Effects of the Phenylalanine-22 → Leucine, Glutamic Acid-49 → Methionine, Glycine-234 → Aspartic Acid, and Glycine-234 → Lysine Mutations on the Folding and Stability of the α Subunit of Tryptophan Synthase from *Escherichia coli*†

A. M. Beasty,‡ M. R. Hurle, J. T. Manz,§ T. Stackhouse,‖ J. J. Onuffer, and C. R. Matthews*
Department of Chemistry, The Pennsylvania State University, University Park, Pennsylvania 16802
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ABSTRACT: The effects of four single amino acid replacements on the stability and folding of the α subunit of tryptophan synthase from *Escherichia coli* have been investigated by ultraviolet difference spectroscopy. In previous studies [Miles, E. W., Yutani, K., & Ogasahara, K. (1982) *Biochemistry 21*, 2586], it had been shown that the urea-induced unfolding at pH 7.8, 25 °C, proceeds by the initial unfolding of the less stable carboxyl domain (residues 189–268) follwed by the unfolding of the more stable amino domain (residues 1–188). The effects of the Phe-22 → Leu, Glu-49 → Met, Gly-234 → Asp, and Gly-234 → Lys mutants on the equilibrium unfolding process can all be understood in terms of the domain unfolding model. With the exception of the Glu-49 → Met replacement, the effects on stability are small. In contrast, the effects of three of the four mutations on the kinetics of interconversion of the native form and one of the stable partially folded intermediates are dramatic. The results for the Phe-22 → Leu and Gly-234 → Asp mutations indicate that these residues play a key role in the rate-limiting step. The Glu-49 → Met mutation increases the stability of the native form with respect to that of the intermediate but does not affect the rate-limiting step. The Gly-234 → Lys mutation does not affect either the stability or the kinetics of folding for the transition between native and intermediate forms. The changes in stability calculated from the unfolding and refolding rate constants agree quantitatively with those obtained from the equilibrium data. When considered with the results from a previous study on the Gly-211 → Glu replacement [Matthews, C. R., Crisanti, M. M., Manz, J. T., & Gepner G. L. (1983) *Biochemistry 22*, 1445], it can be concluded that the rate-limiting step in the conversion of the intermediate to the native conformation involves either domain association or some other type of molecule-wide phenomenon.

It is now generally accepted that the amino acid sequence of a protein determines its unique native conformation (Anfinsen, 1973). However, the mechanism by which the one-dimensional information in the sequence is translated into three-dimensional structures is not known in detail for any protein. The opportunity to selectively replace individual amino acids using recombinant DNA technology (Itakura et al., 1984; Craik, 1985) provides a new approach to the solution of the folding problem by permitting the identification of amino acids that play key roles in folding. Information on the involvement of these essential amino acids in the native conformation may provide the clues required to determine the mechanism of folding.

Several protein systems are currently being studied for the effects of single amino acid replacements on folding and stability: T4 lysozyme (Grutter et al., 1979; Hawkes et al., 1984), staphylococcal nuclease (D. Shortle, personal communication), and the α subunit of tryptophan synthase from *Escherichia coli* (Matthews et al., 1980, 1983; Yutani et al., 1984). Previous work in our laboratory on the α subunit of Trp synthase has focused on the elucidation of the mechanism of folding (Matthews & Crisanti, 1981; Crisanti & Matthews, 1981) and the effect of amino acid replacements at position 211 on the thermal (Matthews et al., 1980) and urea-induced (Matthews et al., 1983) unfolding transitions. The replacement of Gly-211 by Glu demonstrated that single amino acid replacements can selectively affect the rates of individual kinetic phases in folding and improved the understanding of the structural basis of several kinetic phases (Matthews et al., 1983). Extremely significant for the interpretation of the folding mechanism of the α subunit was the observation that the Gly-211 → Glu replacement slows the rates of *both* unfolding and refolding between the native form and one of the stable intermediates. Thus, position 211 appears to play a critical role in one of the rate-limiting steps of folding.

Several questions can be posed on the basis of these previous studies: (1) Is the rate-limiting step that is affected by the Glu-211 → Glu replacement due to carboxyl domain folding in this two-domain protein (Higgins et al., 1979) or to the association of the prefolded amino and carboxyl domains? (2) Do amino acid replacements at other sites in the α subunit have an effect similar to that of the Gly-211 → Glu mutant on this rate-limiting step? (3) Are there sites at which replacements have alternative effects on the kinetics of unfolding and refolding? (4) On a more general theme, can one develop a format for classifying the effects of mutations on the stability and kinetics of folding in a way that will be useful in understanding the role of a given amino acid in the folding mechanism?

This paper reports the results of equilibrium and kinetic studies on the reversible unfolding of four single amino acid replacements in the α subunit of tryptophan synthase: Phe-22

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‡ Present address: Centocor, Malvern, PA 19355.
§ Present address: Department of Immunology, Univeristy of Washington, Seattle, WA 98195.
‖ Present address: Department of Biochemistry, University of California, Davis, Davis, CA 95616.

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→ Leu and Glu-49 → Met in the amino domain and Gly-234 → Asp and Gly-234 → Lys in the carboxyl domain. When considered with the previous results for Gly-211 → Glu, the data make it possible to obtain preliminary answers to the above questions and clearly demonstrate the potential of the mutagenic approach to elucidate the mechanism of folding of globular proteins.

Experimental Procedures

α Subunit of Tryptophan Synthase. The wild-type α subunit of tryptophan synthase from *E. coli*[1] (EC 4.2.1.20) and the Phe-22 → Leu, Glu-49 → Met, Gly-211 → Glu, Gly-234 → Asp and Gly-234 → Lys mutant α subunits were isolated from strains pBN55, trpA (Leu-22), A33, A46, A58 and pST54-3, respectively, by using the method of Kirschner et al. (1975) with previously discussed modifications (Matthews et al., 1983). The activities of the wild-type α subunit and the mutant proteins were determined by measuring their abilities to activate the wild-type β_2 subunit in the indole to tryptophan condensation reaction (Yanofsky & Crawford, 1972). The maximum activity of wild-type α subunit is 5500 units/mg (Kirschner et al., 1975). The wild-type α subunit and the mutant proteins Phe-22 → Leu, Glu-49 → Met, Gly-211 → Glu, Gly-234 → Asp, and Gly-234 → Lys had activities of 4500, 2000, 3200, 4000, 2700, and 2000 units/mg, respectively, with errors of ±10%. The purity of the protein was demonstrated by the appearance of a single band on both native and $NaDodSO_4$–polyacrylamide gel electrophoresis.

Protein concentration was determined by using the specific absorption $E^{1\%}_{278nm} = 4.4$ (Adachi et al., 1974). Using a molecular weight of 28 700 (Yanofsky et al., 1967; Li & Yanofsky, 1972), the molar extinction coefficient was calculated as 12 600 M^{-1} cm^{-1}. The reversibility of the mutant α subunits always exceeded 90%, as monitored by the recovery of the difference absorbance spectrum.

Chemicals. Ultrapure urea was purchased from Schwarz/Mann and used without further purification. All other chemicals were reagent grade.

Methods. (*A*) *Spectroscopy.* Ultraviolet difference spectroscopy measurements at 286 nm were made by using the tandem cell technique (Herskovits, 1967) on a Cary 118CX spectrophotometer. Unfolding (refolding) was initiated by using microliter syringes to add accurately measured volumes of protein (protein and urea) to premeasured volumes of a urea/buffer solution. The solutions were manually mixed, and the time dependence of the absorbance change was recorded by a Digital PDP-11 computer interfaced to the spectrophotometer. These changes were fit with a nonlinear least-squares computer program to a sum of exponentials, $A(t) = \sum_i A(i) \exp(-t/\tau_i) + A_\infty$ where $A(t)$ is the absorbance at time t, $A(t)$ is the absorbance of phase i at zero time, τ_i is the relaxation time for phase i, and A_∞ is the absorbance at infinite time. Equilibrium measurements were made after letting the protein/urea solution incubate until no further changes in absorbance were observed, generally 1–2 h at 25 °C. The protein concentration was in the range of 0.6–1.5 mg/mL.

