Nauseef, W. M., & Malech, H. L. (1986) *Blood 67*, 1504–1507.
Olsen, R. L., & Little, C. (1984) *Biochem. J. 222*, 701–709.
Olsson, I., Persson, A., & Strömberg, K. (1984) *Biochem. J. 223*, 911–920.
Parmley, R. T., Rice, W. G., Kinkade, J. M., Jr., Gilbert, C., & Barton, J. C. (1987) *Blood 70*, 1630–1638.
Pember, S. O., Fuhrer-Krüsi, S. M., Barnes, K. C., & Kinkade, J. M., Jr. (1982) *FEBS Lett. 140*, 103–108.
Pember, S. O., Shapira, R., & Kinkade, J. M., Jr. (1983) *Arch. Biochem. Biophys. 221*, 391–403.
Rice, W. G., Kinkade, J. M., Jr., & Parmley, R. T. (1986) *Blood 68*, 541–555.
Rice, W. G., Ganz, T., Kinkade, J. M., Jr., Selsted, M. E., Lehrer, R. I., & Parmley, R. T. (1987) *Blood 70*, 757–765.
Schultz, J. (1980) in *The Reticuloendothelial System* (Sbarra, A., & Strauss, R., Eds.) Vol. 2, pp 231–254, Plenum Publishing Corp., New York.
Sibbett, S. S., & Hurst, J. K. (1984) *Biochemistry 23*, 3007–3013.
Spitznagel, J. K., Dalldorf, F. G., Leffell, M. S., Folds, J. D., Welsh, I. R. H., Cooney, M. H., & Martin L. E. (1974) *Lab. Invest. 30*, 774–785.
Strömberg, K., Persson, A., & Olsson, I. (1985) *Eur. J. Cell Biol. 39*, 424–431.
Suzuki, K., Yamada, M., Akashi, K., & Fujikura, T. (1986) *Arch. Biochem. Biophys. 245*, 167–173.
Svensson, B. E., Domeij, K., Lindvall, S., & Rydell, G. (1987) *Biochem. J. 242*, 673–680.
Taylor, K. L., Burgess, C. A., Guzman, G. S., & Kinkade, J. M., Jr. (1988) *FASEB J. 2*, 1596.
Taylor, K. L., Winton, E. F., & Kinkade, J. M., Jr. (1989) *FASEB J. 3*, 561.
Weil, S. C., Rosner, G. L., Reid, M. S., Chisholm, R. L., Farber, N. M., Spitznagel, J. K., & Swanson, M. S. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 2057–2061.
Wright, D. G., & Gallin, J. I. (1977) *J. Immunol. 119*, 1068–1076.
Wright, J., Yoshimoto, S., Offner, G. D., Blanchard, R. A., Troxler, R., & Tauber, A. I. (1987) *Biochim. Biophys. Acta 915*, 68–76.
Yamada, M. (1982) *J. Biol. Chem. 257*, 5980–5982.
Yamada, M., Mori, M., & Sugimura, T. (1981a) *Biochem. Biophys. Res. Commun. 98*, 219–226.
Yamada, M., Mori, M., & Sugimura, T. (1981b) *Biochemistry 20*, 766–771.

Multiple Replacements at Position 211 in the α Subunit of Tryptophan Synthase as a Probe of the Folding Unit Association Reaction†

Neil B. Tweedy,‡ Mark R. Hurle,§ Boris A. Chrunyk, and C. Robert Matthews*
Department of Chemistry, The Pennsylvania State University, University Park, Pennsylvania 16802
Received July 5, 1989; Revised Manuscript Received October 3, 1989

