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Amino Acid Replacements Can Selectively Affect the Interaction Energy of Autonomous Folding Units in the α Subunit of Tryptophan Synthase†

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ABSTRACT: Amino acid replacements were made at the interface between two autonomous folding units in the α subunit of tryptophan synthase from *Salmonella typhimurium* to test their mutual interaction energy. The results of equilibrium studies of the urea-induced unfolding reaction of the wild-type and mutant proteins in which phenylalanine 22 is replaced by leucine, isoleucine, and valine can be understood in terms of a selective decrease in the interaction energy between the two folding units; the intrinsic stability of each folding unit is not significantly altered. Kinetic studies of the rate-limiting step in unfolding show that the interaction energy appears in the transition state preceding the native conformation. Comparisons of the individual effects of these nonpolar side chains show that both hydrophobic and steric effects play important roles in the interaction energy between the folding units. The implication of these results is that the high cooperativity observed in the folding of many globular proteins may be reduced by appropriate amino acid replacements.

One of the most serious impediments to the solution of the protein folding problem is the high cooperativity of the folding reaction. When unfolded by chemical denaturants or temperature, only the native and fully denatured forms are significantly populated at equilibrium for many globular proteins (Jaenicke, 1987). The absence of stable, partially folded species, i.e., folding intermediates, has precluded the approach of monitoring the development of substructure formation as the solution conditions are progressively changed to favor the native conformation.

That structures smaller than the entire protein are capable of independent folding has been clearly demonstrated by studies of peptides derived from naturally occurring proteins. Under appropriate conditions, the C-peptide from ribonuclease A spontaneously adopts the α-helical conformation which is found in the intact protein (Bierzynski et al., 1982). Nativelike folding has also been observed for fragments of bovine pancreatic trypsin inhibitor (Oas & Kim, 1988; Staley & Kim, 1990) and for a leucine zipper peptide from a transcriptional activation factor (O'Shea et al., 1991). Given these data, it seems reasonable to suppose that if these sequences can fold to a native or nativelike conformation in isolation, they also do so in the context of the intact protein.

If substructures, or autonomous folding units, are stable, why is the folding reaction so cooperative? One possibility is that the high cooperativity reflects strong interactions between two or more autonomous folding units. If these interactions are sufficiently strong, the unfolding equilibrium transition may appear to follow a two-state model when probed by chemical denaturants or heat. Both of these perturbants are nonspecific and would be expected to disrupt both intra- and interfolding unit stabilizing interactions.

Site-directed mutagenesis provides a potential method for perturbing selectively the interfolding unit stabilization forces and, thereby, breaking down the cooperativity of the folding reaction. To test the feasibility of this approach, amino acid substitutions were made at the interface between two folding units in the α subunit of tryptophan synthase from *Salmonella typhimurium* (ST).[1] This α/β barrel protein, as well as its highly homologous counterpart from *Escherichia coli* (EC), is thought to unfold via a stable intermediate in which the first six strands and five helices (of the canonical α_8/β_8 set), residues 1–188, remain folded while the final two strands and three helices, residues 189–268, become disordered (Beasty & Matthews, 1985; Miles et al., 1982; Stackhouse et al., 1988). Examination of the circular dichroism spectra of the isolated fragments from the EC α subunit, under native conditions, showed that each retains substantial secondary structure which is similar to that found in the intact protein (Higgins et al., 1979). A key piece of the evidence supporting this structural model for the partially folded species is that the midpoint of the denaturant-induced unfolding of the amino-terminal fragment is very similar to that for the transition from the intermediate to the unfolded form of the intact protein (Miles et al., 1982). The occurrence of a stable folding intermediate in the α subunit has been a curiosity because the native conformation is a single structural domain (Hyde et al., 1988).

The effects of multiple replacements at position 22 in strand 1 at the folding unit interface show that the interfolding unit stabilization energy can be selectively altered. Kinetic studies of the slow folding reaction leading to and from the native conformation demonstrate that this interaction is established in the preceding transition state.