(*B*) *Circular dichroism* experiments were performed on a JASCO J-20 spectrophotometer. Spectra were recorded over the 200–350-nm wavelength range, as described previously (Matthews et al., 1983).

The buffer used in all experiments was 10 mM potassium phosphate, pH 7.8, 0.2 mM EDTA, and 1 mM 2-mercaptoethanol.

[1] Abbreviations: *E. coli*, *Escherichia coli*; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; UV, ultraviolet; $\Delta\epsilon^{286}$, extinction coefficient change at 286 nm.

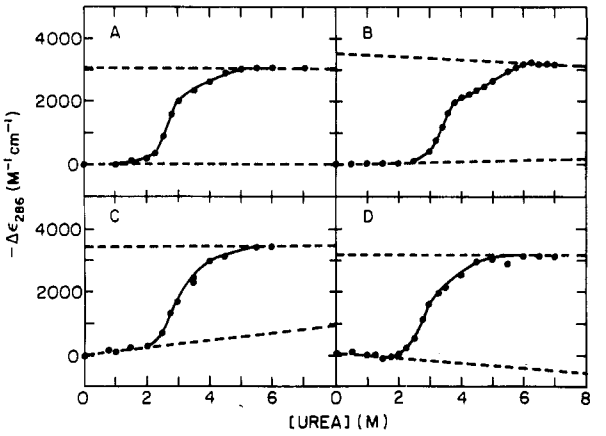

FIGURE 1: Dependence of the difference extinction coefficient at 286 nm, $\Delta\epsilon_{286}$, on urea concentration in 10 mM potassium phosphate, pH 7.8, 0.2 mM EDTA, and 1 mM 2-mercaptoethanol at 25 °C for the mutant α-subunit mutants (A) Phe-22 → Leu, (B) Glu-49 → Met, (C) Gly-234 → Asp, and (D) Gly-234 → Lys. Solid lines are drawn to aid the eye. Dotted lines are extrapolations of the base lines in the native and unfolded region.

Results

Comparison of the equilibrium and kinetic properties of the wild-type and mutant α subunits depends critically on the assumption that the amino acid replacements do not significantly alter the conformation of the protein. The validity of this assumption was tested by comparing the circular dichroism spectra in the far-UV region for these proteins. The spectra were virtually coincident (data not shown), demonstrating that the mutations do not alter the secondary structure in any appreciable way. Also, the selection procedure for these mutant proteins requires that they bind to and activate the β_2 subunit of Trp synthase in the conversion of indole to tryptophan (Yanofsky & Crawford, 1972). Thus, the tertiary structure is also not significantly affected by the mutations.

Equilibrium Studies. The urea-induced unfolding reaction for the wild-type α subunit and four point mutants was monitored by difference UV spectroscopy (Herskovits, 1967; Donovan, 1973). The decrease in extinction coefficient at 286 nm reflects changes in solvent exposure of buried tyrosine residues as the protein unfolds. The results for the Phe-22 → Leu, Glu-49 → Met, Gly-234 → Asp, and Gly-234 → Lys mutant proteins at pH 7.8, 25 °C, are shown in Figure 1A–D. The unfolding transitions are qualitatively similar in the following respects: (1) There are either small linear changes or no changes in $\Delta\epsilon^{286}$ up to approximately 2 M urea. The native conformation is stable in this region. (2) The sigmoidal decrease in $\Delta\epsilon^{286}$ between 2 and 3 M urea indicates that a cooperative unfolding transition occurs in this region. Previous studies assign this reaction to the conversion of the native form to stable intermediate species (Matthews & Crisanti, 1981). (3) The more gradual decrease in $\Delta\epsilon^{286}$ between 3 and 6 M urea shows that a second, less cooperative unfolding transition occurs in this region. This reaction has been assigned to the conversion of the intermediates to unfolded forms (Matthews & Crisanti, 1981). (4) Above 6 M urea, there are again either small linear changes or no changes in $\Delta\epsilon^{286}$. The unfolded forms are stable in this region.

These transition curves can best be compared by normalizing the data in terms of the apparent fraction unfolded, F_{app}:

$$F_{app} = \frac{\epsilon^{286}_{obsd} - \epsilon^{286}_{nat}}{\epsilon^{286}_{unf} - \epsilon^{286}_{nat}}$$

where ϵ^{286}_{obsd} refers to the observed extinction coefficient at

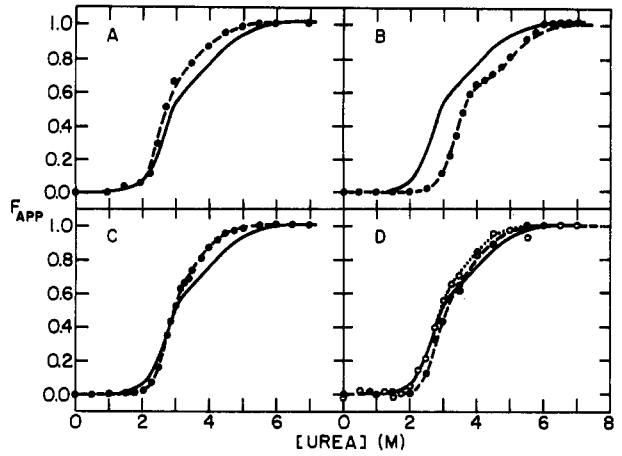

FIGURE 2: Dependence of the fractional change, F_{app}, in $\Delta\epsilon_{286}$ on urea concentration as determined from the data in Figure 1 and from previously published data (Matthews et al., 1983) for the wild-type α subunit (—) and for the mutant α-subunit mutants (A) Phe-22 → Leu (---), (B) Glu-49 → Met (---), (C) Gly-211 → Glu (---), and (D) Gly-234 → Asp (---) and Gly-234 → Lys (···). Closed and open circles represent data points for the appropriate mutant, and lines represent fits to data as discussed in the paper.

286 nm and ϵ_{nat} and ϵ_{unf} refer to the extinction coefficient for the native and unfolded forms at the appropriate urea concentration. These latter values were obtained by linear extrapolation of ϵ_{nat} and ϵ_{unf} from the native and unfolded base-line regions, respectively, into the transition zone (Figure 1).

The plots of F_{app} as a function of urea concentration for the wild-type α subunit and the four point mutants are shown in Figure 2A–D. Also included for comparison is the result for the Gly-211 → Glu mutant studied previously (Matthews et al., 1983). For the Phe-22 → Leu α subunit (Figure 2A), the transition from native to intermediates is not significantly affected; however, the transition from intermediates to unfolded forms is shifted to lower urea concentration, indicating that this replacement decreases the stabilities of the intermediates relative to the unfolded forms. For the Glu-49 → Met α subunit (Figure 2B), both transitions are shifted to higher urea concentration, indicating that substantial increases in the stabilities of native relative to intermediates and of intermediates relative to unfolded forms have occurred. For the Gly-211 → Glu mutant (Figure 2C), the transition from native to intermediates is displaced to higher urea concentration, indicating a greater stability of the native form relative to the intermediates. The transition from intermediates to unfolded forms occurs at a slightly lower urea concentration than wild type, suggesting a small decrease in stability of the intermediates relative to unfolded forms. For the Gly-234 → Asp α subunit (Figure 2D), the transition from native to intermediates shifts to slightly higher urea concentration while that for the conversion of intermediates to unfolded forms shifts to a slightly lower urea concentration. The associated changes in stability are expected to be small. For the Gly-234 → Lys α subunit (Figure 2D), the transition from native to intermediate forms is coincident with that from wild-type protein while that for the conversion of intermediates to unfolded terms is shifted to a slightly lower urea concentration. A quantitative analysis of these results will be presented under Discussion.