ABSTRACT: Equilibrium and kinetic studies on the folding of a series of amino acid replacements at position 211 in the α subunit of tryptophan synthase from *Escherichia coli* were performed in order to determine the role of this position in the rate-limiting step in folding. Previous studies [Beasty, A. M., Hurle, M. R., Manz, J. T., Stackhouse, T., Onuffer, J. J., & Matthews, C. R. (1986) *Biochemisty 25*, 2965–2974] have shown that the rate-limiting step corresponds to the association/dissociation of the amino (residues 1–188) and carboxy (residues 189–268) folding units. In terms of the secondary structure, the amino folding unit consists of the first six strands and five α helices of this α/β barrel protein. The carboxy folding unit comprises the remaining two strands and three α helices; position 211 is in strand 7. Replacement of the wild-type glycine at position 211 with serine, valine, and tryptophan at most alters the rate of dissociation of the folding units; association is not changed significantly. In contrast, glutamic acid and arginine dramatically decelerate and accelerate, respectively, both association and dissociation. The difference in effects is attributed to long-range electrostatic interactions for these charged side chains; steric effects and/or hydrogen bonding play lesser roles. When considered with previous data on replacements at other positions in the α subunit [Hurle, M. R., Tweedy, N. B., & Matthews, C. R. (1986) *Biochemistry 25*, 6356–6360], it is clear that β strands 6 (in the amino folding unit) and 7 (in the carboxy folding unit and containing position 211) dock late in the folding process.

The determination of the mechanism of protein folding requires detailed information on the structures of partially folded forms and the transition states which link these forms and the native conformation. Structures of folding intermediates are beginning to become available through advances in technology such as the recent combination of hydrogen-exchange and two-dimensional nuclear magnetic resonance (2D NMR)[1] spectroscopy (Udgaonkar & Baldwin, 1988; Roder et al., 1988) and peptide synthesis (Oas & Kim, 1988; Marqusee &

† This work was supported by a grant from the U.S. Public Health Service (GM 23303).
‡ Present address: Department of Biochemistry, Duke University School of Medicine, Durham, NC 27710.
§ Present address: Department of Pharmaceutical Chemistry, University of California, San Francisco, School of Pharmacy, San Francisco, CA 94143.

[1] Abbreviations: 2D NMR, two-dimensional nuclear magnetic resonance; $NaDodSO_4$, sodium dodecyl sulfate; G211X (where X = D, E, R, S, V, and W), single amino acid code with the first letter designating the wild-type residue followed by the position and the second letter designating the mutation, i.e., G211D referes to the replacement of glycine by aspartic acid at position 211, and E, R, S, V, and W refer to glutamic acid, arginine, serine, valine, and tryptophan, respectively; C_{mxy}, urea concentration at the midpoint of the transition between species x and y.

0006-2960/90/0429-1539$02.50/0

Baldwin, 1987). Information on the structures of transition states for the rate-limiting steps in folding can be obtained by observing the effects of single amino acid replacements on the folding kinetics (Matthews, 1987). Application of this mutagenic approach to the folding of the α subunit of tryptophan synthase (Beasty et al., 1986) and dihydrofolate reductase (Perry et al., 1987) has shown that some positions play key roles in the respective transition states while others are involved in establishing stabilizing interactions in the native or intermediate conformations.

The α subunit of tryptophan synthase has been a particularly intriguing case because the folding involves a stable intermediate whose conversion to the native form is the rate-limiting step in folding (Beasty et al., 1986). The solution of the X-ray crystal structure (Hyde et al., 1988) has led to the startling discovery that this intermediate corresponds to a folded conformation for the first six strands and five α helices of this α/β barrel motif protein; the remaining two strands and three helices are apparently unfolded. Folding studies (Matthews et al., 1983; Beasty et al. 1986) on a collection of randomly generated mutants in the α subunit (Yanofsky et al., 1969; Murgola & Yanofsky, 1974) showed that replacements in strands 1 (F22L), 7 (G211E), and 8 (G234D) alter the energy of the transition state relative to the energies of the folding intermediate and the native conformation. In contrast, replacements in strands 2 (E49M) and 6 (Y175C) (Hurle et al., 1987) appear to alter the energies of the intermediate and the native conformations, respectively. As noted above, mutants which perturb the energy of the transition state are of special interest in understanding its structure.

Examination of the X-ray structure reveals that positions 22 and 234 are in adjacent strands in the barrel and contiguous, as are positions 175 and 211 (Hyde et al., 1988). On the basis of the above mutagenic results, one would conclude that the interaction between side chains at positions 22 and 234 plays a key role in the transition state. However, the apparently conflicting responses at positions 175 and 211 (equilibrium effect at 175 and kinetic effect at 211)[2] prevent a definitive assignment of the role of their interaction in the folding of the α subunit.