Materials and Methods

Mutagenesis. A single plasmid/phage system, pRR2, which contains the entire structural gene encoding the ST α subunit of tryptophan synthase (Nichols & Yanofsky, 1979), was

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[1] Abbreviations: EC, *Escherichia coli*; ST, *Salmonella typhimurium*; F22L, F22I, and F22V, the Phe 22 to Leu, Ile, and Val mutations, respectively; K_2EDTA and Na_2EDTA, ethylenediaminetetraacetic acid, dipotassium and disodium salts, respectively; $NaDodSO_4$, sodium dodecyl sulfate; PMSF, phenylmethanesulfonyl fluoride; DTE, dithioerythritol.

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Table I: Thermodynamic Parameters for the Wild-Type and Mutant ST α Subunits[a]

protein	$\Delta G_{NI}^{H_2O}$	$-A_{NI}$	Cm_{NI}	$\Delta G_{IU}^{H_2O}$	$-A_{IU}$	Cm_{IU}	$\Delta G_{total}^{H_2O}$	$\Delta\Delta G_{total}^{H_2O}$[b]
ST F22 (WT)[c]	7.35 ± 0.67	2.68 ± 0.26	2.75	5.81 ± 0.74	1.60 ± 0.19	3.64	13.17 ± 1.00	0
ST F22L	6.38 ± 0.90	2.95 ± 0.42	2.16	5.73 ± 0.50	1.70 ± 0.14	3.38	12.11 ± 1.03	1.05 ± 1.44
ST F22I	3.16 ± 0.38	1.57 ± 0.20	2.01	5.43 ± 0.22	1.51 ± 0.06	3.61	8.58 ± 0.44	4.58 ± 1.09
ST F22V	3.85 ± 0.29	2.04 ± 0.16	1.89	5.87 ± 0.33	1.74 ± 0.10	3.38	9.72 ± 0.44	3.44 ± 1.09

[a] $\Delta G_{xy}^{H_2O}$ = free energy difference between x and y in the absence of urea (in kcal/mol). A_{xy} = dependence of the free energy difference on urea concentration [in kcal mol^{-1} (mol of urea)$^{-1}$]. Cm_{xy} = midpoint for the $x \leftrightarrow y$ transition; $Cm_{xy} = -\Delta G_{xy}^{H_2O}/A_{xy}$. Errors are the standard deviations. [b] $\Delta\Delta G_{total}^{H_2O} = \Delta G_{total}^{H_2O}$(wild type) $- \Delta G_{total}^{H_2O}$(mutant). [c] WT = wild type.

constructed for both mutagenesis and gene expression. The plasmid is based upon the pTZ system (Pharmacia, Inc., Piscataway, NJ). Site-directed mutagenesis was performed according to the protocol from the Bio-Rad kit (Bio-Rad, Richmond, CA) designed for the pTZ system. Three mutations, F22I, F22V, and F22L, were confirmed by DNA sequencing of the entire *trpA* gene.

Protein Purification. *E. coli* strain CB 149, lacking the wild-type α-subunit gene (a gift from Dr. Edith Miles), was used to express the ST α subunit. A high level of expression resulted in the formation of inclusion bodies in the cells. The wild-type α subunit, purified from such precipitates, has comparable specific activity and stability to denaturants as that purified from the supernatant using a different expression system (Chen and Matthews, unpublished results).

The cells were opened by repeated sonication in an opening buffer which contained 100 mM potassium phosphate, pH 7.8, 5 mM K_2EDTA, 1 mM DTE, 0.2 mM PMSF, and 2 mg of lysozyme/g of wet cells. After centrifugation at 10000*g* for 30 min, the precipitate was dissolved in the above buffer containing 6 M urea. The solution was vigorously stirred and slowly diluted 10× to a final condition of 10 mM potassium phosphate, pH 7.8, 5 mM K_2EDTA, and 1 mM DTE. This solution was centrifuged again to remove any precipitate formed during dilution (T. Tsuji, personal communication). The supernatant was further purified as described previously (Matthews et al., 1983). The wild-type, F22L, F22I, and F22V α subunits had specific activities of 3800, 3500, 3250, and 3320 units/mg, respectively, in the standard assay (Kirschner et al., 1975) and migrated as single bands on NaDodSO$_4$–polyacrylamide gels.

