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†

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ABSTRACT: A series of single and double amino acid replacements in four β strands of the α subunit of tryptophan synthase from *Salmonella typhimurium*, an α/β barrel protein, was made to study the interior packing of the barrel and to clarify its folding mechanism. The urea-induced unfolding of the α subunit is thought to involve a stable intermediate in which the amino folding unit (residues 1–188; helices 0–5, strands 1–6) remains folded while the carboxy folding unit (residues 189–268; helices 6–8, strands 7–8) becomes disordered [Beasty, A. M., & Matthews, C. R. (1985) *Biochemistry 24*, 3547; Miles, E. W., Yutani, K., & Ogasahara, K. (1982) *Biochemistry 21*, 2586]. Mutations in strands 1 (A18G and A18V), 6 (Y175Q), 7 (L209V), and 8 (G230A, G230V, and I232V) at the interface between these two folding units show that the effects on the stabilities of the native and intermediate conformations critically depend on the site of the replacement. Although all of these mutations decrease the stability of the native conformation, only the replacements in strand 6, Y175Q, and possibly strand 8, I232V, also perturb the intermediate. Comparisons of the effects of three pairs of double mutants with the effects of the constituent single mutants on stability show that strands 6 and 7 interact in both the intermediate and native conformations, while strands 1 and 8 interact only in the native conformation. Kinetic studies of unfolding indicate that the interactions which occur in the native conformation arise in the preceding transition state. These results demonstrate that the carboxy folding unit adopts an organized structure in the intermediate, contrary to our previous interpretation. The general implication is that the state of folding of one segment of a protein can depend on the presence of another, more stable element of structure.

One of the basic structural motifs found in globular proteins is the α/β barrel. This highly symmetrical form, which has now been observed in some 18 different cases (Farber & Petsko, 1990), consists of eight parallel β strands arranged sequentially in an approximately cylindrical shape. These internal "staves" alternate in sequence with amphipathic α helices, which dock on the exterior of the barrel in a β–α–β connectivity. The β strands are stabilized by a network of backbone hydrogen bonds and by van der Waals interactions between hydrophobic side chains in the interior of the barrel. The interactions between the helices and the barrel are dominated by nonpolar contacts. Lesk and co-workers (1989) have analyzed the packing within the interior of six α/β barrel proteins and concluded that the interior can be described as alternating "layers" of four hydrophobic side chains. The layers are composed of residues from either the odd (1, 3, 5, and 7) or even (2, 4, 6, and 8) numbered strands in an alternating fashion, a result of the right-handed "twist" of the β strands (Figure 1). The relatively regular and interlocking nature of this arrangement makes the α/β barrel motif an interesting target for a mutagenic study of the role of packing in folding and stability.

A favorable candidate for this type of analysis is the α subunit of tryptophan synthase. The tryptophan synthase holoenzyme is an α_2/β_2 tetramer which catalyzes the condensation of indole (from an activated form, indole-3-glycerol phosphate) and serine to form tryptophan (Yanofsky & Crawford, 1972). The structure of this complex, which has been solved at 2.5-Å resolution, shows that the α subunit is a member of the α/β barrel class of proteins (Hyde et al., 1988). In addition to the canonical set of eight helices and eight strands, the α subunit has another helix at the amino terminus, 0, and two more embedded in the sequence, 2′ and 8′. The α subunit can be isolated from the complex in a folded, active conformation, indicating that the structure is likely to be very similar. The urea-induced unfolding reactions of the wild-type α subunit of tryptophan synthase and all active mutants examined thus far are consistent with a three-state equilibrium model involving a native form, N, a stable intermediate, I, and an unfolded form, U. This intermediate is thought to have a folded amino folding unit (residues 1–188), containing helices 0–5 and strands 1–6, and a disorganized if not completely unfolded carboxy folding unit (residues 189–268), containing helices 6–8 and strands 7–8 (Miles et al., 1982; Matthews et al., 1983; Beasty & Matthews, 1985).

The equilibrium results reported in the present article demonstrate that the tolerance for amino acid replacements inside the barrel of the *Salmonella typhimurium* α subunit can vary substantially from one position to another. Comparison of the stability effects of several double mutations, located in the strands which form the interface between the folding units, with the sum of the effects of the component single mutations shows that interactions between some of the strands arise in the folding intermediate and persist into the native conformation. Others only appear in the native conformation. Kinetic studies demonstrate that the inter-

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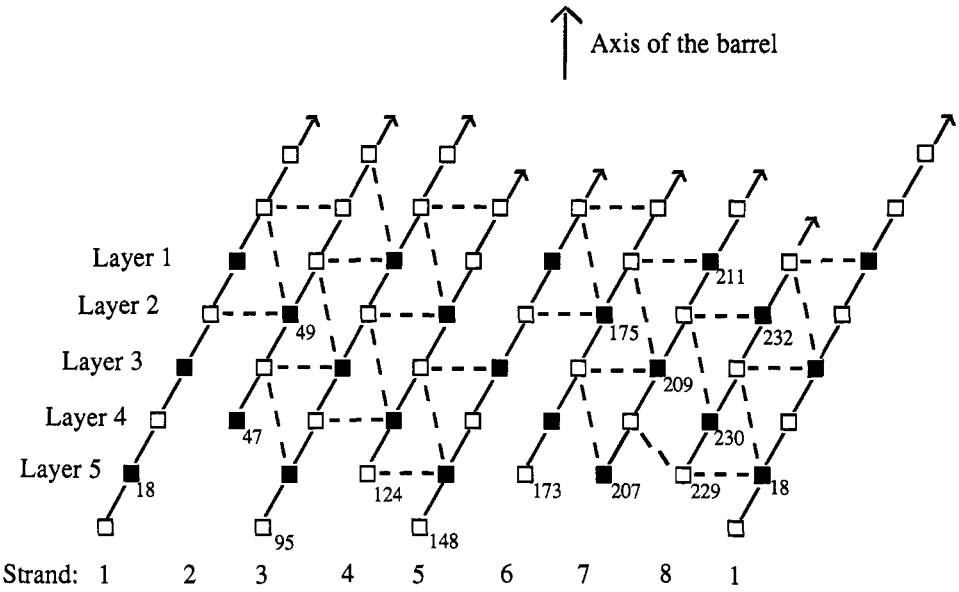

FIGURE 1: Two-dimensional representation of the β strands in the α/β barrel motif of the α subunit of tryptophan synthase. ■ indicates positions whose side chains penetrate into the interior of the barrel. - - - indicates interstrand hydrogen bonds with a donor and an acceptor distance <3.6 Å. Strand 1 was drawn twice to illustrate contacts with both strands 2 and 8.

Table I: Sequence of Oligonucleotides Used for Site-Directed Mutagenesis[a]

mutant	sequence
A18G	GCCGGGAAGGT#GG*TTTTGTCCCC
A18V	GCCGGGAAGGT#GT*TTTTGTCCCC
Y175Q	CGGTTACACCC*AG*CTGCTTTCGC
L209V	CGCGCCTGCA#G*TACAGGGCTTC
G230A	GCCGGGGCT#GCTGC*CGCTATCTCC
G230V	CCGGGGCT#GCTGT*CGCTATCTC
I232V	GGCTGGCGCC#G*TCTCCGGCTC

[a] Nucleotides with # are mismatched bases introduced for changes of restriction sites with silent mutations, while nucleotides with * are mismatched bases for amino acid replacements.

actions which occur in the native conformation arise in the transition state for the preceding, rate-limiting step in folding.

MATERIALS AND METHODS

The α Subunit of Tryptophan Synthase. A phage/plasmid system pRR2, previously constructed in this lab (Chen et al., 1992), was used for both mutagenesis and expression of the α subunit. *Escherichia coli* strain CB149 (a gift from Dr. Edith Miles), which lacks the entire *trp* operon (Kawasaki et al., 1987), was transformed with pRR2 and served as the host for expressing wild-type and mutant α subunits.