To resolve this ambiguity, the effects of a series of amino acid replacements at position 211 on the folding and stability of the α subunit were investigated. The results indicate that long-range electrostatic effects can play a significant role in the behavior of the mutants at this position and are the primary reason that the replacement of glycine by glutamic acid acts as a kinetic mutant; steric effects appear to play a lesser role. When the unique effect of the electrostatic interaction of glutamic acid 211 is taken into account, the principle effect of replacements at position 211 is to alter the energy of the native conformation. The similar behavior of mutations at positions 175 and 211 suggests that these two positions dock late in folding, *after* the transition state for the rate-limiting step in folding.

Materials and Methods

Protein Isolation and Purification. The wild-type α subunit was isolated from *Escherichia coli* strain W3110 (ΔtonB-trp)BA17 *his* containing plasmid pBN55 (a gift from Brian Nichols). The G211D, G211E, G211R, G211S, G211V, and G211W mutant proteins used in this study were isolated from bacterial strains A46^{ASP}, A46, A23, A23FR2, A46PR9, and 2102, respectively. These bacterial strains were a generous gift from Dr. Charles Yanofsky. The proteins were isolated from *Escherichia coli* cultures of the respective strains and purified according to procedures described previously (Beasty et al., 1986). The purity of the proteins was ascertained by the presence of a single band on Coomassie Blue stained native and $NaDodSO_4$–polyacrylamide gel electrophoresis. The activities of all the isolated α subunit proteins were determined by measuring their ability to enhance the activity of the β_2 subunit of tryptophan synthase in the condensation of indole and serine to form tryptophan; the maximum specific activity in this assay is 5500 units/mg (Kirschner et al., 1975). The activities of the G211D, G211E, G211R, G211S, G211V, and G211W mutants were 4900, 4100, 2486, 5033, 1954, and 5101 units/mg, respectively, with an error of ±10%. Protein concentrations were held constant in any given experiment and were in the range of 0.6–1.5 mg/mL as ascertained by using the extinction coefficient of 12600 M^{-1}.

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

Methods. The equilibrium unfolding data were obtained by measuring the ultraviolet difference spectrum at 287 nm using the tandem cell technique (Herskovits, 1967) on a Cary 118CX spectrophotometer. The data were fit to a three-state model as described previously (Beasty et al., 1986). Unfolding (refolding) kinetic jumps were initiated by adding measured volumes of protein (protein and urea) to premeasured volumes of urea/buffer solution. The solutions were manually mixed, and the time dependence of the absorbance change at 287 nm was recorded by a Digital PDP-11 computer interfaced to the spectrophotometer. These changes were fit to a sum of exponentials as described previously (Beasty et al., 1986). The buffer used in all experiments was 10 mM potassium phosphate, 0.2 mM EDTA, and 1 mM 2-mercaptoethanol, pH 7.8. The equilibrium and kinetic studies were performed at 25 °C for the wild-type and all mutant proteins except G211R which was studied at 4 °C.

Results

Equilibrium Unfolding Experiments

The urea-induced unfolding reactions of the wild-type α subunit of tryptophan synthase and all 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 (Matthews & Crisanti, 1981; Beasty et al., 1986). The intermediate can be detected in optical experiments using absorbance spectroscopy at 287 nm (monitoring the solvent exposure of buried tyrosines) and circular dichroism spectroscopy at 222 nm (monitoring the secondary structure). In both cases, an inflection in the plot of the apparent fraction of unfolded protein, F_{app}, as a function of the urea concentration indicates the presence of the intermediate.

Neutral Side-Chain Replacements. The transition curves for the wild-type α subunit (Gly211) and the mutant proteins containing serine (G211S), valine (G211V), and tryptophan (G211W) at position 211 are shown in Figure 1. These neutral replacements vary in size, hydrophobicity, and hydrogen bonding capability. The entire F_{app} curve for the G211S protein is displaced slightly to lower urea concentrations while that for the G211V protein occurs at slightly higher urea

[2] The designation of equilibrium and kinetic effects is derived from transition-state theory and is explained more extensively elsewhere (Beasty et al., 1986; Matthews, 1987). A kinetic mutation is defined as one in which the rates of both unfolding and refolding are affected equally without any significant change in stability. An equilibrium mutation is defined as affecting *either* refolding or unfolding with an accompanying change in stability.