Kinetic Studies. The transient responses of the α subunit during unfolding and refolding jumps were monitored by following the time dependence of $\Delta\epsilon^{286}$. The data were fit to one or more exponentials and the relaxation times and amplitudes extracted. This kinetic study focuses on the slow interconversions between the native and intermediate forms,

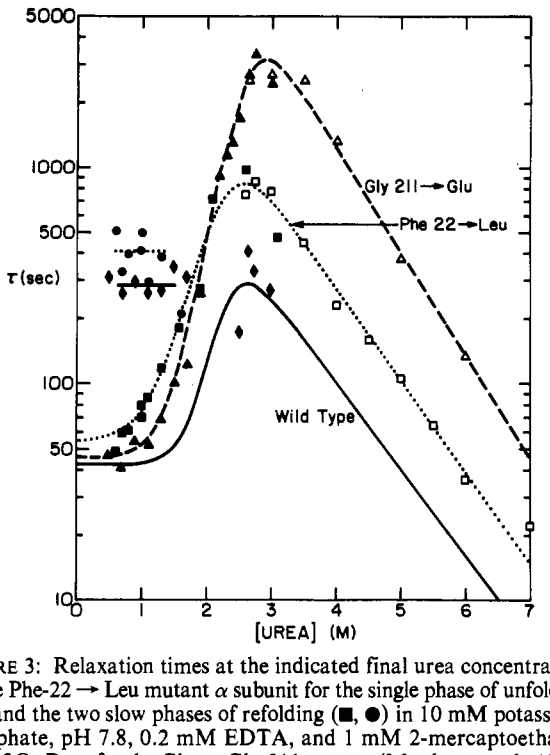

FIGURE 3: Relaxation times at the indicated final urea concentration of the Phe-22 → Leu mutant α subunit for the single phase of unfolding (□) and the two slow phases of refolding (■, ●) in 10 mM potassium phosphate, pH 7.8, 0.2 mM EDTA, and 1 mM 2-mercaptoethanol at 25 °C. Data for the Gly → Glu-211 mutant (Matthews et al., 1983) are also shown (△, ▲, ♦). Theoretical fits done as described in the paper are shown for wild-type (—), Phe-22 → Leu (···), and Gly-211 → Glu (---). Unfolding jumps were initiated from 0 M urea and refolding jumps from 5 or 6 M urea.

where the relaxation times exceed 10 s (Matthews et al., 1983). NMR studies (C. Froebe and C. R. Matthews, unpublished experiments) have shows that the conversion of intermediates to unfolded forms occurs in the submillisecond time range, precluding the use of either manual mixing or stopped-flow methods.

A semilogarithmic plot of the observed relaxation times for unfolding and refolding of the Phe-22 → Leu mutant as a function of the final urea concentration at pH 7.8, 25 °C, is shown in Figure 3. For reference, the fitted curve for the wild-type protein is also shown. Similar to the wild-type protein, the unfolding of the Phe-22 → Leu mutant is well described by a single exponential that accounts for all the absorbance change expected from equilibrium studies (Figure 1A). The logarithm of the relaxation time for this phase decreases linearly with increasing urea concentration similar to the wild-type protein; however, this phase is 4-fold slower than that for the wild-type protein at 6 M urea.

The response in refolding is more complex, with a substantial amount of absorbance change occurring in the dead time of mixing. This fast phase was followed by two slower phases designated τ_1(slower) and τ_2(faster). The relaxation times for the τ_1 phase in the Phe-22 → Leu mutant protein at a series of final urea concentrations are somewhat scattered but appear to be independent of urea concentration and have an average value of 400 s. The relaxation time for the τ_2 phase clearly depends upon the final urea concentration, with the log of τ_2 decreasing linearly with decreasing urea concentration. The general behavior of this phase is similar to that of the wild-type protein; however, the relaxation time is 2.5 times longer than that of the wild type at 2 M urea. Unlike the wild-type protein, however, τ_2 does not appear to become independent of urea concentration at low urea.

Also shown in Figure 3 are the results for the Gly-211 → Glu mutant published previously (Matthews et al., 1983). The

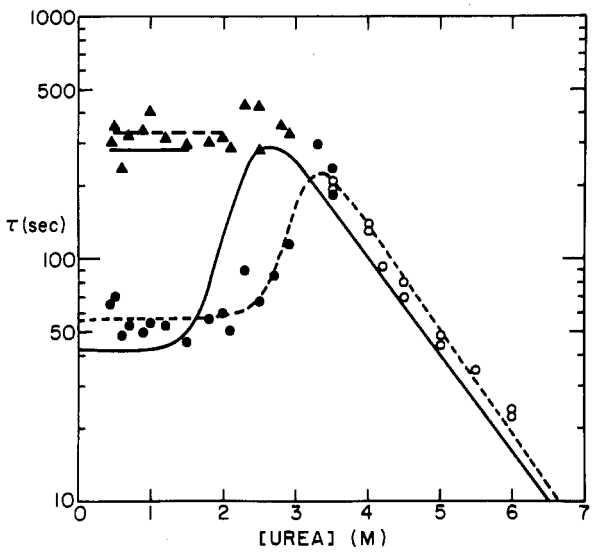

FIGURE 4: Relaxation times of the Glu-49 → Met mutant α subunit at the indicated final urea concentration for unfolding (○) and refolding (●, ▲). Theoretical fits for the wild-type (—) and Glu-49 → Met mutant (---) α subunits are also shown. Experiments were performed as described in Figure 3.

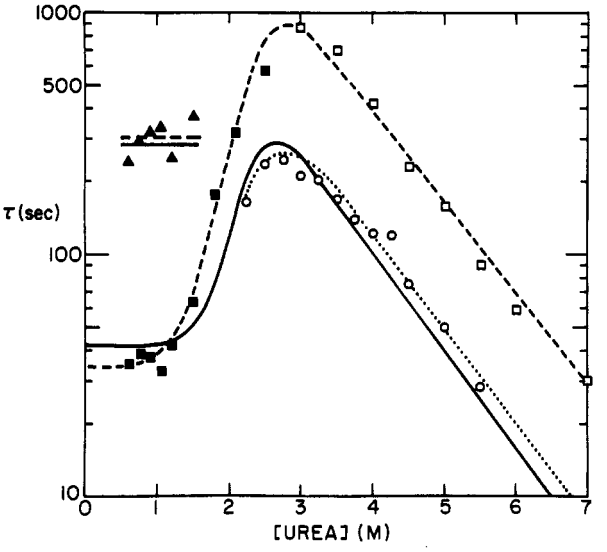

FIGURE 5: Relaxation times at the indicated final urea concentration of the Gly-234 → Asp mutant α subunit for unfolding (□) and refolding (■, ▲), along with unfolding data for the Gly-234 → Lys mutant (○). Theoretical fits to the data for the Gly-234 → Asp (---) and wild-type (—) α subunits are shown. The fit to the Gly-234 → Lys (···) α-subunit mutant was calculated in the range 4–6 M urea; the line is extended to lower urea concentrations to aid the eye.

unfolding relaxation time increases by a factor of 8 at 6 M urea while the refolding relaxation time for the τ_2 phase increases by a factor of 4 at 2 M urea. At low urea concentration, both τ_1 and τ_2 phases have urea-independent relaxation times that are identical with those of wild-type protein. The relaxation time for the τ_1 phase is 282 ± 22 s, and that for the τ_2 phase is 44 ± 5 s.

For the Glu-49 → Met mutant protein, the unfolding relaxation time is very similar to that of wild-type protein (Figure 4). The ~20% increase in relaxation time is probably within experimental error. For refolding, the τ_2 phase is twice as fast as that for the wild type above 2 M urea. Below 2 M urea, this phase becomes urea independent and has an average value of 50 ± 5 s. The τ_1 phase is urea independent between 0.5 and 3 M urea and has an average value of 320 ± 25 s. Both of these values are also within experimental error of those for wild-type protein.

The effect of the Gly-234 → Asp replacement on the urea-dependent region of the τ_2 phase is similar to that observed for the Gly-211 → Glu mutation (Figure 5). For unfolding, the relaxation time increases by a factor of 4 at 6 M urea while for refolding the relaxation times are approximately twice as long as those for wild-type protein at 2 M urea. At urea concentrations less than 1 M, the τ_2 phase again becomes independent of urea concentration and has an average value of 40 ± 5 s. The τ_1 phase is urea independent over the range from 0.5 to 1.5 M urea and has an average value of 320 ± 25 s. Both τ_1 and τ_2 values below 1 M urea are within experimental error of those for the wild type.