Oligonucleotide-Directed Mutagenesis. Mutagenesis was carried out by the method described by Kunkel (1987), except that the single-strand template of pRR2 was produced by superinfection with helper phage M13K07 (McClay et al., 1989). The oligonucleotides used for mutagenesis are listed in Table I. They were designed to mutate the target amino acid as well as to remove or introduce a restriction site by a silent mutation. The mutant plasmids were screened first by restriction enzyme digestions. The entire sequence of the *trpA* gene of the positive plasmids from the initial screen was then sequenced by the dideoxy nucleotide method. Double mutants were constructed from a single-strand DNA derived from a plasmid containing one of the single mutations. The oligonucleotides were purchased from the Biotechnology Center of the Pennsylvania State University.

Protein Purification. E. coli CB149 harboring the wild-type or mutant plasmid was grown in 5 L of LB medium supplemented with 50 mg/mL ampicillin at 37 °C for 16 h with shaking. Cells were collected by centrifugation, suspended in 50 mL of 100 mM potassium phosphate buffer (pH 7.8) containing 2 mM EDTA,[1] 2 mM DTE, 0.2 mM PMSF, and chicken lysozyme (1 mg/mL). After incubation on ice for 30 min, the cells were disrupted by sonication and the extract was centrifuged at 20000*g* for 10 min. The pellet, which contained inclusion bodies as well as cellular debris, was suspended thoroughly in 50 mL of the above buffer but without lysozyme and centrifuged again. The pellet was washed again with 30 mL of the same buffer and then suspended in 50 mL of 100 mM potassium phosphate buffer (pH 7.8) containing 2 mM EDTA, 2 mM DTE, 0.2 mM PMSF, and 6 M urea (ultrapure grade, Schwarz/Mann, Cleveland, OH). The suspension was mixed until uniform and then centrifuged at 20000*g* for 10 min. The pellet was then resuspended in 30 mL of the above urea–buffer solution and centrifuged. The supernatants from the two urea-extraction steps were combined and poured into 9 vol of distilled water containing 2 mM DTE. The pH of the resultant solution was adjusted to 7.8, and after it was stirred for 30 min at 4 °C, the solution was applied to a DE-52 column (5 × 40 cm) equilibrated with 10 mM potassium phosphate buffer (pH 7.8) containing 2 mM EDTA and 2 mM DTE. The column was washed with 4 bed vol of the same buffer, and the protein was then eluted with a 4-L linear gradient from 10 to 50 mM potassium phosphate (pH 7.8) containing 2 mM EDTA and 2 mM DTE. The α subunit usually eluted at about 20-30 mM potassium phosphate. Fractions containing the α subunit were pooled and the protein was stored as an ammonium sulfate suspension (70% saturation) at 4 °C. The typical yield of protein was 150–250 mg from 5 L of culture. The purity of the protein was confirmed by the presence of a single band on both $NaDodSO_4$ and native polyacrylamide gel electrophoresis.

The wild-type protein is mostly soluble in the first extraction step of cell debris, and the supernatant from this step was used for further purification. In this case, extracts were extensively dialyzed against the equilibration buffer used for

[1] Abbreviations: DTE, dithioerythritol; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; PMSF, phenylmethanesulfonyl fluoride.

Table II: Specific Activities for the α Subunit of Tryptophan Synthase Mutants from *Salmonella typhimurium*[a]

plasmid	mutation	activity (units/mg)
pRR-2	wild-type	3800
	Group A	
pTT-1	A18G	3360
pTT-2	A18V	3430
pTT-3	L209V	3830
pTT-12	Y175Q	3350
pTT-13	Y175Q+L209V	3000
pTT-14	I232V	3940
pTT-15	A18G+I232V	3520
pTT-16	A18V+I232V	3300
	Group B	
pTT-4	G230A	80
pTT-5	G230V	320
pTT-6	A18G+G230A	380
pTT-7	A18G+G230V	80
pTT-8	A18V+G230A	100
pTT-9	A18V+G230V	50
pTT-10	L209V+G230A	850
pTT-11	L209V+G230V	60
pTT-17	L209V+I232V	80

[a] Specific activities for the mutant proteins in group A and for the G230A mutant in group B were measured using purified proteins, with errors of ±10%. Those for the mutants in group B, except the G230A mutants, were estimated using crude materials with errors of ±25%. The amounts of the α subunit mutants were estimated by the intensity of the bands in $NaDodSO_4$ polyacrylamide gel electrophoresis.

the DE-52 column, and elution was performed in the same way as described above.

The G230A[2] mutant was extracted from the precipitant with 10 mM MES buffer (pH 7.8) containing 2 mM EDTA, 2 mM DTE, and 6 M urea, and the extract was applied to a CM-Sephadex C-50 column equilibrated with the same buffer. After sufficient washing, the protein was eluted with a linear salt gradient using the equilibration buffer and 0.2 M potassium chloride in the equilibration buffer (both containing 6 M urea). Urea was removed by dialysis, and the purified, soluble protein solution was finally obtained by a centrifugation step which removed a precipitant formed during dialysis.

Activity Assay. The activities of the mutant α subunit proteins were determined by measuring their ability to enhance the activity of the β_2 subunit of tryptophan synthase from *E. coli* (Dicamelli & Balbinder, 1976) in the condensation reaction of indole and serine to form tryptophan (Kirschner et al., 1975). The specific activities for each of the proteins studied are listed in Table II.

Equilibrium and Kinetic Unfolding Reactions. The equilibrium unfolding data were obtained by measuring the ultraviolet difference spectrum at 286 nm using the tandem cell technique (Herskovits, 1967) on an AVIV 118DS spectrophotometer. The buffer used in all experiments was 10 mM potassium phosphate (pH 7.8), 0.2 mM EDTA, and 1 mM 2-mercaptoethanol. All experiments were performed at 25 °C. The protein concentrations were constant for a given titration in the range of 0.6–0.9 mg/mL, as ascertained using $E^{1\%}_{278} = 4.4$ for the wild-type and mutant proteins with tyrosine at position 175 (Adachi et al., 1974). The value of $E^{1\%}_{278} = 3.3$ was used for the mutants which replace Tyr 175 with nonaromatic amino acids (Hurle et al., 1986).

[2] Mutant proteins are designated using the single letter amino acid codes, with the first letter designating the wild-type residue, the number indicating position of the residue, and the second letter designating the mutation, e.g., A18G indicates alanine 18 mutated to glycine. Double mutants are designated as A18G+I232V, indicating that two mutations are introduced in the same protein.

Kinetic data on the slow unfolding and refolding reactions were obtained by manual mixing methods as described previously (Finn et al., 1991). The same protein concentration ranges were used as in the equilibrium titration.

Data Analysis. As described previously (Beasty et al., 1986), the equilibrium unfolding results were fit to a three-state model:

$$N \overset{K_{NI}}{\leftrightarrow} I \overset{K_{IU}}{\leftrightarrow} U$$

where N, I, and U are native, intermediate, and unfolded forms, respectively, $K_{NI} = [I]/[N]$, and $K_{IU} = [U]/[I]$. The free energy of unfolding, which is calculated from the equilibrium constant by $\Delta G^{\circ}_{XY} = -RT \ln K_{XY}$, was assumed to depend linearly on the urea concentration (Schellman, 1978).

The kinetic data were fit to one or more exponentials as appropriate, and the relaxation times and associated amplitudes were extracted as described previously (Beasty et al., 1986). On the basis of transition-state theory, the activation free energies for unfolding were obtained by fitting the logarithm of the relaxation times obtained for jumps from zero molar denaturant into the unfolded base-line region (≥4 M urea) to a linear dependence on the denaturant concentration (Matthews, 1987). Both equilibrium and kinetic data were fit with a nonlinear least-squares fitting program, NLIN (SAS Institute, Cary, NC). The detailed procedures and equations were described elsewhere (Finn et al., 1991).