Table I: Equilibrium Parameters for Urea-Induced Unfolding of α Subunit Proteins at pH 7.8[a]

temp (°C)	protein	$\Delta G_{NI}^{H_2O}$	A_{NI}	$\Delta G_{IU}^{H_2O}$	A_{IU}	C_{mNI}	$\Delta\Delta G_{NI}$[b]	C_{mIU}	$\Delta\Delta G_{IU}$[c]
25	wild type	5.7 (0.4)	2.2 (0.2)	5.0 (0.6)	1.2 (0.2)	2.62 (0.02)		4.18 (0.06)	
	G211E	8.1 (0.6)	3.0 (0.2)	5.5 (0.5)	1.5 (0.1)	2.73 (0.02)	0.3 (0.1)	3.72 (0.04)	−0.7 (0.1)
	G211D	5.4 (0.9)	2.0 (0.4)	4.2 (0.5)	1.2 (0.3)	2.70 (0.09)	0.2 (0.2)	3.61 (0.15)	−0.7 (0.1)
	G211S	5.9 (0.8)	2.4 (0.3)	5.2 (0.8)	1.3 (0.2)	2.46 (0.04)	−0.4 (0.1)	3.97 (0.07)	−0.3 (0.1)
	G211V	5.4 (0.9)	1.9 (0.4)	6.9 (1.7)	1.6 (0.4)	2.78 (0.06)	0.3 (0.1)	4.30 (0.09)	0.2 (0.2)
	G211W	4.1 (1.8)	1.8 (0.8)	3.9 (2.0)	1.1 (0.5)	2.25 (0.15)	−0.7 (0.5)	3.62 (0.20)	−0.5 (0.2)
4	wild type	5.0 (1.7)	3.1 (1.1)	6.0 (0.9)	1.9 (0.3)	1.63 (0.07)		3.17 (0.09)	
	G211R	2.2 (0.5)	1.6 (0.4)	7.3 (3.3)	2.2 (1.0)	1.40 (0.08)	−0.4 (0.2)	3.28 (0.11)	0.3 (0.3)

[a] Units are as follows: $\Delta G_{xy}^{H_2O}$ (free energy of unfolding in the absence of denaturant), kilocalories per mole; A (urea dependence of the free energy of unfolding), kilocalories per mole per molar (urea); $\Delta\Delta G$, kilocalories per mole; C_m (transition midpoint), molar urea; errors represent 95% confidence limits and are shown in parentheses. [b] Calculated at the wild-type N ↔ I midpoint, 2.62 M urea (25 °C) and at 1.63 M urea (4 °C), where $\Delta G = \Delta G^{H_2O} + A$[urea at C_m] and $\Delta\Delta G = \Delta G$(mutant) − ΔG(wild type). [c] Calculated at the wild-type I ↔ U midpoint, 4.18 M urea (25 °C) and at 3.17 M urea (4 °C) as above.

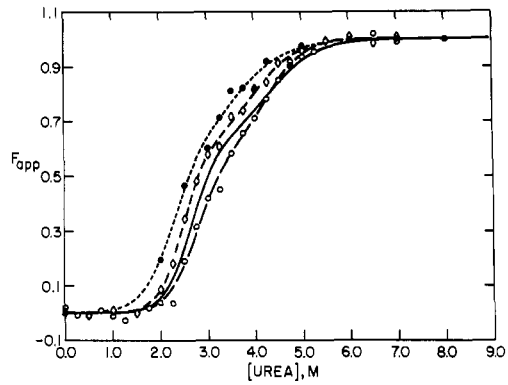

FIGURE 1: Apparent fraction of unfolded protein, R_{app}, as a function of the urea concentration for the neutral replacements at position 211 in the presence of 10 mM potassium phosphate, 0.2 mM EDTA, and 1 mM 2-mercaptoethanol, pH 7.8, 25 °C, for the wild-type (glycine) (—) and the G211S (◇--◇), G211V (○—○), and G211W (●---●) mutant proteins. Lines represent computer fits to the data using a three-state model as described previously (Beasty et al., 1986).