The results of a kinetic study on the Gly-234 → Lys mutant are also shown in Figure 5. For this mutant only, unfolding jumps could be quantitatively analyzed since refolding was invariably accompanied by light-scattering effects attributed to aggregation. The relaxation time of the single τ_2 phase observed in unfolding was virtually identical with that of wild-type protein at all urea concentrations studied.

The single amino acid replacements examined in this study selectively alter the relaxation times of the urea-dependent folding phase; the relaxation times of the urea-independent phases detected at low urea concentrations in refolding are largely unaffected. The variations in the urea-dependent phase include increases in the relaxation times of both unfolding and refolding (Phe-22 → Leu, Gly-211 → Glu, and Gly-234 → Asp) and a decrease in the relaxation time of refolding while leaving unfolding unchanged (Glu-49 → Met). The implications of these changes in the kinetics of refolding and unfolding will be considered under Discussion.

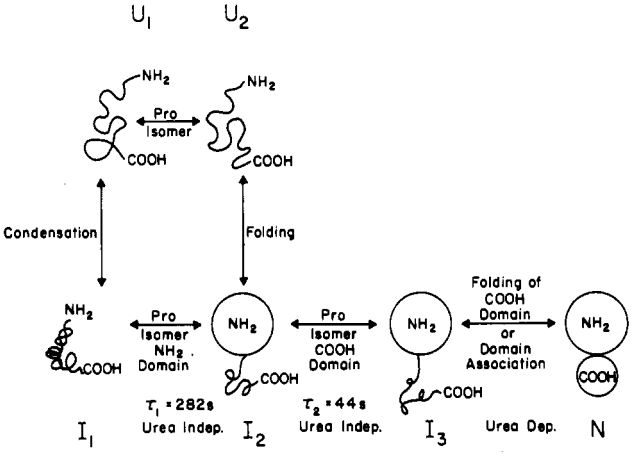

FIGURE 6: Proposed structural folding model for the α subunit.

Discussion

Quantitative Analysis of Equilibrium Unfolding Data. The equilibrium results can be put on a quantitative basis by fitting the data to a three-state model involving a native form, N, an intermediate, I, and an unfolded form, U:

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

where $K_{NI} = [I]/[N]$ and $K_{IU} = [U]/[I]$ are the appropriate equilibrium constants. This model is a simplification of the complete model which involves three stable intermediates and two unfolded forms (Figure 6). The justification for the three-state approximation is based upon our current view that the unfolded forms only differ from each other in the cis/trans isomeric configuration of the peptide bond at one or more X–Pro peptide bonds. Since the cis/trans ratio is not expected to be affected by denaturant (Nall et al., 1978), the two un-

Table I: Thermodynamic and Kinetic Parameters for Urea-Induced Unfolding Transitions at 0 M Urea[a]

species	ΔG°_{NI}	A_{NI}	ΔG°_{IU}	A_{IU}	$\Delta G^{\ddagger\circ}_{12}$	A_{12}	$\Delta G^{\ddagger\circ}_{21}$	A_{21}	$\Delta G^{\circ K}_{12}$	A^K_{12}
wild type	5.7 ± 0.4	−2.2 ± 0.2	5.0 ± 0.6	−1.2 ± 0.2	22.3 ± 0.2	−0.54 ± 0.03	15.1 ± 1.0	2.4 ± 0.5	7.2 ± 1.0	−2.9 ± 0.5
Phe-22 → Leu	7.9 ± 1.4	−3.1 ± 0.6	4.2 ± 1.2	−1.2 ± 0.3	22.9 ± 0.3	−0.52 ± 0.05	17.4 ± 0.5	1.7 ± 0.3	5.4 ± 0.4	−2.2 ± 0.3
Glu-49 → Met	9.4 ± 1.1	−2.8 ± 0.3	7.0 ± 0.9	−1.4 ± 0.2	22.5 ± 0.3	−0.53 ± 0.06	11.6 ± 5.0	2.9 ± 2.6	10.9 ± 4.4	−3.5 ± 1.6
Gly-211 → Glu	8.1 ± 0.6	−3.0 ± 0.2	5.4 ± 0.5	−1.5 ± 0.1	24.3 ± 0.5	−0.65 ± 0.11	16.3 ± 0.6	2.2 ± 0.3	8.0 ± 0.6	−2.8 ± 0.3
Gly-234 → Asp	7.1 ± 2.4	−2.6 ± 0.9	7.0 ± 3.0	−1.8 ± 0.8	23.0 ± 0.3	−0.51 ± 0.05	16.3 ± 0.6	2.0 ± 0.4	6.7 ± 0.6	−2.6 ± 0.4
Gly-234 → Lys	6.2 ± 1.4	−2.3 ± 0.6	4.7 ± 1.7	−1.2 ± 0.4	22.4 ± 0.4	−0.53 ± 0.08				
wild type, 0.5 M NaCl	5.7 ± 1.0	−2.2 ± 0.4	3.3 ± 1.0	−0.9 ± 0.2						
Gly-211 → Glu, 0.5 M NaCl	8.2 ± 1.2	−2.9 ± 0.4	4.2 ± 0.8	−1.1 ± 0.2						

[a] Units are as follows: ΔG, kilocalories per mole; A, kilocalories per mole per molar (urea).

Table II: Midpoints and Changes in Free Energy of Unfolding at Standard States Defined by the Wild-Type Protein[a]

species	$C_{m_{NI}}$	$C_{m_{IU}}$	$C_{m_{12}}{}^K$	$\Delta\Delta G_{NI}$[b]	$\Delta\Delta G_{IU}$[c]	$\Delta\Delta G_K$[d]
wild type	2.62 ± 0.02	4.18 ± 0.06	2.48 ± 0.13			
Phe-22 → Leu	2.54 ± 0.05	3.62 ± 0.13	2.43 ± 0.20	−0.3 ± 0.2	−0.6 ± 0.2	−0.1 ± 0.5
Glu-49 → Met	3.33 ± 0.02	4.92 ± 0.06	3.15 ± 0.23	2.0 ± 0.2	1.1 ± 0.2	2.3 ± 0.5
Gly-211 → Glu	2.73 ± 0.02	3.72 ± 0.04	2.81 ± 0.17	0.3 ± 0.1	−0.7 ± 0.1	0.9 ± 0.4
Gly-234 → Asp	2.78 ± 0.07	3.99 ± 0.19	2.65 ± 0.17	0.4 ± 0.2	−0.3 ± 0.4	0.4 ± 0.4
Gly-234 → Lys	2.65 ± 0.08	3.81 ± 0.16		0.1 ± 0.2	−0.4 ± 0.2	
wild type, 0.5 M NaCl	2.65 ± 0.07	3.86 ± 0.16		0.0 ± 0.2	−0.3 ± 0.1	
Gly-211 → Glu, 0.5 M NaCl	2.80 ± 0.04	3.97 ± 0.07		0.5 ± 0.1	−0.2 ± 0.1	

[a] Units are as follows: $\Delta\Delta G$, kilocalories per mole; C_m, [urea]. [b] 2.62 M urea. [c] 4.18 M urea. [d] 2.48 M urea.

folded forms should behave as a single species in an equilibrium model. The three intermediates are also thought to differ in the isomeric configuration at one or more X–Pro peptide bonds; however, the relative ratios of these species could depend upon the urea concentration. The spectral properties of the intermediates are such that I_1 resembles unfolded protein in its exposure of Tyr residues to solvent while I_2 and I_3 have approximately three Tyr residues buried in a folded amino domain (Crisanti & Matthews, 1981). The α subunit has six Tyr residues in the amino domain and one in the carboxyl domain. The consistently good fit of the data to a three-state model suggests that this approximation is valid.