RESULTS

Selection of Mutational Sites

The stable folding intermediate of the α subunit is proposed to contain strands 1–6 and helices 0–5 in a folded conformation, while the remaining strands (7–8) and helices (6–8) are thought to be disorganized (Miles et al., 1982; Beasty & Matthews, 1985). To enhance the possibility of obtaining information not only on the packing in the native conformation but also on the intermediate and the transition state which links them, replacements were made in those strands which form the interface between the two folding units, i.e., strands 1, 6, 7, and 8. The specific sites were selected so as to have proximal side chains in strands 1 (position 18) and 8 (position 230), 6 (position 175) and 7 (position 209), and 7 (position 209) and 8 (position 232). As will be shown below, even the most minimal replacement at position 230, i.e., G230A, resulted in a major conformational change and a virtually inactive protein. Therefore, to investigate possible interactions between strands 1 and 8, a mutation was also made at position 232. The side chains at positions 18 and 232 are separated by about 6 Å in the wild-type protein. A previous study on a pair of replacements in strands 6 (Y175C) and 7 (G211E) of the *E. coli* α subunit has shown that these side chains interact with each other in the native conformation but not in the intermediate (Hurle et al., 1986). The current set of mutants extends this analysis to another pair of sites in strands 6 and 7 and a pair in strands 1 and 8.

Protein Production and Purification

The mutant α subunit proteins constructed for this study were found to be predominantly insoluble in the host *E. coli* cells, probably forming inclusion bodies (Harris, 1983). Only the wild-type enzyme, when expressed in the same system, was found in the soluble fraction. The mutant enzymes were prepared for purification by solubilization of the pellet in 6

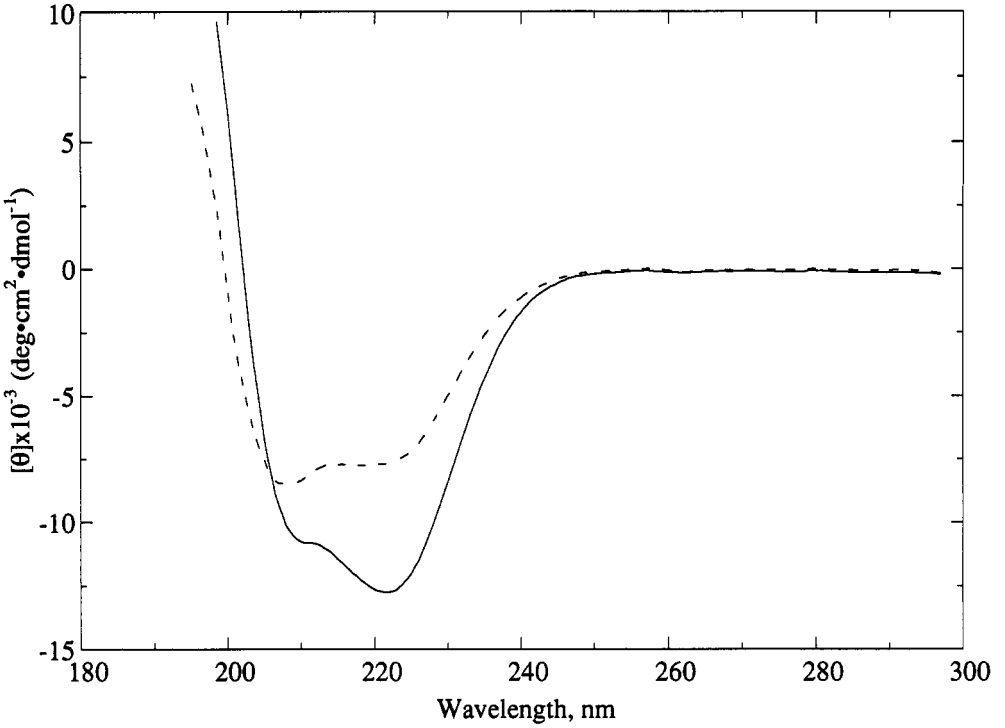

FIGURE 2: Circular dichroism spectra for wild-type (—) and G230A (- - -) mutant α subunits. The protein concentration for both samples was 0.4 mg/mL in 10 mM potassium phosphate, 0.2 mM EDTA, and 1 mM 2-mercaptoethanol (pH 7.8, 25 °C).

M urea and renaturation by dilution with buffer (see Materials and Methods). One class of mutants (Group A in Table II) was then purified in a manner similar to that used for the wild-type protein using anion-exchange chromatography. A second class of mutants (Group B in Table II) showed quite different behavior. They only bound to the anion-exchange resin at low protein concentration (0.5–1.0 mg/mL) and were eluted over a very wide range of ionic strength relative to those in group A.

The specific activities of all of the α subunit mutants are summarized in Table II. The group A mutants have activities almost identical to that of the wild-type enzyme. In contrast, those in group B have remarkably less activity. All but one of these group B mutants, the double mutant L209V+I232V, contain a replacement for glycine at position 230. Because the activity assay employed in this study monitors the enhancement of the β_2 activity of Trp synthase by the α subunit (Yanofsky & Crawford, 1972), the loss in activity could reflect a substantial change either in the structure for the α subunit or in its ability to form a complex with β_2 or both. The fact that these sites are buried and not in direct contact with the β_2 subunit strongly suggests that the native conformation is altered.

This supposition was supported by collecting a far-UV circular dichroism spectrum of the G230A mutant protein. The content of secondary structure in the mutant protein is significantly reduced compared to that of the wild-type enzyme (Figure 2). Analysis of this protein by gel-filtration chromatography also shows that the protein readily forms aggregates (T. Tsuji, unpublished results). Although no detailed stability studies were performed on this mutant, it is clear that the insertion of even a single methyl group in certain interior positions in the barrel can cause a drastic change in the structure and, presumably, the stability of the α subunit.

Equilibrium Results

Single Mutations. The equilibrium unfolding transition curves for the wild-type (Ala 18) and mutant α subunits containing either Gly or Val at position 18 in strand 1 are shown in Figures 3 and 4, respectively. Comparison of these curves makes it clear that these replacements destabilize the native state relative to the intermediate; the stability of the intermediate relative to the unfolded form appears to be little altered. Graphically, the N ↔ I transition is shifted to lower urea concentration while the I ↔ U transition is not significantly affected. The replacement of Leu 209 in strand 7 by Val (Figure 5) or Ile 232 in strand 8 by Val (Figure 3) also lowers the midpoint of the N ↔ I transition. For the I232V mutant, the I ↔ U transition may be affected as well (Figure 3). In contrast to the nearly selective effects of these replacements in strands 1, 7, and 8 on the N ↔ I transition, the unfolding curve of the Y175Q mutant in stand 6 shows that the midpoints of both the N ↔ I and I ↔ U transitions are shifted to lower urea concentration (Figure 5).

The thermodynamic parameters corresponding to the fitted curves in Figures 3–5 are shown in Table III. The errors in the estimates of the stabilities in the absence of denaturant are sufficiently large, especially for $\Delta G^\circ_{IU}(H_2O)$, that comparisons of the effects of these mutations cannot be made with confidence. To avoid the ambiguities introduced by extrapolation to the absence of denaturant, the free energy differences between N and I and between I and U can be compared at the urea concentration corresponding to the midpoint of the appropriate transition for the wild-type protein (Beasty et al., 1986; Cupo & Pace, 1983). At 2.75 M urea, the decrease in the free energy difference for the N ↔ I transition, $\Delta\Delta G^\circ_{NI}$(2.75 M), ranges from 0.7 (I232V) to 2.1 (Y175Q) kcal/mol. In contrast, the stability of I relative to U, $\Delta\Delta G^\circ_{IU}$(3.63 M), is virtually unchanged for all of the single mutants with the exception of Y175Q, the stability of I with respect to U is decreased by 1.9 kcal/mol. The apparent decrease in stability for I232V is slightly greater than the estimated error (0.5 ± 0.4 kcal/mol) and may be significant.