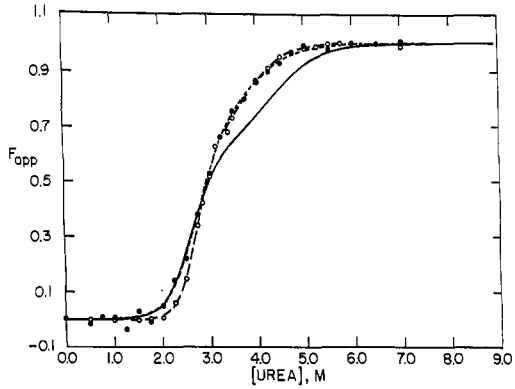

FIGURE 2: Apparent fraction of unfolded protein, F_{app}, as a function of the urea concentration for the wild-type (—) and the G211E (○--○) and G211D (●---●) mutant proteins. Lines represent computer fits to the data. Conditions were as described in Figure 1.

concentrations, relative to wild-type α subunit. The G211W curve shows more significant differences from the wild-type protein in both the N ↔ I and I ↔ U transitions; both occur at lower concentrations of urea.

These data can be fit to the three-state model and the relevant thermodynamic parameters extracted (Beasty et al., 1986). The values for the free energy of folding in the absence of denaturant, ΔG^{H_2O}, the midpoint of the transition, C_m, and the dependence of the apparent free energy of folding on the denaturant concentration, A, are shown in Table I. When the errors in each of these parameters are taken into account, these replacements appear to have rather little effect on the stability or cooperativity of the unfolding induced by urea.

In considering the effects of amino acid replacements on stability, it appears to be more reasonable to compare the free energy differences at the urea concentration corresponding to the midpoint of the wild-type protein, $\Delta\Delta G$, than in the absence of denaturant, ΔG^{H_2O} (Cupo & Pace, 1983). As can be seen above, the extrapolation required to obtain the value in the absence of urea introduces errors which are comparable to the changes in stability caused by the replacements. According to the latter approach, the G211S mutant is seen to be 0.4 kcal/mol less stable in the N ↔ I transition and 0.3 kcal/mol less stable in the I ↔ U transition than the wild-type protein (Table I, columns 6 and 8). The G211V mutant is 0.3 kcal/mol more stable than wild type in the N ↔ I transition and of the same stability in the I ↔ U transition. The replacement of glycine-211 by tryptophan has the most noticeable effect on the N ↔ I transition: the stability decreases by 0.8 kcal/mol. The stability between I and U decreases by 0.4 kcal/mol. Overall, with the exception of the G211W mutant protein, there is rather little effect of these neutral replacements on the stability of the α subunit.

Charged Side-Chain Replacements. The replacement of glycine with anionic aspartate or glutamate side chains again has little effect on the N ↔ I transition; however, there is a pronounced effect on the I ↔ U transition (Figure 2 and Table I). The N ↔ I transition for the G211E mutant is slightly stabilized, 0.3 kcal/mol, while that for the G211D is unchanged within experimental error. However, the I ↔ U transitions for both mutants are destabilized by 0.7 kcal/mol (Table I, columns 6 and 8).

The introduction of a positive charge at position 211 was examined with the G211R mutant. In preliminary experiments at 25 °C, the G211R mutant was found to unfold and refold substantially faster than wild-type α subunit. Because only very small quantities of this mutant could be purified, limited equilibrium and kinetic studies were performed at 4 °C. The lower temperature slowed folding kinetics sufficiently so that manual mixing methods could be used on the amounts of protein available. The equilibrium unfolding transitions for the G211R and wild-type proteins at 4 °C are shown in Figure 3. The midpoint of the N ↔ I transition is lowered by 0.23 M urea and the stability by 0.4 kcal/mol, relative to the wild-type protein at the same temperature (Table I, columns 5 and 6). The decreased slope for this transition indicates that the cooperativity is lowered as well. There is no significant effect on the I ↔ U transition. For comparison, the equilibrium unfolding transition for the G211E mutant at 4 °C was investigated; the results are also shown in Figure 3. The midpoint of the N ↔ I transition is increased to 2.0 M urea and the stability by 1.3 kcal/mol, relative to wild-type protein (Table I). As is the case for the G211R mutant, the I ↔ U