The free energy of folding was assumed to have a linear dependence on the urea concentration (Pace, 1975; Schellman, 1978):

$$\Delta G_{xy} = \Delta G^\circ_{xy} + A_{xy}[\text{urea}]$$

where ΔG_{xy} is the free energy difference between species x and y at a given urea concentration, ΔG°_{xy} is the free energy difference in the absence of urea, and A_{xy} describes the cooperativity of the transition. In this formalism, $A_{xy} < 0$.

By use of the linear dependence of the free energy changes in each of the two transitions in the three-state model, the F_{app} vs. [urea] data were fit to the equation (M. R. Hurle et al., unpublished results):

$$F_{app} = \frac{K_{NI}(Z + K_{IU})}{1 + K_{NI} + K_{IU}}$$

where $Z = (\epsilon_I - \epsilon_N)/(\epsilon_U - \epsilon_N) = 0.59$ and ϵ_N, ϵ_I, and ϵ_U are the extinction coefficients of the native, intermediate, and unfolded forms, respectively.

The free energy differences for the N ↔ I and I ↔ U reactions for the wild-type α subunit and five point mutants are shown in Table I. The values for ΔG°_{NI} vary from 5.7 ± 0.4 kcal mol^{-1} for the wild-type protein to 9.4 ± 1.1 kcal mol^{-1} for the Glu-49 → Met mutant protein. The values for ΔG°_{IU} vary from 4.2 ± 1.2 kcal mol^{-1} for the Phe-22 → Leu mutant to 7.0 ± 0.9 kcal mol^{-1} for the Glu-49 → Met mutant. Although the effects of the amino acid replacements on stability could be considered in terms of the differences between ΔG°_{xy} values, it is our opinion that the large extrapolations required to obtain these values decrease their accuracy. The error of concern is that associated not only with the statistical fitting of the data but also with the validity of a linear dependence of the free energy of folding on denaturant concentration outside the transition region. Alternative fitting procedures use different mathematical forms for the extrapolation (Tanford, 1970), but the validity of such forms far from the transition zone is equally uncertain.

For example, ΔG°_{NI} values for the wild-type α subunit and the Phe-22 → Leu mutant protein differ by 2.2 kcal mol^{-1} (Table I), yet the unfolding curves are nearly identical in the region between 2 and 3 M urea where the transition from native to intermediate occurs (Figure 2A). It appears that a better comparative method involves using the concentration of urea at the midpoint of the transition for the wild-type protein as a reference state instead of 0 M urea. The midpoint of the transition, $C_{m_{xy}}$, is defined as the concentration of urea at which $\Delta G_{xy} = 0$, yielding

$$C_{m_{xy}} = -\Delta G^\circ_{xy}/A_{xy}$$

At 25 °C, pH 7.8, these reference states are 2.62 and 4.18 M urea for the transitions from native to intermediates and intermediates to unfolded forms, respectively (Table II). The effect of a given mutation on stability is then computed by using the fitted values of ΔG°_{xy} and A_{xy} for that mutant and the urea concentration for the appropriate reference state. Cupo and Pace (1983) have evaluated these procedures and have suggested that the latter provides the more accurate measure of the differences in stability for a series of chemically modified proteins.

The effects of the mutations on ΔG_{NI} at 2.62 M urea ($\Delta\Delta G_{NI}$) and ΔG_{IU} at 4.18 M urea ($\Delta\Delta G_{IU}$) are also shown in Table II. In this notation, $\Delta\Delta G_{NI} = \Delta G_{NI}$(mutant) − ΔG_{NI}(wild type) at 2.62 M urea and $\Delta\Delta G_{IU} = \Delta G_{IU}$(mutant) − ΔG_{IU}(wild type) at 4.18 M urea. The values for $\Delta\Delta G_{NI}$ range from −0.3 kcal mol^{-1} for the Phe-22 → Leu mutant to +2.0 kcal mol^{-1} for the Glu-49 → Met mutant. The values for $\Delta\Delta G_{IU}$ range from −0.7 kcal mol^{-1} for Gly-211 → Glu to +1.1 kcal mol^{-1} for Glu-49 → Met. With the exception of the Glu-49 → Met protein, the effects of amino acid re-

placements on stability are relatively small.

The guanidine hydrochloride induced unfolding of the Glu-49 → Met mutant protein has been studied by Yutani et al. (1984). These workers found that this replacement increases the stability of the native form with respect to the intermediate at pH 9.0, but not at pH 7.0. The urea-induced folding results reported in the present paper show that this influence of pH on the difference in stabilities is appreciable by pH 7.8. Yutani et al. (1984) also found that this substitution increases the stability of the intermediate with respect to the unfolded form at both pH 7 and pH 9, in agreement with the results presented here.

Given the hypothesis that the N → I transition reflects the unfolding of the carboxyl domain and the I → U transition the unfolding of the amino domain, it is pertinent to inquire about which of these transitions a particular mutation will affect. Inspection of the data in Table II shows that the Phe-22 → Leu mutant only significantly alters the stability of the amino domain, Glu-49 → Met increases the stability of both the amino and carboxyl domains, Gly-211 → Glu also appears to alter the stability of both domains, Gly-234 → Asp slightly changes the stability of the carboxyl domain, and Gly-234 → Lys only has a small effect on the stability of the amino domain, considering the error limits. These results are in agreement with the domain unfolding hypothesis with the apparent exception of the effect of Glu-49 → Met on the N → I transition and that of Gly-211 → Glu and Gly-234 → Lys on the I → U transition.

If the N → I equilibrium is formally considered as the dissociation of the two domains followed by the unfolding of the carboxyl domain, the Glu-49 → Met result can be thought of as an effect on the dissocation constant. The residue at position 49 may have either a direct or an indirect effect on this dissociation constant, and replacements at this site would then appear to alter the stability of the carboxyl domain.

The Gly-211 → Glu and Gly-234 → Lys results are not easily explained in terms of the domain unfolding model. Because a variety of experiments which directly examine the conformation of the intermediate all support the model (Miles et al., 1982; Crisanti & Matthews, 1981; Beasty & Matthews, 1985), one must postulate that the unfolded carboxyl domain may still be capable of interacting with and thereby influencing the stability of the amino domain. This possibility was tested by comparing the equilibrium unfolding curves for the wild-type and Gly-211 → Glu mutant proteins in the presence of 0.5 M NaCl. Although the mutant protein is still more stable in the N → I transition, the I → U unfolding transitions are now nearly coincident (Table II). This result suggests that repulsive electrostatic interactions between the anionic glutamic acid side chain and negatively charged groups in the folded amino domain may be responsible for the decreased stability in the mutant. A similar explanation can be postulated for the Gly-234 → Lys mutant protein.

Correlation between Equilibrium and Kinetic Effects of Point Mutations. Comparison of the effects of the point mutations in the α subunit on the midpoints of the equilibrium unfolding transition between the native and intermediate forms (Figure 2A–D) and the maxima in the relaxation times for the urea-dependent τ_2 phase (Figures 3–5) shows that a good correlation exists. A quantitative analysis of the kinetics of the appropriate steps in the folding model (Figure 6) provides a mathematical basis for this correlation.

The urea dependence of the τ_2 kinetic phase has been attributed to the involvement of two separate processes (Matthews et al., 1983). The urea-dependent portion which resembles an inverted V reflects the interconversion of the native form N and the intermediate I_3 while the urea-independent portion at low urea concentrations is due to the transition between I_2 and I_3 (Figure 6). The relevant steps, including microscopic rate constants, are

$$N \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} I_3 \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} I_2$$

This simplification of the complete model is appropriate because the conversion of unfolded to intermediate forms in refolding is orders of magnitude faster than the subsequent conversion of intermediates to the native form and because the $I_1 \rightarrow I_2$ reaction is more than 4-fold slower than $I_2 \rightarrow I_3$ or $I_3 \rightarrow N$ at low urea concentrations.