Double Mutants. To investigate possible interactions between adjacent strands in the barrel, four sets of double mutants were also constructed. The A18G+I232V and

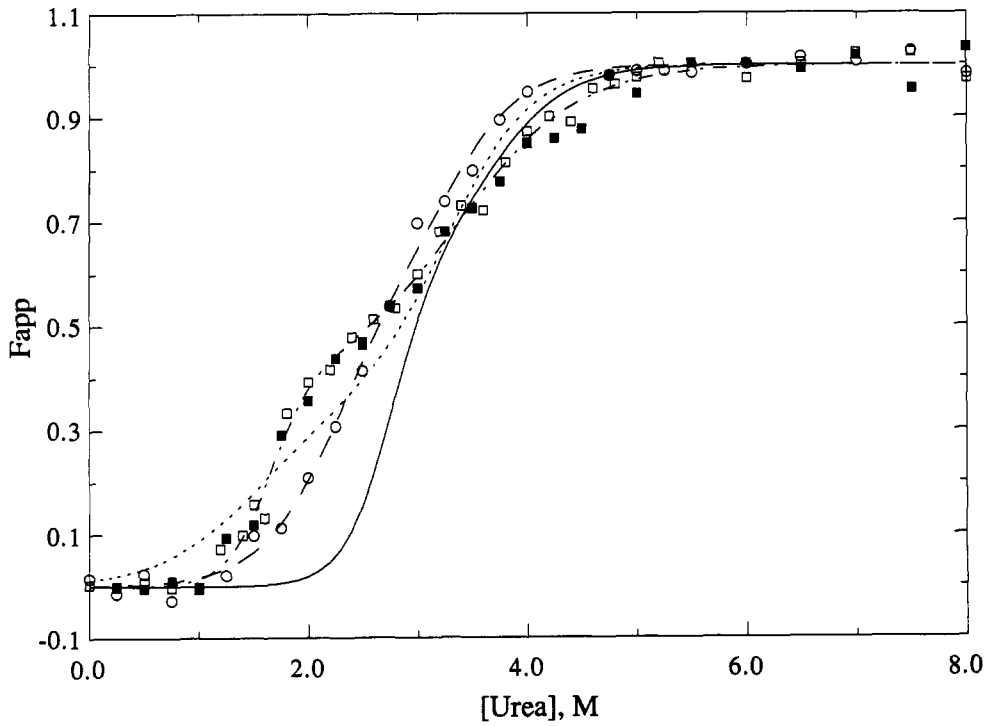

FIGURE 3: Apparent fraction of unfolded protein, F_{app}, as a function of the urea concentration for the wild-type (—), A18G (□, – - –), I232V (○, – –), A18G+I232V (- - - -) (all by absorbance), and A18G (■) (by circular dichroism) mutant α subunits in the presence of 10 mM potassium phosphate, 0.2 mM EDTA, and 1 mM 2-mercaptoethanol (pH 7.8, 25 °C). Lines represent the fits to a three-state model. Data for the double mutant protein were omitted for clarity.

A18V+I232V mutants probe interactions of strands 1 and 8, the Y175Q+L209V mutant probes interactions of strands 6 and 7, and the L209V+I232V mutant probes interactions of strands 7 and 8. As is shown in Figure 3, the A18G+I232V mutations result in a substantial decrease in the cooperativity, i.e., the slope, of the N ↔ I transition. For the A18V+I232V mutant, the entire transition curve is almost identical to that for the A18V single mutant (Figure 4). The N ↔ I transition for the Y175Q+L209V double mutant is similar to that of the Y175Q mutant, but the I ↔ U transition is displaced to higher urea concentration than those of either constituent single mutant (Figure 5). An equilibrium unfolding curve for the L209V+I232V mutant was not obtained because of difficulties in obtaining sufficient quantities of protein. The very low specific activity (Table II) suggests that, like the G230A mutant, the structure of this double mutant is drastically altered.

Thermodynamic analyses of these data (Table III) show that, like the constituent single mutations, these double replacements decrease the stability of N with respect to I. At 2.75 M urea, this decrease varies from 0.6 (A18G+I232V) to 1.3 (A18V+I232V) kcal/mol. The relative energies of I and U are perturbed significantly only for the Y175Q+L209V double mutant. In this instance, the stability of I with respect to U increases by 1.6 kcal/mol at 3.63 M urea. Comparison of the perturbations observed for the double mutants with the sum of the perturbations for the constituent single mutants shows that the A18G+I232V and Y175Q+L209V pairs are nonadditive (interaction occurs) for the N ↔ I transition. The interaction energy for A18G+I232V is –1.6 kcal/mol, and that for Y175Q+L209V is –2.0 kcal/mol (Table III). The negative signs indicate that both of these double mutants are more stable than predicted on the basis of the sum of the effects of the single mutants. Curiously, the A18V+I232V pair of mutations are additive for both the N ↔ I and I ↔ U transitions within experimental error (no evidence for interaction). For the I ↔ U transition, only the Y175-Q+L209V double mutant displays the nonadditivity characteristic of interacting side chains. In this case, the interaction energy is –3.7 kcal/mol.

Inspection of the fitted slopes for the N ↔ I transitions in the mutant α subunits (A_{NI}, column 4 in Table III) shows a measureable decrease in most cases. To test the possibility that these changes reflect a change in the equilibrium folding model, e.g., the appearance of additional folding intermediates, the urea-induced unfolding of the A18G and Y175Q+L209V mutants was also examined by far-UV circular dichroism spectroscopy. The normalized changes in ellipticity at 222 nm followed the same pattern as the apparent fraction of unfolding protein determined by the absorbance spectroscopy (Figures 3 and 5). The thermodynamic parameters derived from the fits of these CD data to a three-state model agree within experimental error with those from the absorbance transition curve (data not shown). Thus, the three-state model continues to provide an accurate description of the equilibrium behavior of these two mutants and, presumably, of the remainder of the collection. A discussion of the possible source of the decrease in slope for the N ↔ I transition can be found in the Discussion section.

The Z value for the Y175Q+L209V double mutant, 0.75, suggests that the exposure of tyrosine side chains to solvent in the intermediate is markedly increased over that for the other mutants. This phenomenon may reflect the fact that Tyr 4 and Tyr 173 are located in the interior of the barrel where their solvent exposure could be affected by simultaneous replacements at positions 175 and 209. Single replacements at either position are not sufficient to cause this effect.

Kinetic Results

The unfolding of the wild-type *S. typhimurium* α subunit has been shown to be controlled by a single, slow reaction which leads directly out of the native conformation (Stackhouse et al., 1988). The logarithm of the relaxation times decreases

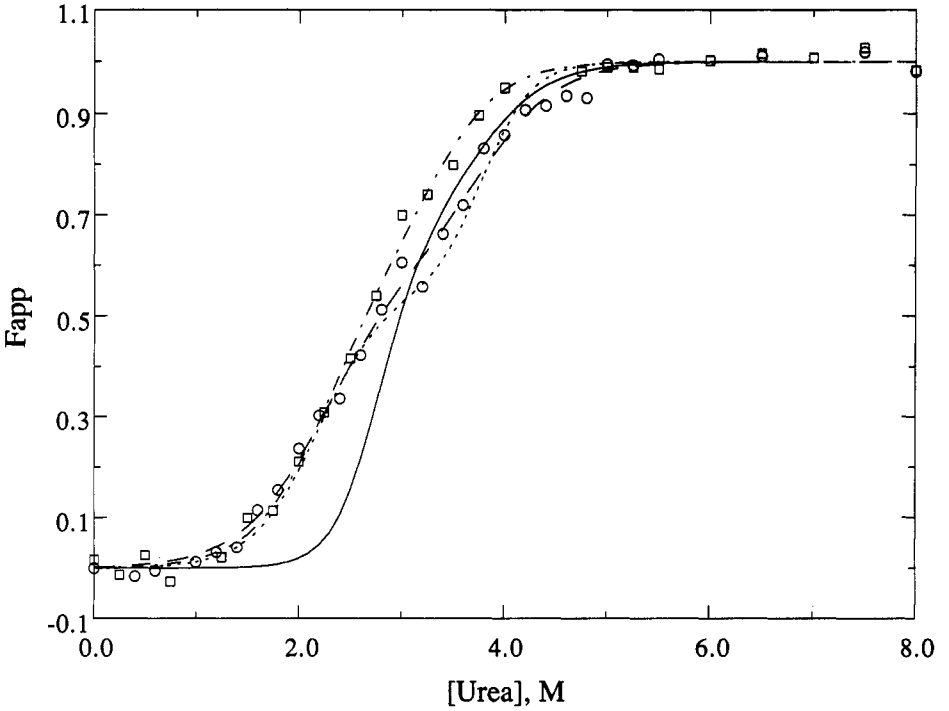

FIGURE 4: Apparent fraction of unfolded protein, F_{app}, as a function of the urea concentration for the wild-type (—), A18V (○, – –), I232V (□, – - –), and A18V+I232V (- - - -) mutant α subunits. Conditions were as described for Figure 3. Lines represent the fits to a three-state model. Data for the double mutant protein were omitted for clarity.