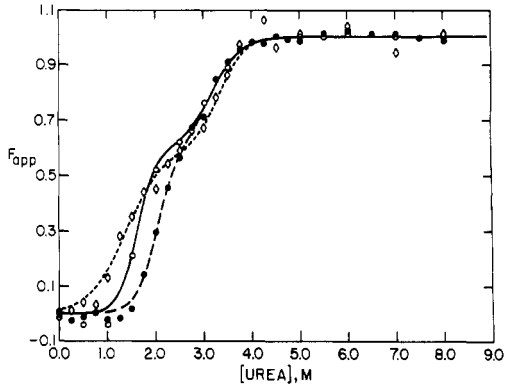

FIGURE 3: Apparent fraction of unfolded protein, F_{app}, as a function of the urea concentration for the wild-type (O—O) and the G211R (◊---◊) and G211E (●—●) mutant proteins at 4 °C. Lines represent computer fits to the data.

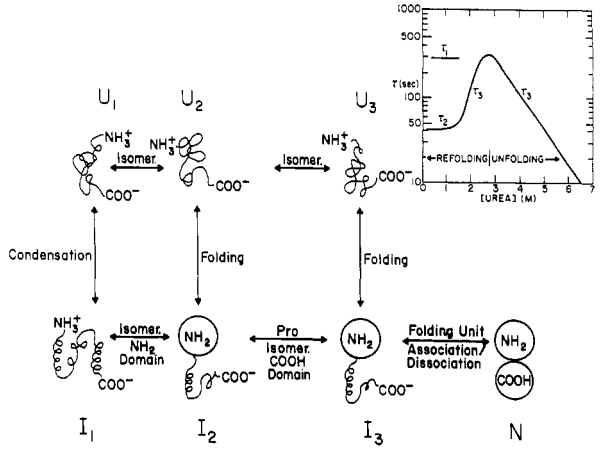

FIGURE 4: Proposed folding model for the subunit of tryptophan synthase from *Escherichia coli*. N, I, and U represent native, intermediate, and unfolded forms, respectively. Inset illustrates the urea concentration dependence of the three kinetic folding phases observed for the wild-type protein at pH 7.8 and 25 °C.

transition is coincident with that for wild-type α subunit.

Kinetic Folding Experiments

A kinetic folding model has been proposed for the α subunit (Hurle & Mathews, 1987). The model includes a native conformation, N, three intermediates, I_1–I_3, and three unfolded forms, U_1–U_3 (Figure 4). The kinetic intermediates I_2 and I_3 correspond to the equilibrium intermediate I and the kinetic intermediate I_1, and the three unfolded forms U_1–U_3 correspond to the equilibrium species U. I_1 is considered as an unfolded form because it has spectroscopic properties similar to those for the unfolded form (Matthews et al., 1983). The rate-limiting steps in refolding involve the interconversion of the intermediates, I_1, I_2, and I_3, described by relaxation times τ_1 and τ_2, and the folding of species I_3 to the native form with relaxation time τ_3. The τ_1 and τ_2 relaxation times are only detected in refolding and are independent of the urea concentration. The rate-limiting step in unfolding is the N ↔ I_3 reaction, the microscopic reverse of the I_3 ↔ N reaction. Semilog plots of the urea dependences of these relaxation times reveal characteristic behavior which has been interpreted in terms of slow isomerization reactions for τ_1 and τ_2 and the association of the amino and carboxy folding units for τ_3 (Beasty et al., 1986; Hurle & Matthews, 1987).

The reaction of interest in this study is the rate-limiting step which appears in both unfolding and refolding, N ↔ I_3. The relaxation time τ_3 proceeds though a maximum, τ_{max}, near the midpoint of the N ↔ I equilibrium transition and shows a

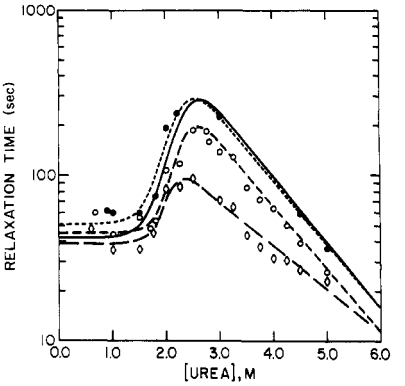

FIGURE 5: Relaxation times as a function of urea concentration for the wild-type (—) and the G211S (●---●), G211V (O--O), and G211W (◊—◊) mutant proteins at pH 7.8, 25 °C. Lines represent computer fits to the data. Data at and above the urea concentration of τ_{max} for each protein were obtained by unfolding while those at and below τ_{max} were obtained by refolding.