The urea concentration dependence of k_{12} and k_{21} can be obtained by fitting the τ_2 relaxation time data to the exact solution for the above three-species model (Eigen & DeMaeyer, 1963). The fit also requires information on the urea dependence of k_{23} and k_{32}, the Pro isomerization step. The urea independence of k_{32} is demonstrated by the observation that τ_2 is independent of urea concentration below 1.5 M urea for wild-type α subunit (Figure 3A); under these conditions, $\tau_2^{-1} \cong k_{32}$. Since $\tau_2 = 44$ s, $k_{32} = 2.3 \times 10^{-2}$ s^{-1}. There is no direct evidence for the urea independence of k_{23}; however, the assignment of the $I_2 \leftrightarrow I_3$ equilibrium to Pro isomerization (Crisanti & Matthews, 1981) supports this assumption. The Pro isomerization equilibrium is expected to be independent of urea concentration, implying that both of the associated rate constants will also be urea independent. The value for k_{23} can then be calculated from the equilibrium constant linking I_2 and I_3 ($K_{23} = [I_2]/[I_3]$) and the rate constant k_{23}. The value of K_{23} was assumed to be 1, based upon experimental measurements of the cis/trans ratio of X–Pro peptide bonds in dipeptides (Brandts et al., 1975) and proteins (Lin & Brandts, 1984). From these values, $k_{23} = 2.3 \times 10^{-2}$ s^{-1}. Variation of the value of K_{23} over the range observed for cis/trans isomers in dipeptides, i.e., by a factor of 2–4, had little effect on the values of k_{12} and k_{21} as a function of urea concentration.

An explicit expression for the urea dependence of k_{12} and k_{21} can be developed by using the Eyring formalism:

$$k_{12} = (k_B T/h) \exp(-\Delta G_{12}^{\ddagger}/RT)$$

$$k_{21} = (k_B T/h) \exp(-\Delta G_{21}^{\ddagger}/RT)$$

where k_B and h are the Boltzmann and Planck constants, respectively, R is the gas constant, T is the absolute temperature, and $\Delta G_{12}^{\ddagger}$ ($\Delta G_{21}^{\ddagger}$) is the activation free energy between the native (intermediate) conformation and the hypothetical transition state.

The urea dependence of k_{12} (k_{21}) can then be introduced by assuming $\Delta G_{12}^{\ddagger}$ ($\Delta G_{21}^{\ddagger}$) depends linearly on the urea concentration:

$$\Delta G_{12}^{\ddagger} = \Delta G_{12}^{\ddagger\circ} + A_{12}[\text{urea}]$$

$$\Delta G_{21}^{\ddagger} = \Delta G_{21}^{\ddagger\circ} + A_{21}[\text{urea}]$$

These equations are formally identical with that used above to describe the urea dependence of the free energy difference between the native and intermediate states for the equilibrium study. The application of this treatment to kinetics is based upon the assumption from transition state theory that the stable state and the transition state are at equilibrium. Note that the urea dependence of the unfolding rate is such that $A_{12} < 0$; for refolding, $A_{21} > 0$.

Fitting the data for the τ_2 phase in Figure 3A–D to this three-species kinetic model yields the parameters shown in

Table I and the solid lines in the respective figures. The value for the free energy difference between N and I_3 in the absence of urea, $\Delta G_{12}^{\circ K}$, can be computed from these kinetic data since $\Delta G_{12}^{\circ K} = \Delta G_{12}^{\ddagger\circ} - \Delta G_{21}^{\ddagger\circ}$. These values are shown in Table I as well as the values for the cooperativity parameter, A_{12}^{K}, calculated from $A_{12}^{K} = A_{12} - A_{21}$. The transition midpoints, $C_{m_{12}}{}^{K}$, determined from $\Delta G_{12}^{\circ K}$ and A_{12}^{K} as described earlier for the equilibrium case, are shown in Table II.

Comparison of these values for $\Delta G_{12}^{\circ K}$ with the free energy differences in the absence of urea obtained from the equilibrium data for the wild-type and mutant proteins, $\Delta G^{\circ}{}_{NI}$ (Table I, columns 2 and 10), shows that the agreement is rather poor. In contrast, the agreement between the midpoints of the transition from N to I_3 determined by equlibrium and kinetic experiments for all of the proteins is very good (Table II, columns 2 and 4). As discussed under Results, the poor agreement for the free energy values in the absence of urea may be due to the necessity of extrapolating data obtained in the transition region, i.e., 2–5 M urea, to 0 M urea. The transition midpoints, C_m and $C_m{}^{K}$, appear to be a better comparative parameter since the midpoints have a smaller relative error than the ΔG° values. This occurs because the errors in ΔG° and A are correlated in such a way that the error in the ratio is relatively small (Table I, columns 2 and 3, and Table II, column 2). By use of a value of 2.48 M urea for $C_m{}^{K}$, the midpoint of the transition between native and intermediate forms for the wild-type protein obtained from the kinetic data as the standard state, and the appropriate values for $\Delta G_{12}^{\circ K}$ and A_{12}^{K} for each mutant, the differences in the free energy of unfolding relative to that of wild type at 2.48 M urea ($\Delta\Delta G_K$) can be calculated as described above for the equilibrium case. These values are also shown in Table II. Comparison with the free energy differences obtained from the thermodynamic data in almost exactly the same standard state (2.62 M urea for the equilibrium data) shows reasonable agreement between these two methods, considering the errors predicted from a propagation of errors analysis.

This agreement is somewhat surprising since the equilibrium constant determined by kinetic methods is that for the N $\leftrightarrow$ I_3 reaction while that determined by equilibrium methods involves two of the three stable intermediates, I_2 and I_3 (Figure 6). That is, for

$$N \overset{K_{12}}{\leftrightarrow} I_3 \overset{K_{23}}{\leftrightarrow} I_2 \overset{K_{34}}{\leftrightarrow} I_1$$

the apparent equilibrium constant, K_{app}, extracted from the equilibrium data is

$$K_{app} = ([I_2] + [I_3])/[N] = K_{12}(1 + K_{23})$$

where $K_{12} = [I_3]/[N]$ and $K_{23} = [I_2]/[I_3]$. The species I_1 is not included in K_{app} since it resembles the unfolded forms in terms of its exposure of Tyr residues to solvent and, therefore, its extinction coefficient at 286 nm.

Converting to free energy:

$$-RT \ln K_{app} = -RT \ln K_{12} - RT \ln (1 + K_{23})$$

$$\Delta G_{app} = \Delta G_{12} - RT \ln (1 + K_{23})$$

Assuming that the value of K_{23}, which represents Pro isomerization equilibrium, is unity and independent of the urea concentration:

$$\Delta G_{app} = \Delta G_{12} - 0.4 \text{ kcal mol}^{-1}$$

The 0.4 kcal mol^{-1} difference in the free energy calculated from the equilibrium data, ΔG_{app}, and that calculated from the kinetic data, ΔG_{12}, is close to the experimental measuring error,

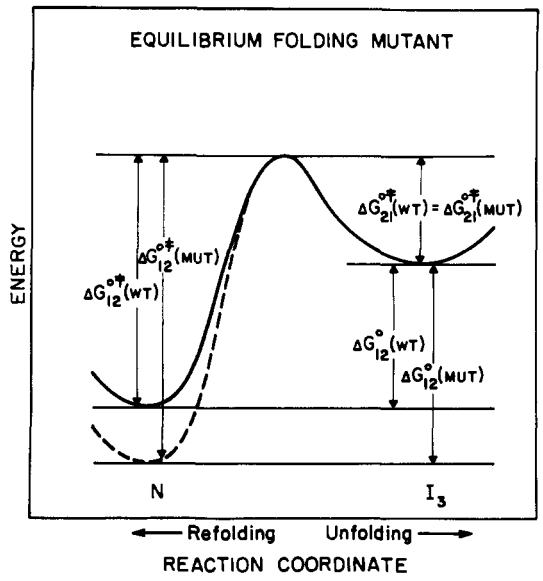

FIGURE 7: Reaction coordinate diagram for the N $\leftrightarrow$ I_3 reaction for the wild-type (—) and hypothetical equilibrium mutant (---) α subunits in the absence of urea. Axes are not drawn to scale.

accounting for the good agreement. If K_{23} is assumed to be 0.2, a value observed for the cis/trans equilibrium constant in model peptides (Brandts et al., 1975), the difference between ΔG_{app} and ΔG_{12} is reduced to −0.1 kcal mol^{-1}.