Table III: Thermodynamic Parameters for Urea-Induced Unfolding Transitions of the α Subunit of Tryptophan Synthase from *Salmonella typhimurium* at 25 °C, pH 7.8[a]

	Z^b	$\Delta G^\circ_{NI}(H_2O)$	A_{NI}	$\Delta G^\circ_{IU}(H_2O)$	A_{IU}	$C_{m_{NI}}$	$\Delta\Delta G^{\circ\ c}_{NI}$	$C_{m_{IU}}$	$\Delta\Delta G^{\circ\ d}_{IU}$	$\Delta G^{\circ\ e}_{NI,int}$	$\Delta G^{\circ\ e}_{IU,int}$
WT	0.59	7.4 ± 1.4	2.7 ± 0.5	5.8 ± 1.5	1.6 ± 0.4	2.75 ± 0.05		3.63 ± 0.05			
A18G	0.59	3.3 ± 0.6	1.7 ± 0.3	5.8 ± 1.5	1.5 ± 0.4	1.88 ± 0.07	1.5 ± 0.3	3.77 ± 0.10	–0.2 ± 0.4		
A18V	0.59	3.3 ± 0.5	1.5 ± 0.3	5.9 ± 1.5	1.6 ± 0.4	2.24 ± 0.07	0.8 ± 0.2	3.78 ± 0.10	–0.2 ± 0.4		
I232V	0.59	3.5 ± 0.8	1.5 ± 0.4	4.3 ± 1.9	1.3 ± 0.5	2.31 ± 0.15	0.7 ± 0.4	3.28 ± 0.19	0.5 ± 0.4		
L209V	0.59	4.0 ± 0.6	1.7 ± 0.3	3.8 ± 0.9	1.1 ± 0.2	2.30 ± 0.07	0.8 ± 0.1	3.44 ± 0.11	0.2 ± 0.2		
Y175Q	0.52	6.0 ± 1.8	2.9 ± 0.9	7.9 ± 2.7	2.7 ± 0.9	2.05 ± 0.06	2.1 ± 0.7	2.91 ± 0.07	1.9 ± 0.2		
A18G+I232V	0.59	1.9 ± 0.6	0.9 ± 0.4	5.6 ± 3.8	1.6 ± 1.0	2.08 ± 0.27	0.6 ± 0.5	3.46 ± 0.27	0.3 ± 0.8	–1.6 ± 0.7	0.0 ± 1.0
A18V+I232V	0.50	4.4 ± 1.6	2.1 ± 0.8	6.8 ± 2.6	1.8 ± 0.7	2.12 ± 0.11	1.3 ± 0.6	3.73 ± 0.12	–0.2 ± 0.6	–0.2 ± 0.8	–0.5 ± 0.8
Y175Q+L209V	0.75	3.6 ± 0.4	1.6 ± 0.2	9.7 ± 3.5	2.2 ± 0.8	2.23 ± 0.04	0.9 ± 0.1	4.32 ± 0.11	–1.6 ± 1.1	–2.0 ± 0.7	–3.7 ± 1.1

[a] Units are as follows: $\Delta G^\circ_{XY}(H_2O)$ (the free energy of unfolding in the absence of denaturant), kcal/mol; A_{XY} (urea dependence of the free energy of unfolding), kcal/mol/mol(urea); $C_{m_{XY}}$ (transition midpoint), molar urea; errors represent 95% confidence limits. [b] Z is the factional change in extinction coefficient for the intermediate, normalized to the difference between the native and unfolded forms. [c] Calculated at the wild-type N ↔ I midpoint, 2.75 M urea, where $\Delta\Delta G^\circ_{XY} = \Delta G^\circ_{XY}$(wild-type) – ΔG°_{XY}(mutant). In this convention, a positive value indicates the destabilization of the X ↔ Y transition upon mutation. [d] Calculated at the wild-type I ↔ U midpoint, 3.63 M urea, as defined above. [e] Interaction energy: $\Delta G^\circ_{XY,int} = \Delta\Delta G^\circ_{XY,DM} - (\Delta\Delta G^\circ_{XY,M1} + \Delta\Delta G^\circ_{XY,M2})$. DM: double mutant; M1: single mutant 1; M2: single mutant 2.

linearly with increasing urea concentration, as expected for protein unfolding reactions (Matthews, 1987). Refolding is more complex, consisting of two slow phases and one or more faster phases which are complete within the deadtime of manual mixing, 10–15 s. The slowest, τ_1, phase is urea independent and is thought to involve a non-proline-type isomerization reaction (Hurle & Matthews, 1987). Because the focus of the present study is on folding reactions and not isomerizations, this phase will not be considered further. The relaxation time for the τ_2 phase, the faster of the two slow phases, has a more complex dependence on the urea concentration. The relaxation time decreases as the denaturant concentration decreases from 2.8 to 1.8 M urea and becomes independent of denaturant concentration below 1.8 M urea. In the region near 2.8 M urea, which corresponds to the midpoint of the folding transition of the N ↔ I reaction, the unfolding and refolding relaxation times connect smoothly (Figure 6). This behavior has been interpreted to mean that the interconversion of N and I is the rate-limiting step in the unfolding and refolding of the α subunit above 1.8 M urea.

The effects of the A18G, I232V, and the A18G+I232V mutations on the rate-limiting unfolding and refolding reaction for the α subunit are also shown in Figure 6. For all three mutants, the unfolding reaction is accelerated compared to that of the wild-type protein. The urea dependence of the unfolding relaxation times is also seen to vary. The refolding relaxation times for the A18G and I232V mutants are somewhat longer than those of the wild-type protein between 1 and 2 M urea; however, they display the same urea-independent behavior at lower denaturant concentration. The refolding of the double mutant, A18G+I232V, only shows a weak dependence on urea concentration. The decrease in the concentration of urea at which these relaxation times proceed through a maximum reflects the decrease in the midpoints of the N ↔ I equilibrium transitions seen in Figure 3 (Matthews, 1987). The plots of the urea dependence of the relaxation times for the other single and double mutants constructed in this study are similar and are omitted for brevity.

The simplicity of the unfolding reaction and the complexities of the refolding reactions for the α subunit (Beasty et al.,

