *Biochem J* 323:259–264.
- Beasty AM, Matthews CR. 1985. Characterization of an early intermediate in the folding of the α subunit of tryptophan synthase by hydrogen exchange measurements. *Biochemistry* 24:3547–3553.
- Bilsel O, Yang L, Zitzewitz JA, Beechem JM, Matthews CR. 1999b. Time-resolved fluorescence anisotropy study of the refolding reaction of the α -subunit of tryptophan synthase reveals a nonmonotonic behavior of the rotational correlation time. *Biochemistry* 38:4177–4187.
- Bilsel O, Zitzewitz JA, Bowers KE, Matthews CR. 1999a. Folding mechanism of the α -subunit of tryptophan synthase, an α/β barrel protein: Global analysis highlights the interconversion of multiple native, intermediate, and unfolded forms through parallel channels. *Biochemistry* 38:1018–1029.
- Chaffotte AF, Cadieux C, Guillo Y, Goldberg ME. 1992. A possible initial folding intermediate: The C-terminal proteolytic domain of tryptophan synthase beta chains folds in less than 4 milliseconds into a condensed state with non-native-like secondary structure. *Biochemistry* 31:4303–4308.
- Chaffotte AF, Guillo Y, Delepierre M, Hinz H-J, Goldberg ME. 1991. The isolated C-terminal (F2) fragment of the *E. coli* tryptophan synthase β_2 -subunit folds into a stable, organized nonnative conformation. *Biochemistry* 30:8067–8074.
- Chaffotte AF, Guijarro I, Guillo Y, Delepierre M, Goldberg ME. 1997. The “pre-molten globule,” a new intermediate in protein folding. *J Protein Chem* 16:433–439.
- Chen X, Matthews CR. 1994. Thermodynamic properties of the transition state for the rate-limiting step in the folding of the α subunit of tryptophan synthase. *Biochemistry* 33:6356–6362.
- Constans AJ, Mayer MR, Sukits SF, Lecomte JTI. 1998. A test of the relationship between sequence and structure in proteins: Excision of the heme binding site in apocytochrome *b*₅. *Protein Sci* 7:1983–1993.
- Corbett RJ, Roche RS. 1986. Independent folding of autolytic fragments of thermolysin and their domain-like properties. *Int J Pept Protein Res* 28:549–559.
- de Prat-Gay G. 1996. Association of complementary fragments and the elucidation of protein folding pathways. *Protein Eng* 9:843–847.
- de Prat-Gay G, Ruiz-Sanz J, Neira JL, Itzhaki LS, Fersht AR. 1995. Folding of a nascent polypeptide chain in vitro: Cooperative formation of structure in a protein module. *Proc Natl Acad Sci USA* 92:3683–3686.
- Eder J, Kirschner K. 1992. Stable substructures of eightfold $\beta\alpha$ -barrel proteins: Fragment complementation of phosphoribosylanthranilate isomerase. *Biochemistry* 31:3617–3625.
- Finn BE, Chen X, Jennings PA, Saalau-Bethell SM, Matthews CR. 1992. Principles of protein stability. Part 1—Reversible unfolding of proteins: Kinetic and thermodynamic analysis. In: Rees AR, Sternberg MJE, Wetzel R, eds. *Protein engineering: A practical approach*. Oxford: IRL Press. pp 167–189.
- Gegg CV, Bowers KE, Matthews CR. 1997. Probing minimal independent folding units in dihydrofolate reductase by molecular dissection. *Protein Sci* 6:1885–1892.
- Gill S, von Hippel P. 1989. Calculation of protein extinction coefficients from amino acid sequence data. *Anal Biochem* 182:319–326.
- Godzik A, Skolnick J, Kolinski A. 1992. Simulations of the folding pathway of triose phosphate isomerase-type α/β barrel proteins. *Proc Natl Acad Sci USA* 89:2629–2633.
- Gualfetti PJ, Bilsel O, Matthews CR. 1999. The progressive development of structure and stability during the folding of the α subunit of tryptophan synthase from *E. coli*. *Protein Sci*. Forthcoming.
- Higgins W, Fairwell T, Miles EW. 1979. An active proteolytic derivative of the α subunit of tryptophan synthase. Identification of the site of cleavage and characterization of the fragments. *Biochemistry* 18:4827–4835.
- Hyde CC, Admed SA, Padlan EA, Miles EW, Davies DR. 1988. Three-dimensional structure of the tryptophan synthase $\alpha_2\beta_2$ multienzyme complex from *Salmonella typhimurium*. *J Biol Chem* 263:17857–17871.
- Jaenicke R. 1991. Protein folding: Local structures, domains, subunits, and assemblies. *Biochemistry* 30:3147–3161.
- Jasanoff A, Davis B, Fersht AR. 1994. Detection of an intermediate in the folding of the ($\beta\alpha$)8-barrel N-(5'-phosphoribosyl)anthranilate isomerase from *E. coli*. *Biochemistry* 33:6350–6355.
- Jones BE, Matthews CR. 1995. Early intermediate in the folding of dihydrofolate reductase from *E. coli* detected by hydrogen exchange and NMR. *Protein Sci* 4:167–177.
- Kamtekar S, Schiffer JM, Xiong H, Babik JM, Hecht MH. 1993. Protein design by binary patterning of polar and nonpolar amino acids. *Science* 262:1680–1685.
- Kim PS, Baldwin RL. 1982. Specific intermediates in the folding reactions of small proteins and the mechanism of protein folding. *Annu Rev Biochem* 51:459–489.