The significant conclusion to be drawn from this analysis is that the urea-dependent τ_2 kinetic phase corresponds closely to the equilibrium transition between the native and intermediate forms. Therefore, the effects of the amino acid replacements on both the equilibrium and kinetic properties of an actual folding reaction can be determined. This information is extremely useful in classifying the effects of mutations (see below).

Classification of the Effects of Point Mutations on Folding and Stability. One of the questions posed in the introduction to this paper involved the possibility of classifying mutant proteins according to their effects on the kinetic and equilibrium properties of the folding transition. A simple way to describe these effects is in terms of a reaction coordinate diagram. Hypothetically, four classes of folding mutants exist.

(*A*) *Equilibrium Mutation.* The relaxation time for either unfolding or refolding is altered while the opposing relaxation time is unchanged relative to wild-type protein; the stability also changes. Four different cases are possible; however, for brevity consider the case where the relaxation time for unfolding is increased while that for refolding is unchanged. The appropriate reaction coordinate diagrams for the wild-type and mutant proteins are shown in Figure 7. This alignment suggests that an additional stabilizing interaction in the native conformation which does not exist in the transition state or the intermediate state has been introduced by the amino acid replacement. The effect of the mutation appears to alter the stability of the protein without any real effect on the kinetics of the rate-limiting step.

(*B*) *Kinetic Mutation.* The unfolding and refolding relaxation times for the mutant protein are either both increased or both decreased by the same amounts relative to the wild-type protein; the stability is unaffected. The reaction coordinate diagrams for the case where both relaxation times are increased are shown in Figure 8. The energy of the transition state increases relative to that of the two stable states, implying that the mutation effects the rate-limiting step in folding.

(*C*) *Mixed Equilibrium–Kinetic Mutation.* The relaxation times for unfolding and refolding are affected in opposite ways;

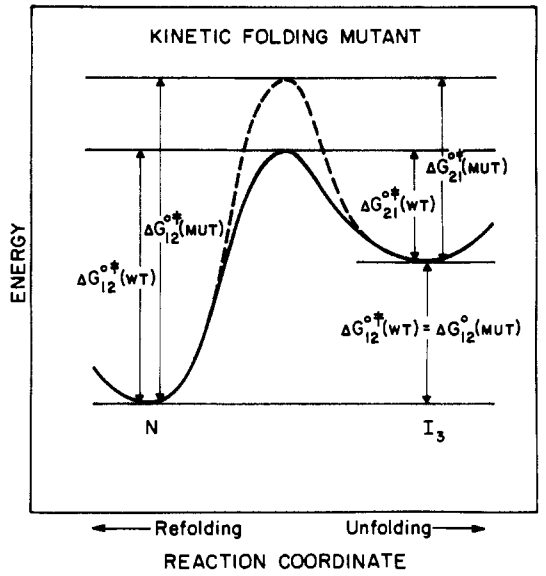

FIGURE 8: Reaction coordinate diagram for the N ↔ I_3 reaction for the wild-type (—) and hypothetical kinetic mutant (- - -) α subunits in the absence of urea. Axes are not drawn to scale.

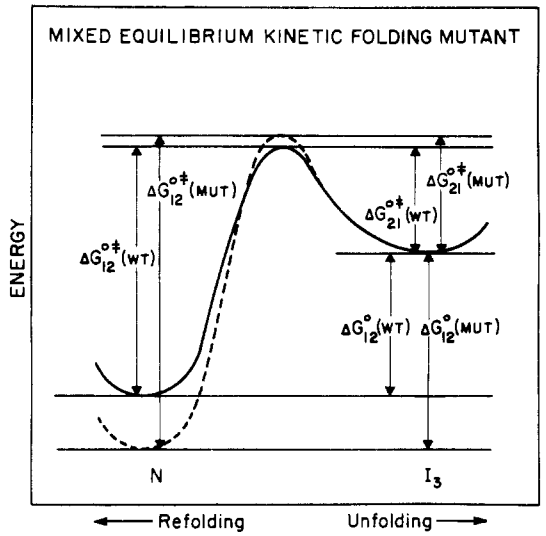

FIGURE 9: Reaction coordinate diagram for the N ↔ I_3 reaction for the wild-type (—) and hypothetical mixed equilibrium–kinetic mutant (- - -) α subunits in the absence of urea. Axes are not drawn to scale.

e.g., one is increased while the other is decreased, thus changing the stability. Another possibility is that the relaxation times of unfolding and refolding are both increased or both decreased; however, in contrast to the kinetic mutation described above, the magnitudes of the changes are not identical; the stability is altered. The reaction coordinate diagrams for the case where the relaxation time for unfolding is increased by a greater amount than that of refolding are shown in Figure 9. The energies of the native and transition states are altered relative to each other and to that of the intermediate. The mutation affects the rate-limiting step in folding as well as the stability of the protein.

(D) Silent Mutation. The relaxation times for unfolding and refolding are unaffected, as is the stability. If the energies of the I_3 species in the wild-type and mutant proteins are aligned, the reaction coordinate diagrams are superimposable. Such results do not necessarily rule out a role for this amino acid in folding or stability. The fortuitous cancellation of competing noncovalent interactions could give rise to such an effect.

The comparisons of the reaction coordinate diagrams shown in Figures 7–9 were made by arbitrarily aligning the energies of the intermediates, I_3, in the wild-type and mutant proteins. There is no a priori reason why this alignment should be correct both because I_3 is a partially folded species whose free energy of folding relative to the unfolded form may be affected by the mutation and because the energies of the unfolded forms themselves will be affected by the amino acid replacement.

Therefore, one is compelled to ask whether meaningful information can be extracted from a comparison of reaction coordinate diagrams if there is no basis for alignment. To answer this question, consider again the reaction coordinate diagram for an equilibrium mutant (Figure 7). An alternative alignment of the two diagrams would have the energies of the native forms coincident while the energies of the transition state and intermediate forms are both increased by the same amount. Instead of introducing a stabilizing interaction in the native form, the mutation destabilizes both the transition state and intermediate forms. Such a situation would still be understood as an equilibrium effect because the destabilization of the transition state is actually due to the destabilization of the intermediate form. In other words, the effect of the mutation cannot be uniquely assigned to the energy of the transition state relative to the energies of the stable states.

Similarly, in the case of a kinetic mutation, realignment of the two reaction coordinate diagrams in Figure 8 so as to equate the energies of the transition states still leads to the conclusion that a mutation which alters the relaxation times for unfolding and refolding in a similar fashion is involved in the rate-limiting step in folding. In this latter case, the mutation stabilizes the native and intermediate conformations by an equal amount, but the effect is relieved in the transition state since its energy is unchanged. The selective effect of the mutation on the energy of the transition state relative to those of the stable states is the key property of a kinetic mutation.

Extension of this argument to the case of a mixed equilibrium–kinetic mutant leads to the same conclusion reached above, namely, that when the relaxation times for unfolding and refolding are both altered, but by different amounts, the relevant amino acid position is involved both in the transition state and in one or both of the stable states. Of importance in identifying the rate-limiting step in folding is the involvement of the amino acid with the transition state.

Mechanistic Implications of Equilibrium Mutations. Further consideration of the reaction coordinate diagram for an equilibrium mutation (Figure 7) leads to the conclusion that mechanistic information can be obtained from such mutants. For the specific case shown in Figure 7, the differential effect of the mutation on the energy of the native form is only realized *after* the transition state has been passed in the folding direction. For mutations in which only the rate of refolding is altered, e.g., Glu-49 → Met, the differential effect must occur *before* the transition state is passed in the folding direction. Recalling that kinetic mutations have their differential effect on the transition state, it appears that mutations that fall into either the equilibrium or the kinetic class will provide a means of determining at precisely which point along the reaction coordinate that residue becomes involved in folding. All that can be concluded from mixed equilibrium–kinetic mutations is that the residue involved plays a key role in the rate-limiting step in folding; it cannot be determined if the effect on stability occurs before or after the transition state.