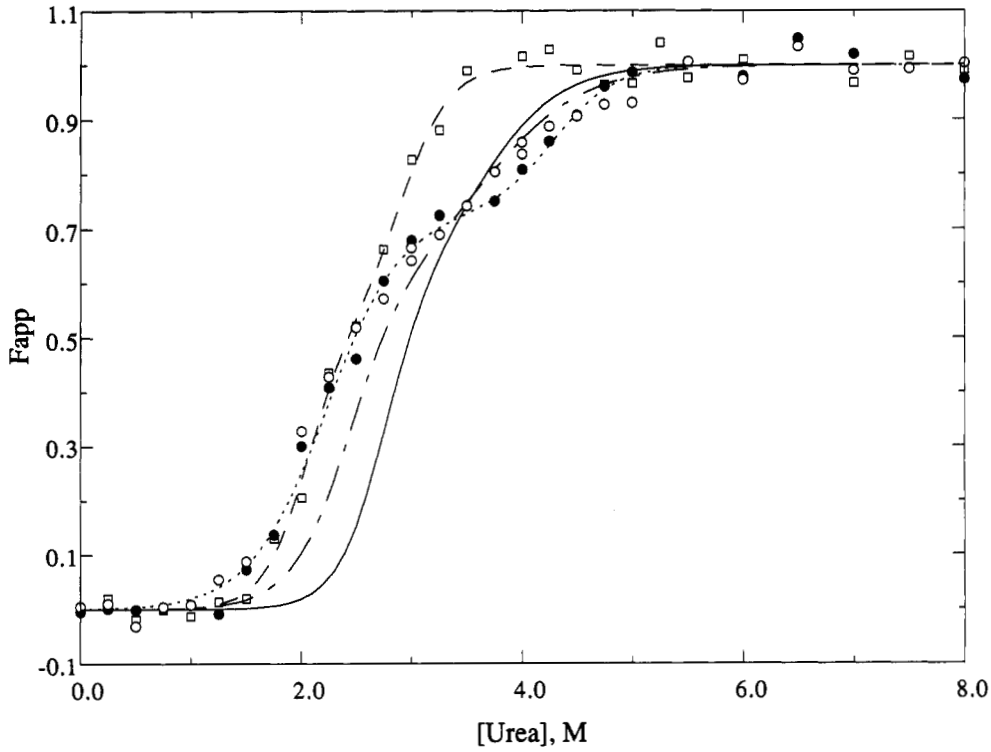

FIGURE 5: Apparent fraction of unfolded protein, F_{app}, as a function of the urea concentration for the wild-type (—), Y175Q (□, – –), L209V (– - –), Y175Q+L209V (●, - - -) (all by absorbance), and Y175Q+L209V (○) (by circular dichroism) mutant α subunits. Conditions were as described for Figure 3. Lines represent the fits to a three-state model. Data for the L209V mutant protein were omitted for clarity.

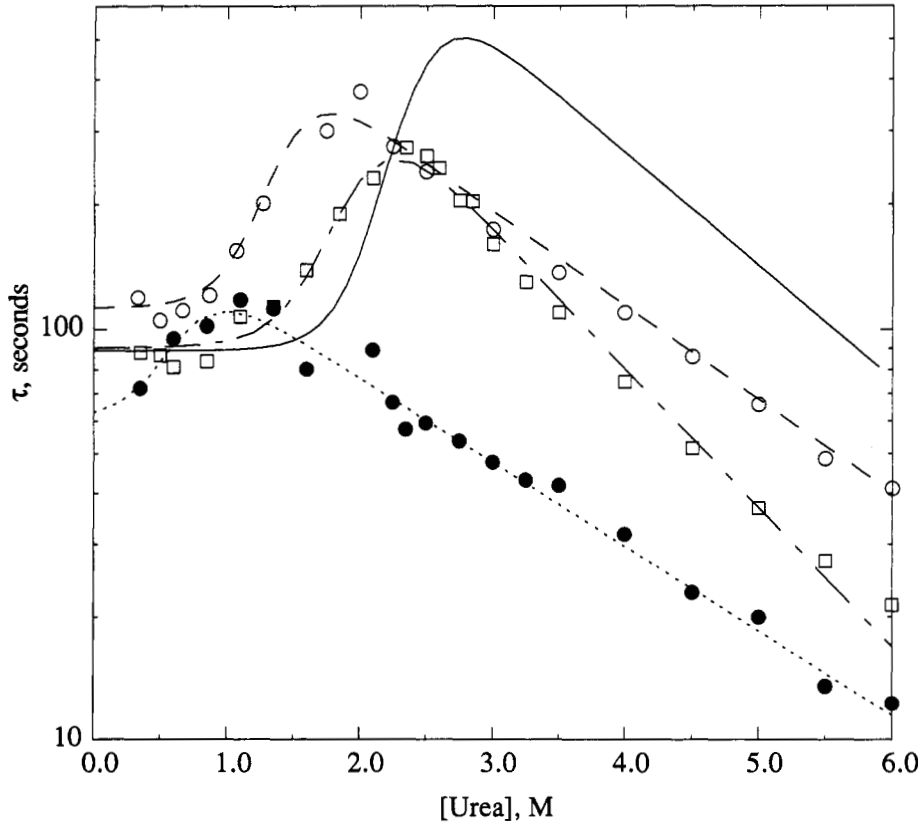

FIGURE 6: Plot of the logarithm of the relaxation times as a function of the urea concentration for the wild-type (—), A18G (○, – –), I232V (□, – - –), and A18G+I232V (●, - - -) mutant α subunits. Conditions were as described for Figure 3. Lines were drawn to aid the eye.

1986) suggest that a more accurate measurement of the relative free energy of the transition state for the rate-limiting step in folding can be obtained from the unfolding relaxation times. At denaturant concentration greater than 4 M urea, the equilibrium unfolding constant for N ↔ I transition, $K_{NI} = k_u/k_r$, is greater than 100 (calculated from the thermodynamic parameters listed in Table III). Under these conditions, the contribution to the relaxation time from the refolding microscopic rate constant, k_r, is negligible, and the unfolding microscopic rate constant, k_u, can be calculated with accuracy from the relaxation time according to the equation $\tau = 1/(k_u + k_r) \approx 1/k_u$ (Matthews, 1987).

Table IV: Transition-State Theory Parameters for the Urea-Induced Unfolding of Wild-Type and Mutant α Subunit of Tryptophan Synthase from *Salmonella typhimurium* at 25 °C, pH 7.8[a,b]

	$\Delta G_u^{\ddagger}(H_2O)$	$A_u^{\ddagger}$	$\Delta G_u^{\ddagger}$(2.75 M)[c]	$\Delta\Delta G_u^{\ddagger}$(2.75 M)	$\Delta G_{int}^{\ddagger}$(2.75 M)[d]
WT	22.25 ± 0.04	0.39 ± 0.01	21.18 ± 0.02		
A18G	21.44 ± 0.22	0.30 ± 0.04	20.61 ± 0.10	0.56 ± 0.10	
A18V	20.57 ± 0.21	0.30 ± 0.04	19.74 ± 0.10	1.43 ± 0.10	
I232V	21.47 ± 0.28	0.37 ± 0.05	20.45 ± 0.13	0.72 ± 0.13	
L209V	21.42 ± 0.07	0.32 ± 0.02	20.55 ± 0.04	0.63 ± 0.04	
Y175Q	22.19 ± 0.09	0.41 ± 0.02	21.07 ± 0.04	0.10 ± 0.04	
A18G+I232V	20.63 ± 0.47	0.29 ± 0.09	19.84 ± 0.22	1.35 ± 0.22	0.04 ± 0.27
A18V+I232V	20.15 ± 0.95	0.26 ± 0.20	19.43 ± 0.42	1.74 ± 0.42	–0.42 ± 0.45
Y175Q+L209V	21.30 ± 0.12	0.29 ± 0.02	20.51 ± 0.05	0.67 ± 0.05	–0.07 ± 0.08

[a] Units are follows: $\Delta G_u^{\ddagger}$ (the activation free energy of unfolding), kcal/mol; $A_u^{\ddagger}$ (urea dependence of the activation free energy of unfolding), kcal/mol/mol(urea); errors represent 95% confidence limits. [b] $\Delta\Delta G_u^{\ddagger} = \Delta G_u^{\ddagger}$(wild-type) – $\Delta G_u^{\ddagger}$(mutant). [c] 2.75 M urea is the midpoint of the N ↔ I transition. [d] Interaction energy: $\Delta G_{int}^{\ddagger} = \Delta\Delta G_{DM}^{\ddagger} - (\Delta\Delta G_{M1}^{\ddagger} + \Delta\Delta G_{M2}^{\ddagger})$. DM: double mutant; M1: single mutant 1; M2: single mutant 2.

The values of the activation free energy for unfolding in the absence of denaturant and of the urea dependence of the activation free energy of unfolding were obtained by assuming that the unfolding activation free energy linearly depends on the urea concentration, as described previously (Matthews, 1987). These parameters are listed in Table IV and allow the computation of the activation free energy of unfolding at any urea concentration. With the exception of the Y175Q mutant, all of these replacements decrease the free energy of the transition state relative to that of the native conformation; the Y175Q replacement does not alter this energy to any significant extent. Note that the greater accuracy of the measurement of the activation free energy allows the comparison in the absence of denaturant.

To make a direct comparison with the equilibrium results for the N ↔ I reaction discussed above, it is useful to compute the effects of mutations on the activation free energy for unfolding at 2.75 M urea, the midpoint for the N ↔ I equilibrium unfolding transition. These values and the perturbations relative to the wild-type protein, $\Delta\Delta G^{\ddagger}$(2.75 M), are also shown in Table IV. In all cases, the effect of the mutation is to decrease the free energy difference between the native state and the transition state. From a kinetic perspective, all of the mutants unfold more rapidly than the wild-type α subunit.