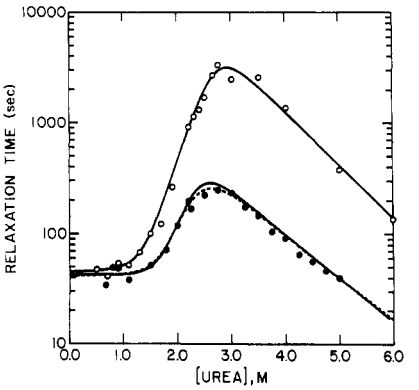

FIGURE 6: Relaxation times as a function of urea concentration for the wild-type (—) and the G211E (O—O) and G211D (●---●) mutant proteins. Lines represent computer fits to the data.

logarithmic dependence on the urea concentration. Below 1.5 M urea in refolding, the τ_2 phase becomes rate limiting. This behavior is shown in the inset to Figure 4. At high urea concentration, the relaxation time for the τ_3 phase is very nearly equal to the reciprocal of the unfolding rate constant and, at low urea concentration prior to the onset of the urea-independent τ_2 phase, the reciprocal of the refolding rate constant (Matthews, 1987).

Neutral Side-Chain Replacements. The replacement of Gly-211 with Ser has very little effect on the τ_3 relaxation time (Figure 5). For G211V, the unfolding relaxation times decrease by approximately 33%, while the refolding relaxation times are unaffected, within experimental error. A decrease of 33% is very close to our limits of detection and represents a change in the activation free energy of only 0.2 kcal/mol. The G211W data are more limited, however, it appears that unfolding is accelerated by a factor of 2.6 at 4 M urea and refolding is only marginally, if at all, altered. Thus, the introduction of three neutral side chains which vary considerably in their volumes, hydrophobicities, and hydrogen bonding capabilities has a significant effect only on the kinetics of the unfolding reaction, N ↔ I_3, for one of the three mutations, G211W. There are no significant effects on the τ_1 and τ_2 relaxation times; the magnitudes of the τ_1 relaxation times are not significantly affected by the replacements at position 211 and are omitted from Figure 5 for clarity.

Charged Side-Chain Replacements. As reported previously (Matthews et al., 1983), the replacement of glycine by glutamate at position 211 dramatically increases both the un-

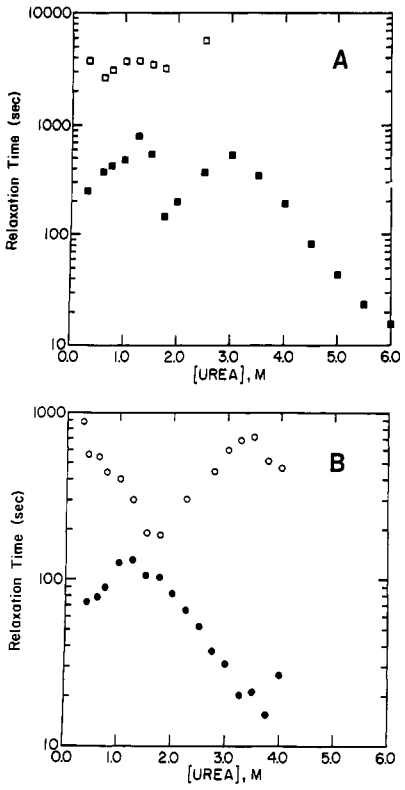

FIGURE 7: Relaxation times as a function of urea concentration for the (A) wild-type and (B) G211R mutant proteins at 4 °C. Symbols represent the observed slow (□, ○) and fast (■, ●) phases. Data at and above 2 M urea in (A) and 1.5 M urea in (B), respectively, were obtained by unfolding while those below 2 M urea (A) and 1.5 M urea (B) were obtained by refolding.