Further studies are required to test the validity of this hypothesis. If it indeed proves to be valid, studies of the effects

of single amino acid replacements on the kinetics of folding will potentially be able to provide a very detailed view of this complex conformational change.

Effect of Point Mutations on the N ↔ I_3 Folding Reaction. With the above considerations in mind, the kinetic and equilibrium results for the N ↔ I_3 transition for the five point mutations described in this paper can be discussed. For the Phe-22 → Leu mutant, the 4-fold increase in relaxation time for unfolding and the 2.5-fold increase for refolding accompanied by very little change in stability suggest that this is very close to a pure kinetic mutation. Therefore, the amino acid at position 22 plays a key role in the rate-limiting step in folding. For the Glu-49 → Met mutant, the selective decrease in the relaxation time of refolding accompanied by an increase in stability identifies this as an equilibrium mutant. The residue at position 49 becomes involved in folding before the transition state is passed in the folding direction; it is not involved in the rate-limiting step. For the Gly-211 → Glu mutant protein, the unequal increase in the relaxation times for both unfolding and refolding and the accompanying increase in stability identify this as a mixed equilibrium–kinetic mutant. Like amino acid position 22, position 211 plays a key role in folding. For the Gly-234 → Asp mutant, the 4-fold increase in the relaxation time for unfolding and the 2-fold increase in the relaxation time for refolding accompanied by an increase in stability identify this as another mixed equilibrium–kinetic mutant. The absence of an effect on either the kinetic or the equilibrium properties for the Gly-234 → Lys mutant classifies this as a silent mutation. Since Asp at this position alters the stability and the kinetics, the absence of an effect by Lys must reflect compensating noncovalent interactions, e.g., electrostatic and van der Waals forces.

One of the questions generated by previous experiments on the folding of the α subunit was the nature of the structural change associated with the I_3 ↔ N transition. The concept of the independent folding of a more stable amino domain and a less stable carboxyl domain had led to the postulate that I_3 has a folded amino domain and an unfolded carboxyl domain (Matthews et al., 1983). Thus, it was suggested that the I_3 to N transition is rate limited either by the folding of the carboxyl domain or by the association of the two prefolded domains. The kinetic results for the Phe-22 → Leu, Gly-211 → Glu, and Gly-234 → Asp mutant proteins permit the correct assignment of this kinetic phase. If the carboxyl domain folding is rate limiting, only amino acid replacements in that domain should affect the rate. If a domain association type reaction is rate limiting, replacements in either domain could affect the rate. The replacements at position 22 in the amino domain and at positions 211 and 234 in the carboxyl domain decrease the rates of *both* unfolding and refolding between N and I_3 and therefore have a real effect on the kinetics of this step. The results for these mutant proteins demonstrate that either a domain association reaction or perhaps some other molecule-wide reaction is rate limiting.

The differences between these two sets of mutants, one set affecting principally the kinetics (Phe-22 → Leu, Gly-211 → Glu, and Gly-234 → Asp) and the other the stability (Glu-49 → Met), may be rationalized in terms of the assignment of this step in the folding model to domain assocation. Mutations at position 211 that inactivate the α subunit can be reverted to activity by second-site amino acid replacements at both position 22 and position 175 (Yanofsky, 1967; Murgola & Yanofsky, 1974). Similarly, an inactivating mutation at position 213 can be reverted by a second change at position 177. It has been suggested that this pattern of second-site revertants implies that these positions are in proximity with each other in the native conformation (Yanofsky, 1967). Thus, in terms of a domain association reaction being rate limiting, positions 22 and 211 may be at the domain interface and able to affect directly the rate. No second site revertant data are available for positions 49 and 234; therefore, it is not known whether they are at the interface between the domains.

The results presented in this paper make it apparent that the mutagenic approach will be very useful in elucidating the importance of particular amino acid positions to one of the slow steps in the folding of the α subunit of Trp synthase. Substitutions at other sites in this protein by either random or site-directed mutagenesis and analysis using the methods described above may allow the formation of general rules that will pertain to the folding of other globular proteins. Such studies are currently in progress in our laboratory.

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Native or Nativelike Species Are Transient Intermediates in Folding of Alkaline Iso-2 Cytochrome *c*†

Barry T. Nall

Department of Biochemistry and Molecular Biology, The University of Texas Medical School at Houston, Houston, Texas 77225

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ABSTRACT: Titration to high pH converts yeast iso-2 cytochrome *c* to an inactive but more stable alkaline form lacking a 695-nm absorbance band [Osterhout, J. J., Jr., Muthukrishnan, K., & Nall, B. T. (1985) *Biochemistry 24*, 6680–6684]. The kinetics of absorbance-detected refolding of the alkaline form have been measured by dilution of guanidine hydrochloride in a stopped-flow instrument. Fast-folding species (τ_2) are detected, as in refolding to the native state at neutral pH. An additional kinetic phase (τ_a) is observed with an amplitude opposite in sign to the fast phase. The amplitude of this phase increases and the rate increases with increasing pH. Comparison to pH-jump measurements of the fully folded protein shows that phase τ_a has the same sign, rate, and pH dependence as the alkaline isomerization reaction, suggesting that this new phase involves isomerization of native or nativelike species following fast folding. Absorbance difference spectra are taken at 5-s intervals during refolding at high pH. The spectra verify that nativelike species—with a 695-nm absorbance band—are formed transiently, before conversion of the protein to the alkaline form. Refolding in the presence of ascorbate shows that the transient, nativelike species are reducible, unlike alkaline iso-2. Thus, (1) refolding to the alkaline form of iso-2 cytochrome *c* proceeds through transient native or nativelike species, and (2) a folding pathway leading to native or nativelike forms is maintained at high pH, where native species are no longer the thermodynamically favored product. For the latter stages of folding, the results argue against growth and merge assembly of structure found *only* in the thermodynamically favored product [Harrison, S. C., & Durbin, R. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 4028–4030] and suggest the existence of unique species which direct folding—perhaps along a sequential pathway [Kim, P. S., & Baldwin, R. L. (1982) *Annu. Rev. Biochem. 51*, 459–489].

Harrison and Durbin (1985) have proposed that protein folding is analogous to putting together a jigsaw puzzle: assembly can be initiated from any element of local structure and proceeds by a "growth and merge mechanism" (Karplus & Weaver, 1976) where the participants (elements of local structure, i.e., puzzle pieces) are limited to aspects of local structure found in the thermodynamically determined product of refolding. The stability of the product determines the folding process in that the structure of intermediates is limited exclusively to local structure or substructures found in the thermodynamic result of the refolding process. Thus, folding proceeds directly to the thermodynamically most stable state.

In contrast, Kim and Baldwin (1982) propose that folding is a sequential process involving a unique series of intermediates which direct folding along the most expedient route to the thermodynamically favored product. The structure of sequential intermediates serves to facilitate folding, but this structure may or may not be related to the structure found in the product of folding.

These two proposals are at opposite extremes. Either might be favored by evolution: the puzzle model by providing a highly adaptable folding process as a hedge against point mutations that lead to polypeptides unable to fold (Harrison & Durbin, 1985), sequential folding by charting a pathway through an otherwise uncountable number of possible conformations (Levinthal, 1968). Refolding processes for particular proteins may exhibit properties of both models, perhaps involving a growth and merge mechanism early on, followed by a sequential pathway in the latter, more highly restricted stages of folding.

The jigsaw puzzle model can be tested for proteins that take on different states under different conditions: the "native" form under physiological conditions and a folded but conformationally distinct "isomeric" form under some other conditions. Cytochrome *c* is such a protein: native and alkaline isomeric forms have been described for both horse (Greenwood & Palmer, 1965; Brandt et al., 1966; Davis et al., 1974) and yeast iso-2 cytochrome *c* (Osterhout et al., 1985). The native (neutral pH) form has a 695-nm absorbance band and is reducible by ascorbic acid while the alkaline (pH 9–10) form

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