When the effects of the double mutations on the unfolding activation free energies at 2.75 M urea are compared to the sum of the constituent single mutations, it is apparent that the effects are additive within experimental error (Table IV). Therefore, the nonadditivity observed in the equilibrium study of the N ↔ I reaction (Table III) must arise principally as the intermediate develops into the transition state.

DISCUSSION

Equilibrium Effects

Single Mutations. The stability effects of single amino acid replacements in the interior of the α/β barrel of the α subunit of tryptophan synthase critically depend on the site and nature of the substitution. The incorporation of a single methyl group at position 230 (G230A) in the *S. typhimurium* α subunit is sufficient to destabilize the protein by at least 4 kcal/mol, on the basis of the observation that destabilizations of this magnitude are detected in other mutants which can still fold to an active conformation (Table III). In contrast, the methyl side chain at an adjacent position in the structure can be deleted (A18G) or replaced with an isopropyl group (A18V), and the protein, although less stable, is fully folded and functional. Because the nonpolar replacements of increasing size at positions 18 and 230 are expected to enhance the contribution of the hydrophobic effect to the stability, the observed decreases in stability must reflect the steric interferences of the side chains or the backbone with neighboring segments of the protein (Sandberg & Terwilliger, 1989; Sauer et al., 1990). Inspection of the backbone dihedral angles for Gly 230 shows that ϕ = 157.7° and ψ = –166.2° (Hyde et al., 1988). These values correspond to a region of the Ramachandran plot in which only glycine can be accommodated (Creighton, 1984). Thus, the dramatic decrease in stability caused by replacing glycine with alanine at position 230 must reflect a rearrangement of the backbone, not just the neighboring side chains. Gly 230 is conserved in all known α subunits, supporting the notion that no other side chain can be accepted at this position. Note that these ϕ/ψ angles for Gly 230 result in a distortion of the backbone such that the amide nitrogen can only make a weak hydrogen bond (3.71 Å) with the carbonyl oxygen of position 208 in strand 7. This distortion also allows the amide nitrogen at position 229 to make a strong hydrogen bond (2.62 Å, Figure 1) to this same oxygen. Thus, the β sheet deviates from the classical structure in this region.

Another interesting aspect of the perturbations in the stability caused by these single mutants is that, with the exception of Y175Q, there is little or no effect on the I ↔ U transition (Table III). These results can be understood, in part, by the structural model for the intermediate which places these sites at the interface between the two autonomous folding units. If these positions have a high exposure to solvent in the intermediate, one might expect a rather small effect on the I ↔ U transition. Similar results were obtained by studying the mutations at position 22 of strand 1 (Chen et al., 1992). The obvious perturbation detected for the Y175Q mutant shows that this position must become at least partially buried in the intermediate. If the effect of replacing Ile 232 with Val is significant, then the carboxy folding unit must not be completely unfolded in the intermediate as previously thought (Beasty & Matthews, 1985). The results of the studies on the double mutants support both of these conclusions (see below).

Double Mutations. Comparison of the results for double mutants with those of the constituent individual mutants provides a powerful tool for probing interactions between specific side chains (Ackers & Smith, 1985; Hurle et al., 1986). The presumption is that nonadditive perturbations in stability reflect direct or indirect structural interactions. The nonadditivity observed in the N ↔ I transition for both A18G+I232V and Y175Q+L209V shows that these pairs of side chains are interacting, but it does not specify whether this interaction arises in the native or intermediate conformations. This issue can be resolved by examining the effects on the I ↔ U transition. Only the Y175Q+L209V double

mutant provides positive evidence for interaction in this latter transition. Therefore, the side chains at positions 175 in strand 6 and 209 in strand 7 interact both in the intermediate and in the native conformations. Nonadditivity for both transitions in Y175Q+L209V implies that a further packing of the side chains occurs as the intermediate converts to the native conformation. The additivity for both A18G+I232V and A18V+I232V mutants in the I ↔ U transition can be interpreted to mean that strands 1 and 8 are not in contact in the intermediate. Therefore, the interaction betewen strands 1 and 8 exists in the native conformation.

The destabilization of the native conformation, relative to that of the intermediate, is reflected not only in a lower transition midpoint, $C_{m_{NI}}$, for all of the mutants but also in a smaller value for the slope, A_{NI}, in most cases (Table III). The coincidence of the normalized absorbance and CD transition curves for two of these mutants (Figures 3 and 5) rules out the possibility that additional intermediate forms are responsible. Because the slope of a denaturant-induced unfolding transition reflects in part differences in the solvent exposure of nonpolar side chains between the two end states (Schellman, 1978; Alonso & Dill, 1991), the slope changes imply that either the native or the intermediate conformation is somewhat loosened in the mutants. That this effect is principally due to a change in the conformation of the native form can be seen in the absence of a significant perturbation in the slope of the I ↔ U transition, with the possible exception of the Y175Q and Y175Q+L209V mutants (Table III). Although the Y175Q and Y175Q+L209V mutants may be exceptions, the errors are too large to draw any firm conclusions. Thus, these replacements in the interior of the barrel generally appear to alter the packing of the native conformation in such a way as to increase the solvent exposure of nonpolar side chains.

There are two curious features in the thermodynamic data presented in Table III. First, if positions 175 and 209 are indeed in contact in the intermediate, why does the single mutation at position 209, L209V, not perturb the relative energies of I and U? Perhaps the absence of a significant effect is due to the coincidental cancellation of competing forces such as packing and hydrophobicity. Also, while the double mutant data demonstrate an interaction with position 175, they do not rule out the possibility that the side chain at position 209 has sufficient remaining solvent exposure to minimize the effect of the replacement of leucine by valine. A second puzzle concerns the additivity observed in the N ↔ I transition for the A18V+I232V double mutant, but not for A18G+I232V. One could argue that competing forces again cancel in A18V+I232V but not in A18G+I232V. Another possibility is that the net loss of two methyl groups in A18G+I232V is sufficient to cause a measurable effect while the net gain of one methyl group for A18V+I232V is not. Perhaps the most important lesson in both of these cases is that the *absence* of an effect accompanying an amino acid replacement becomes significant only if the same results are obtained with additional mutations at the same site (Garvey & Matthews, 1989).

The kinetic studies on this collection of mutant α subunits show that the interaction between positions 18 in strand 1 and 232 in strand 8 arises in the transition state for the rate-limiting step in folding. This result agrees with a previous conclusion based upon the effects of single replacements at positions 22 and 234 in the *E. coli* α subunit (Beasty et al., 1986) and suggests that the strands are behaving in a similar fashion. Newly discovered in the present study is the existence of an interaction between positions 175 in strand 6 and 209 in strand 7 in the intermediate. The additional interaction energy for this pair of amino acids in the N ↔ I transition must also appear in the transition state because the double mutant has an additive effect on the unfolding reaction (Table IV). Therefore, the rate-limiting step in the formation of the barrel must correspond, in part, to the concerted interactions of residues in strands 1 and 8 and residues in strands 6 and 7.

Structure of the Stable Intermediate

The revisions to the structural model of the stable folding intermediate in the α subunit which emerge from this study are (1) that the carboxy folding unit (residues 189-268) is not entirely unfolded and (2) that it associates with the amino folding unit. The interaction detected between positions 175 and 209 in the intermediate implies that these sites, and possibly strands 6 and 7, are in proximity. The absence of such an interaction between positions 175 and 211 in a previous study (Hurle et al., 1986) may be due to the fact that position 211 is in the top layer of the barrel where the side chain has an opportunity to protrude into the solvent (Figure 1). Yanofsky and his colleagues have successfully incorporated 14 other amino acids at this site, suggesting a very high tolerance for the amino acid side chain (Yanofsky & Horn, 1972; Murgola & Yanofsky, 1974). The additivity observed in the I ↔ U transition for pairwise replacements at positions 18 and 232 shows that these positions and, perhaps, strands 1 and 8 do not interact in the intermediate.