Experiments. Chemical reagents, experimental conditions, and procedures were as described previously (Chrunyk & Matthews, 1990; Finn et al., 1991). The wild-type and mutant ST α subunits were studied at concentrations from 0.5 to 1.7 mg/mL, a range where the equilibrium and kinetic properties of folding were found to be independent of protein concentration and fully reversible (Chen and Matthews, unpublished results).

Data Analysis. The equilibrium unfolding data were fit to a three-state model involving a native (N), an intermediate (I), and an unfolding form (U) as described previously (Beasty et al., 1986; Stackhouse et al., 1988). The free energy differences between N and I and between I and U were assumed to depend linearly on the denaturant concentration (Schellman, 1978).

The decay curves obtained from kinetic experiments on the folding of ST α-subunit proteins were fit to one or more exponential terms with a nonlinear least squares statistical program obtained from SAS Institute (Cary, NC). Relaxation times and amplitudes for each phase were obtained. The refolding reactions for the F22I and F22V mutant α-subunit proteins were plagued by slow, linear time-dependent increases in absorbance both at 287 nm and at 350 nm where the protein difference spectrum is zero. These effects were attributed to light scattering from protein aggregates which appear after the completion of the major refolding reaction. To estimate properly the refolding relaxation times, these data were fit to the sum of a linear term and the sum of two exponential terms. The activation free energy in the absence of urea for the unfolding reaction was obtained by fitting the urea dependence of the observed rate constant as described previously and assuming a linear dependence on the denaturant concentration (Finn et al., 1991).

Results

The X-ray structure of the tetrameric $\alpha_2\beta_2$ complex of tryptophan synthase from *S. typhimurium* (Hyde et al., 1988) shows that the phenylalanine side chain at position 22 in the wild-type α-subunit protein is nearly completely buried in the interior of the barrel, at the interface between strand 1 and strand 8/helix 8′. Using the algorithm of Lee and Richards (1971), only 3% of the total accessible surface area is exposed to the solvent for this side chain. If the carboxyl folding unit (residues 189–268) is removed, this exposure increases to 34%.

A previous study of the effects of the replacement of phenylalanine 22 by leucine in the highly homologous EC α subunit showed a small increase in stability which was accompanied by increases in both the slow unfolding and refolding relaxation times (Beasty et al., 1986). This type of behavior implies that the side chain at this position plays a key role in the rate-limiting step in folding (Matthews, 1987) and that the potential development of interfolding unit interaction energy at this site during folding can be determined from kinetic studies. Because the effects of mutations at this buried Phe can be more easily interpreted if perturbations, not drastic changes, are made, Leu, Ile, and Val were selected as replacements.

Thermodynamics. The equilibrium unfolding transitions of the wild-type, F22L, F22I, and F22V mutant ST α subunits obtained by absorbance spectroscopy are shown in Figure 1. The N $\leftrightarrow$ I transitions for all three mutants are clearly shifted to lower urea concentrations, while the I $\leftrightarrow$ U transitions are not appreciably altered. The thermodynamic parameters derived from fitting these data to a three-state model are listed in Table I. The predicted curves based on these fits are also shown in Figure 1.

The principal effects of the amino acid replacements at position 22 are to decrease the stability of N relative to that of I (Table I, column 2). The perturbations for F22I and F22V are comparable, 4.19 and 3.50 kcal/mol relative to wild-type protein, and are significantly larger than that for F22L, 0.97 kcal/mol. This decrease for F22L is actually within the estimated error limit and, if just the free energies in the absence of denaturant were considered, may not be significant. However, in cases where the slopes of the transition curves are similar, a more accurate estimate can be obtained by comparing the free energy differences in the transition zone (Cupo & Pace, 1983). This approach circumvents the necessity of extrapolating the results to 0 M denaturant, a process which results in a larger estimated error. At 2.75 M urea, which is the midpoint of the N $\leftrightarrow$ I transition for the wild-type protein,