- Kippen AD, Sancho J, Fersht AR. 1994. Folding of barnase in parts. *Biochemistry* 33:3778–3786.
- Kirschner K, Wiskocil RL, Foehn M, Rezeau L. 1975. The tryptophan synthase from *E. coli*: An improved purification procedure for the α subunit and binding studies with substrate analogues. *Eur J Biochem* 60:513–523.
- Kraulis PJ. 1991. MOLSCRIPT: A program to produce both detailed and schematic plots of protein structures. *J Appl Cryst* 24:946–950.
- Kuwajima K, Garvey EP, Finn BE, Matthews CR, Sugai S. 1991. Transient intermediates in the folding of dihydrofolate reductase as detected by far-ultraviolet circular dichroism spectroscopy. *Biochemistry* 30:7693–7703.
- Lecomte JTI, Matthews CR. 1993. Unraveling the mechanism of protein folding: New tricks for an old problem. *Protein Eng* 6:1–10.
- Lesk AM, Branden C-I, Chothia C. 1989. Structural principles of α/β barrel proteins: The packing of the interior of the sheet. *Proteins* 5:139–148.
- Llinas M, Marqusee S. 1998. Subdomain interactions as determinants in the folding and stability of T4 lysozyme. *Protein Sci* 7:96–104.
- Matthews CR, Crisanti MM. 1981. Urea-induced unfolding of the α subunit of tryptophan synthase: Evidence for a multistate process. *Biochemistry* 20:784–792.

- Miles EW, Yutani K, Ogasahara K. 1982. Guanidine hydrochloride induced unfolding of the α subunit of tryptophan synthase and of the two α proteolytic fragments: Evidence for stepwise unfolding of the two α domains. *Biochemistry* 21:2586–2592.
- Missiakas D, Betton J-M, Minard P, Yon JM. 1990. Unfolding-refolding of the domains in yeast phosphoglycerate kinase: Comparison with the isolated engineered domains. *Biochemistry* 29:8683–8689.
- Myers JK, Pace CN, Scholtz JM. 1995. Denaturant m values and heat capacity changes: Relation to changes in accessible surface areas of protein unfolding. *Protein Sci* 4:2138–2148.
- Neri D, Billeter M, Wider G, Wüthrich K. 1992. NMR determination of residual structure in a urea-denatured protein, the 434-repressor. *Science* 257:1559–1563.
- Oas TG, Kim PS. 1988. A peptide model of a protein folding intermediate. *Nature* 336:42–48.
- Pace CN, Shirley BA, Thomson JA. 1990. Measuring the conformation stability of a protein. In: Creighton TE, ed. *Protein structure: A practical approach*. Oxford: IRL Press. pp 311–330.
- Parrado J, Conejero-Lara F, Smith RA, Marshall JM, Ponting CP, Dobson CM. 1996. The domain organization of streptokinase: Nuclear magnetic resonance, circular dichroism, and functional characterization of proteolytic fragments. *Protein Sci* 5:693–704.
- Peng ZY, Kim PS. 1994. A protein dissection study of a molten globule. *Biochemistry* 33:2136–2140.
- Saab-Rincon G, Froebe CL, Matthews CR. 1993. Urea-induced unfolding of the α subunit of tryptophan synthase: One dimensional proton NMR evidence for residual structure near histidine-92 at high denaturant concentration. *Biochemistry* 32:13981–13990.
- Saab-Rincon G, Gualfetti PJ, Matthews CR. 1996. Mutagenic and thermodynamic analyses of residual structure in the α subunit of tryptophan synthase. *Biochemistry* 35:1988–1994.
- Sanchez del Pino MM, Fersht AR. 1997. Nonsequential unfolding of the α/β barrel protein indole-3-glycerol-phosphate synthase. *Biochemistry* 36:5560–5565.
- Sarkar G, Sommer SS. 1990. The “megaprimer” method of site-directed mutagenesis. *BioTechniques* 8:404–408.
- Schellman JA. 1978. Solvent denaturation. *Biopolymers* 17:1305–1322.
- Segel DJ, Fink AL, Hodgson KO, Doniach S. 1998. Protein denaturation: A small-angle X-ray scattering study of the ensemble of unfolded states of cytochrome *c*. *Biochemistry* 37:12443–12451.
- Tasayco ML, Carey J. 1992. Ordered self-assembly of polypeptide fragments to form native-like dimeric trp repressor. *Science* 255:594–597.
- Tsai C-J, Nussinov R. 1997. Hydrophobic folding units derived from dissimilar monomer structures and their interactions. *Protein Sci* 6:24–42.
- Tsuji T, Chrzynek BA, Chen X, Matthews CR. 1993. Mutagenic analysis of the interior packing of an α/β barrel protein. Effects on the stabilities and rates of interconversion of the native and partially folded forms of the α subunit of tryptophan synthase. *Biochemistry* 32:5566–5575.
- Vuilleumier S, Sancho J, Loewenthal R, Fersht AR. 1993. Circular dichroism studies of barnase and its mutants: Characterization of the contributions of aromatic side chains. *Biochemistry* 32:10303–10313.
- Yee MC, Horn V, Yanofsky C. 1996. On the role of helix 0 of the tryptophan synthetase α chain of *E. coli*. *J Mol Biol* 271:14754–14763.
- Zitzewitz JA, Bilsel O, Luo J, Jones BE, Matthews CR. 1995. Probing the folding mechanism of a leucine zipper peptide by stopped-flow circular dichroism. *Biochemistry* 34:12812–12819.