Given these new results, two models for the intermediate can be proposed: (1) The amino and carboxy folding units are arranged in an eight-stranded sheet. Strands 7 and 8 are associated with each other and with strand 6 through a hydrogen-bond network. (2) The two folding units are internally hydrogen bonded but associate with each other through noncovalent interactions between nonpolar side chains in strands 6 and 7. The latter model seems more likely on the basis of previous evidence that the amino folding unit provides greater protection against the exchange of amide hydrogens than the carboxy folding unit (Beasty & Matthews, 1985). The highly hydrophobic nature of the interior of the barrel suggests that either of these models should wrap up in a way so as to protect these residues from contact with solvent. Mutations in strand 2 at position 49 (in the interior of the barrel, Figure 1) perturb the stability of the native form and, to a lesser extent, the intermediate (Beasty et al., 1986; Chrunyk & Matthews, 1990), indicating that this side chain is at least partially protected from solvent in the intermediate and that the packing is not as tight as in the native form.

The conclusion that the carboxy folding unit remains at least partially organized and associated with the amino folding unit in the stable intermediate of the α subunit of tryptophan synthase must be confirmed by direct structural studies. If this conclusion proves to be correct, then it demonstrates that the state of folding of an autonomous folding unit can depend not only upon its own sequence but also upon the presence of another folded substructure. Previous studies have shown that the isolated carboxy folding unit is unfolded in 0.5 M guanidine hydrochloride, well below the region in which the intermediate is stable in this denaturant (Miles et al., 1982). Taken together, these results imply that the amino folding unit (strands 1–6) acts as a template for the folding of the carboxy folding unit (strands 7–8). Computer simulation of the folding of the α subunit also reveals a stable intermediate consisting of the first six β strands (Godzik et al., 1992).

The potential generality of the 6 + 2 folding scheme for the α/β barrel proteins is suggested by the results of Eder and Kirschner (1992) on phosphoribosylanthranilate isomerase (PRAI). Like the α subunit, PRAI is also an α/β barrel protein. The amino terminal fragment (containing strands 1–6) is compact and has a native-like far-UV CD spectrum, but a strongly reduced near-UV CD spectrum. This fragment has measurable stability to guanidine hydrochloride induced denaturation and unfolds cooperatively. In contrast, the carboxy terminal fragment is less compact and does not display a cooperative unfolding transition. If these and other proteins of this structural class evolved from a common ancestor (Farber & Petsko, 1990), then it is possible that the 6 + 2 mechanism has also been conserved during evolution.

The general implication for protein folding mechanisms is that the progressive development of native-like substructures is essential for the rapid and efficient folding to the final native conformation. More stable substructures form first and shift the folding reactions of less stable elements to favor their folded forms. These autonomous folding units then dock in the transition state for the rate-limiting step in folding and rearrange to a limited extent to form the native conformation. Similar observations have recently been reported for the tryptophan apo-repressor (Tasayco & Carey, 1992).

REFERENCES

Ackers, G. K., & Smith, F. R. (1985) *Annu. Rev. Biochem. 54*, 597.

Adachi, O., Kohn, L. D., & Miles, E. W. (1974) *J. Biol. Chem. 249*, 7756.

Alonso, D. O. V., & Dill, K. A. (1991) *Biochemistry 30*, 5974.

Beasty, A. M., & Matthews, C. R. (1985) *Biochemistry 24*, 3547.

Beasty, A. M., Hurle, M. R., Manz, J. T., Stackhouse, T., Onuffer, J. J., & Matthews, C. R. (1986) *Biochemistry 25*, 2965.

Chen, X., Rambo, R., & Matthews, C. R. (1992) *Biochemistry 31*, 2219.

Chrunyk, B. A., & Matthews, C. R. (1990) *Biochemistry 29*, 2149.

Creighton, T. E. (1984) in *Proteins: Structures and Molecular Properties*, p 167, W. H. Freeman and Company, New York.

Cupo, J. F., & Pace, C. N. (1983) *Biochemistry 22*, 2654.

Davis, B. J. (1964) *Ann. N. Y. Acad. Sci. 121*, 404.

DiCamelli, R. F., & Balbinder, E. (1976) *Genet. Res. Camb. 27*, 323.

Eder, J., & Kirschner, K. (1992) *Biochemistry 31*, 3617.

Farber, G. K., & Petsko, G. A. (1990) *TIBS 15*, 228.

Finn, B. E., Chen, X., Jennings, P. A., Saalau-Bethell, S. M., & Matthews, C. R. (1991) in *Principles of Protein Stability I–Reversible Unfolding of Proteins–Kinetic and Thermodynamic Analysis* (Rees, A. R., Wetzel, R., & Sternberg, J. E., Eds.) IRL Press, Oxford.

Garvey, E. P., & Matthews, C. R. (1989) *Biochemistry 28*, 2083.

Godzik, A., Skolnick, J., & Kolinski, A. (1992) *PNAS 89*, 2629.

Harris, T. J. R. (1983) in *Genetic Engineering 4* (Williamson, R., Ed) p 127, Academic Press, London.

Hurle, M. R., & Matthews, C. R. (1987) *Biochim. Biophys. Acta 913*, 179.

Hurle, M. R., Tweedy, N. B., & Matthews, C. R. (1986) *Biochemistry 25*, 6356.

Hyde, C. C., Ahmed, S. A., Padlan, E. A., Miles, E. W., & Davies, D. R. (1988) *J. Biol. Chem. 263*, 17857.

Kawasaki, H., Bauerle, R., Zon, G., Ahmed, S. A., & Miles, E. W. (1987) *J. Biol. Chem. 262*, 10678.

Kirschner, K., Wiskocil, R. L., Loehn, M., & Rezeau, L. (1975) *Eur. J. Biochem. 60*, 513.

Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 488.

Laemmli, U. K. (1970) *Nature (London) 227*, 680.

Lasters, I., Wodak, S. J., Alard, P., & Cutsem E. V. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 3338.

Lesk, A. M. Branden, C. I., & Chothia, C. (1989) *Proteins 5*, 139.

Luger, K., Hommel, U., Herold, M., Hofsteenge, J., & Kirschner, K. (1989) *Science 243*, 206.

Matouschek, A., Kellis, J. T., Jr., Serrano, L., & Fersht, A. R. (1989) *Nature (London) 340*, 122.

Matthews, C. R. (1987) *Methods Enzymol. 26*, 498.

Matthews, C. R., & Crisanti, M. M. (1981) *Biochemistry 20*, 784.

Matthews, C. R., Crisanti, M. M., Manz, J. T., & Gepner, G. L. (1983) *Biochemistry 22*, 1445.

McClary, J. A., Witney, F., & Geisselsoder, J. (1989) *BioTechniques 7*, 282.

Messing, J. (1983) *Methods Enzymol. 101*, 20.

Miles, E. W., Yutani, K., & Ogasahara, K. (1982) *Biochemistry 21*, 2586.

Sandberg, W. S., & Terwilliger, T. C. (1989) *Science 245*, 54.

Sauer, R. T., Jordan, S. R., & Pabo, C. O. (1990) *Adv. Protein Chem. 40*, 1.

Schellman, J. A. (1978) *Biopolymers 17*, 1305.

Schneider, W. P., Nichols, B. P., & Yanofsky, C. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 2169.

Schulz, G. E., & Schirmer, R. H. (1978) in *Principles of Protein Structure*, p 27, Springer-Verlag, New York.

Stackhouse, T. M., Onuffer, J. J., Matthews, C. R., Ahmed, S. A., & Miles, E. W. (1988) *Biochemistry 27*, 824.

Tasayco, M. L., & Carey, J. (1992) *Science 255*, 594.

Tweedy, N. B., Hurle, M. R., Chrunyk, B. A., & Matthews, C. R. (1990) *Biochemistry 29*, 1539.

Yutani, K., Ogasawara, K., Suzuki, M., & Sugino, Y. (1979) *J. Biochem. (Tokyo) 95*, 915.

Yutani, K., Ogasahara, K., Aoki, K., Kakuno, T., & Sugino, Y. (1984) *J. Biol. Chem. 259*, 14076.