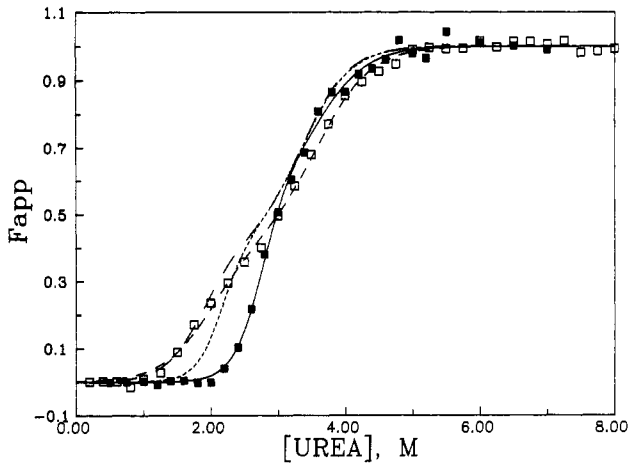

FIGURE 1: Urea-induced equilibrium unfolding transitions for the wild-type (—), F22L (---), F22I (--), and F22V (— —) α subunits at pH 7.8, 25 °C, monitored by difference absorbance spectroscopy at 287 nm. F_{app} is the apparent fraction of unfolded protein and is determined from the data as described elsewhere (Finn et al., 1991). Only the fitted transition curves for the F22L and F22V mutant proteins are shown; the data were omitted for clarity.

the stability of N relative to I decreases by 2.07 ± 0.38 kcal/mol for the F22L mutant. Thus, the perturbations observed for F22L in the absence of denaturant appear to be real. In contrast, the effects any of these three replacements have on the free energy difference between I and U are insignificant (Table I, column 5).

Kinetics. Previous studies have shown that one of the rate-limiting steps in refolding and the single phase in the unfolding of the α subunit is the interconversion of the native and one of three intermediate species (Tweedy et al., 1990). Intermediate I_1 resembles the unfolded protein in terms of absorbance property and appears to be due to some non-proline isomerization reaction (Hurle & Matthews, 1987). Intermediates I_2 and I_3 have similar absorbance properties and energies and are thought to differ in the state of isomerization of an Xaa–Pro peptide bond. In terms of the three-state equilibrium model applied above, I_1 would be included with the unfolded forms and I_2 and I_3 would correspond to the equilibrium intermediate, I. The interconversion of N and I_3 is the rate-limiting step in unfolding and refolding.

To determine if the destabilization detected in the native conformation is also present in the preceding transition state, manual mixing kinetic studies of the slow unfolding and refolding reactions of the wild-type and position 22 mutant α subunits were performed. The unfolding reaction for all three mutants follows a simple exponential which accounts for all the change in absorbance amplitude expected from the equilibrium study (data not shown). The data are well described by the previously proposed N → I_3 reaction (Figure 2). The refolding reactions of the F22I and F22V mutants were plagued by aggregation effects which interfered with the measurement of the slower, τ_1, refolding relaxation times. The data which were available on the urea dependence of the faster, τ_2, refolding relaxation times for the wild-type protein and the F22L, F22I, and F22V mutants are shown along with the unfolding data in Figure 2. The inverted "V" shape for the urea dependence of the unfolding and refolding relaxation times of this set of proteins is similar to that observed in the folding of other proteins (Matthews, 1987).

The unfolding relaxation times for the F22L mutant are consistently longer than those of the wild-type α subunit while those for the F22I and F22V mutants are very similar to the wild-type protein. In contrast, the τ_2 refolding relaxation times

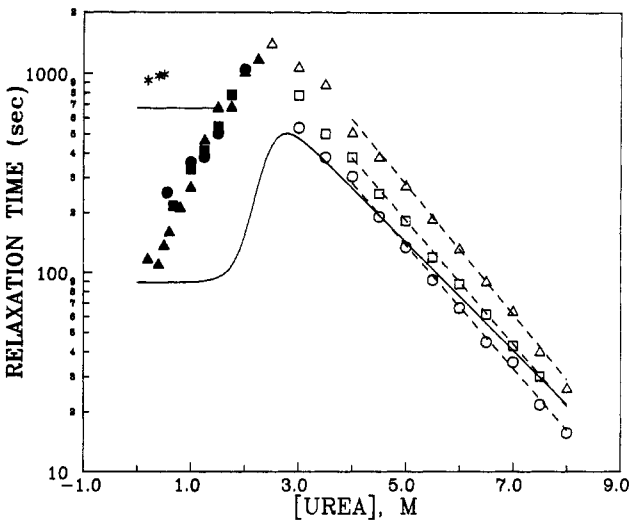

FIGURE 2: Urea dependence of the relaxation times for the single unfolding (open symbols) and the τ_2 refolding (filled symbols) phases of the F22L (△), F22I (□), and F22V (○) mutant α subunits. The τ_1 refolding relaxation times for the F22L mutant are also shown (*). The solid lines represent the behavior of the wild-type α subunit. Dashed lines represent the fits of the log of the unfolding relaxation times to a linear dependence on the urea concentration for the N → I_3 reaction for the three mutant ST α subunits. The unfolding activation free energies can be calculated from these fits as described previously (Finn et al., 1991).

Table II: Kinetic Parameters for the Unfolding Reaction of the Wild-Type and Mutant ST α Subunits[a]

protein	$\Delta G_u^{\ddagger H_2O}$ (kcal/mol)	$-A_u$ [kcal mol^{-1} (mol of urea)$^{-1}$]	$\Delta\Delta G_u^{\ddagger H_2O}$ (kcal/mol)
ST F22 (WT)	22.25 ± 0.01	0.390 ● 0.001	0.00
ST F22L	23.01 ± 0.05	0.445 ± 0.008	−0.76 ± 0.06
ST F22I	22.66 ± 0.02	0.424 ± 0.004	−0.41 ± 0.03
ST F22V	22.48 ± 0.03	0.422 ± 0.006	−0.23 ± 0.04

[a] Errors are the standard deviations. $\Delta\Delta G_u^{\ddagger H_2O} = \Delta G_u^{\ddagger H_2O}$(wild type) − $\Delta G_u^{\ddagger H_2O}$(mutant).

for all three mutants are longer than those of the wild-type protein, and none show the urea-independent behavior at low denaturant concentration seen in the wild-type protein (Figure 2). In the only instance where light scattering did not obscure the measurement, the slower, τ_1, refolding phase was also observed for F22L (Figure 2). A similar phase occurs for the wild-type protein.

Reaction Coordinate Diagram Analysis. The free energy of the transition state for the unfolding reaction in the absence of denaturant relative to that of the native conformation can be determined by fitting the urea dependence of the unfolding relaxation times to equations described previously (Jennings et al., 1991). This procedure is probably more accurate than referencing the transition-state energy to that of the intermediate because the refolding relaxation times can contain contributions from preceding kinetics events. The results are shown in Figure 2 and Table II. The activation free energies for unfolding in the absence of denaturant, $\Delta G_u^{\ddagger H_2O}$, can be combined with the above equilibrium measurements to produce a reaction coordinate diagram depicting the relative free energies of the native, transition-state, intermediate, and unfolded forms. The effects of the mutations on these species can then be determined by comparing their free energy differences to those for the wild-type protein. A histogram summarizing the results of such comparison in the absence of denaturant is shown in Figure 3.

Relative to the free energies of the unfolded forms, none of these replacements at position 22 affect the stability of the

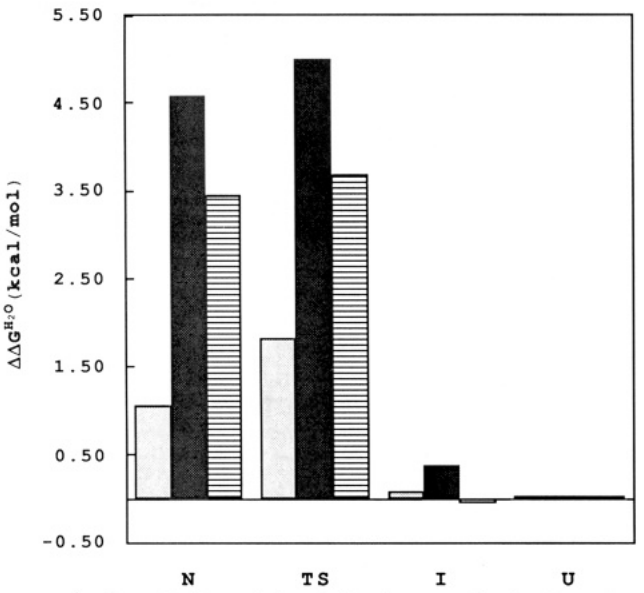

FIGURE 3: Perturbations of the relative free energies for the native (N), transition-state (TS), and intermediate (I) species for the F22L (open bars), F22I (filled bars), and F22V (horizontal bars) mutant α subunits in the absence of denaturant. The free energies of the unfolded forms (U) of all three mutant and wild-type α subunits were aligned to facilitate the comparison. The perturbation for the intermediate form relative to the unfolded form was calculated by $\Delta\Delta G_{I}^{H_2O}$(mutant) = $\Delta G_{IU}^{H_2O}$(wild type) − $\Delta G_{IU}^{H_2O}$(mutant), the perturbation for the native form by $\Delta\Delta G_{N}^{H_2O}$(mutant) = $\Delta G_{total}^{H_2O}$(wild type) − $\Delta G_{total(mutant)}^{H_2O}$, and that for the transition state by $\Delta\Delta G_{TS}^{\ddagger H_2O}$(mutant) = $\Delta\Delta G_{N}^{H_2O}$(mutant) − $\Delta\Delta G_{u}^{\ddagger H_2O}$(mutant) (Table II). A positive sign indicates a destabilizing effect.

intermediate to any significant extent. The predicted high exposure of this side chain to solvent in the intermediate is a plausible explanation for this result. In contrast, these replacements do have a significant effect on the energies of both the native conformations and the rate-limiting transition states. The perturbations of the transition-state energies are consistent with the results obtained for the F22L mutation in the EC α subunit (Beasty et al., 1986). The absence of any significant effect on the unfolding rates for F22I and F22V mutants shows that the perturbations of the energies of the native conformations are very similar to those of the transition states. The smaller effects observed for F22L compared to F22I and F22V suggest that steric interactions from the branched β carbons in Ile and Val play a comparable role to the hydrophobic effect in determining the stability (Pakula & Sauer, 1989; Sandberg & Terwilliger, 1989). The volume, surface area, and free energy of transfer of Leu and Ile from a nonaqueous solvent to water are identical (Creighton, 1984).

Discussion

From a thermodynamic perspective, the unfolding of the α subunit is well described by a three-state model involving N, I, and U forms. Thus the free energy difference between the native and unfolded forms of the protein, $\Delta G_{total}^{H_2O}$, can be calculated from the data in Table I as

$$\Delta G_{total}^{H_2O} = \Delta G_{NI}^{H_2O} + \Delta G_{IU}^{H_2O}$$

Perturbations in the stabilities can be written as

$$\Delta\Delta G_{total}^{H_2O} = \Delta\Delta G_{NI}^{H_2O} + \Delta\Delta G_{IU}^{H_2O}$$

The current view of the folding reaction of the α subunit of tryptophan synthase postulates that the carboxyl folding unit (residues 189–268) becomes disorganized at moderate urea concentrations while the amino folding unit (residues 1–188) retains substantial nativelike structure (Tweedy et al., 1990). At higher urea concentrations, the protein completely unfolds. On the basis of this structural model, the total free energy of folding can be partitioned into three components:

$$\Delta G_{total}^{H_2O} = \Delta G_{A}^{H_2O} + \Delta G_{C}^{H_2O} + \Delta G_{A/C}^{H_2O}$$

where $\Delta G_{A}^{H_2O}$ is the intrinsic free energy developed by the folding of the amino folding unit, $\Delta G_{C}^{H_2O}$ is the intrinsic free energy of folding of the carboxyl folding unit, and $\Delta G_{A/C}^{H_2O}$ is the free energy of the interaction between the folding units. Perturbations in these quantities caused by amino acid substitutions can be written as

$$\Delta\Delta G_{total}^{H_2O} = \Delta\Delta G_{A}^{H_2O} + \Delta\Delta G_{C}^{H_2O} + \Delta\Delta G_{A/C}^{H_2O}$$

As is shown in Table I, the replacement of Phe 22 with Leu, Ile, or Val has no effect on the I ↔ U transition. Because this reaction corresponds principally to the unfolding of the amino folding unit, the data show that these mutations do not alter its intrinsic stability to any significant extent. The expected high exposure of this side chain to solvent in the intermediate is a reasonable explanation for this behavior. Therefore, $\Delta\Delta G_{A}^{H_2O} \approx 0$ for these mutants. Replacements made in the amino folding unit at position 22 cannot affect the intrinsic stability of the carboxyl folding unit. Therefore, $\Delta\Delta G_{C}^{H_2O}$ = 0 as well. One is left to conclude that the observed perturbations in the stability of the folded α subunit, $\Delta\Delta G_{total}^{H_2O}$ (Table I, column 9), can be assigned principally to $\Delta\Delta G_{A/C}^{H_2O}$, the perturbation in the interaction free energy between the amino and carboxyl folding units.

The manner in which this decrease in the interaction energy becomes apparent in the equilibrium unfolding curve for the α subunit is to cause a greater separation of the N ↔ I and I ↔ U transitions. The difference between the midpoints of the N ↔ I and I ↔ U transitions increases from a value of 0.89 M urea for the wild-type protein to 1.22, 1.60, and 1.49 M for the F22L, F22I, and F22V mutants, respectively (Table I, columns 4 and 7). The mutations uncouple the two transitions to a greater extent than is seen for the wild-type protein, resulting in a more pronounced inflection in the unfolding transition curve (Figure 1). In terms of the maximum relative population of the intermediate in the transition zone, this value increase from 70% for the wild-type α subunit to 82%, 80%, and 84% for the F22L, F22I, and F22V mutant proteins, respectively (calculated from thermodynamic parameters in Table I).

The selective perturbation of the interaction energy by the amino acid replacements at the interface between two folding units has an important implication in the solution of the protien folding problem. It suggests that mutations can be used to break the simple cooperative transition observed for many proteins into discrete stages involving stable intermediates. Previous studies on the effects of a double mutation in dihydrofolate reductase (Perry et al., 1987) and a triple mutant in staphylococcal nuclease (Meeker & Shortle, 1986) support this contention. In both cases, noncoincident spectroscopic transition curves denote the presence of equilibrium intermediates which are not seen in the wild-type protein. Although an understanding of the structure of the stable folding intermediate in the α subunit of tryptophan synthase made it a particularly favorable candidate for the disruption of the folding unit interaction energy, the identification of autonomous folding units in other proteins (see above) provides a rational approach for the selection of appropriate mutants in these systems as well.

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Purification and Patch Clamp Analysis of a 40-pS Channel from Rat Liver Mitochondria†

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ABSTRACT: Patch clamp analysis of membranes reconstituted with a fraction isolated from detergent-solubilized mitochondrial membranes by affinity chromatography on immobilized quinine earlier indicated the presence of two classes of ion channels, of about 40- and 140-pS conductance in medium including 150 mM KCl. Now a 57-kDa constituent of the quinine-affinity column eluate has been identified as the 40-pS channel. Protein fractions derived from the quinine-affinity column eluate by preparative isoelectric focusing with a Rotofor cell have been reconstituted into phospholipid vesicle membranes by detergent dialysis, and vesicles have been enlarged for patch clamping by dehydration and rehydration. Voltage clamp analysis has been carried out on excised patches bathed symmetrically in buffered medium containing 150 mM KCl and 100 μM $CaCl_2$. Patches of membrane incorporating the 57-kDa protein exhibit 40-pS conductance transitions. The magnitude of conductance transitions is similar when Na^+ replaces K^+ in the bathing medium, indicating little selectivity of the 40-pS channel for K^+ relative to Na^+. Another fraction derived from the quinine-affinity column eluate is found to contain the larger channel, now estimated to have an average conductance of about 130 pS. Patches of control membrane prepared in the same way but without protein exhibit no channel activity.

The development of patch clamp techniques (Sakmann & Neher, 1983; Hille, 1984) has greatly facilitated the study of ion channels. Since the pioneering work of Sorgato et al. (1987), several laboratories have reported patch clamp studies of mitoplast membranes and of artificial membranes incorporating mitochondrial membrane fragments, indicating the presence of channels of conductance varying from 6 pS to 1.3

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