folding (8-fold at 6 M urea) and refolding (4-fold at 2 M urea) relaxation times for the τ_3 reaction but has no effect on the τ_2 reaction (Figure 6). The τ_1 reaction is also unaltered by this replacement [Figure 3 in Matthews et al. (1983)] and is omitted from Figure 6 for clarity. Surprisingly, the τ_3 relaxation time for the mutant with aspartate at this position is identical with that for the wild-type protein at all urea concentrations (Figure 6). Asp and Glu are obviously quite different in terms of their effects on the kinetics of the $N \leftrightarrow I_3$ reaction.

To provide a basis for comparison for the kinetics of folding of the G211R mutant protein at 4 °C, the kinetics of the wild-type protein were also examined at this temperature. Figure 7A shows that nearly the same qualitative behavior is observed for the τ_3 phases; the quantitative differences reflect the relevant activation energies (Hurle et al., 1987) and the lower stability (Figure 3, Table I). The one significant difference at low temperature is the sudden drop in the τ_3 relaxation time near 2 M urea. Below this point, τ_3 recovers and follows the previously observed pattern. Betton et al. (1985) have also observed this phenomenon in the folding of phosphoglycerate kinase. A second difference is the absence of the urea-independent behavior of the faster phase in refolding at low urea concentrations. Both of these variations from the patterns at 25 °C can be simulated by using the above kinetic model and some plausible alterations in rate constants due to the lower temperature (N. Tweedy, unpublished results).

At 4 °C, the G211R mutant has two detectable kinetic phases in both unfolding and refolding when manual mixing methods are used (Figure 7B). The faster phase has a urea concentration dependence which is similar in most respects to that for the τ_3 phase in the wild-type protein. The τ_{max} occurs at ~1.2 M urea, reflecting the decreased stability (Figure 3, Table I). The slower phase has a very complex urea dependence, demonstrating that a number of elementary processes are involved. The faster phase in the unfolding of the G211R mutant was confirmed as the folding unit dissociation reaction by measuring the activation energies of both phases at 3 M urea. The activation energy of the fast phase is 4.7 kcal/mol, the same as that observed previously for the folding unit dissociation reaction in the wild-type protein (Hurle et al., 1987). The activation energy of the slow phase is 22 kcal/mol, similar to that reported for the τ_1 and τ_2 phases in refolding (Hurle & Matthews, 1987). Therefore, although the decrease in temperature has significant effects on the rate-limiting steps in folding for both the wild-type and G211R proteins, the folding unit association/dissociation reaction is still the dominant phase for both.

Comparison of the relaxation times for the folding unit dissociation/association reaction, i.e., the τ_3 phase, for the wild-type and G211R mutant proteins (Figure 7A,B) shows that both unfolding and refolding are accelerated in the mutant. At 3 M urea, the unfolding relaxation time for the G211R mutant is 17-fold smaller than that for the wild-type protein; for refolding to 1 M urea, the relaxation time is 3.5-fold smaller for the mutant protein.

Discussion

The replacement of glycine at position 211 in the α subunit of tryptophan synthase from *E. coli* with a series of amino acids which vary the volume, charge, hydrophobicity, and hydrogen bonding capability strongly suggests that electrostatic interactions are the predominant reason that the G211E protein was previously observed to behave as a kinetic mutant in the folding unit association reaction (Matthews et al., 1983). Serine and valine have little or no effect on the kinetics of this rate-limiting step in folding, and tryptophan only significantly affects the unfolding reaction. In contrast, glutamic acid and arginine have significant and opposing effects on both unfolding and refolding relaxation times and therefore act as kinetic mutants (Matthews, 1987). The observation that aspartic acid has no effect on the kinetics is puzzling but may reflect a different placement for this side chain compared to that for glutamic acid. Glutamic acid and aspartic acid replacements have been observed to adopt different spatial orientations in T4 lysozyme (Alber et al., 1987) and staphylococcal nuclease (Hibler et al., 1987). Obviously, X-ray structures of these mutants are required to provide a definitive answer to this apparent discrepancy.

The unique effects of arginine and glutamate on the folding unit association reaction most likely reflect their potential for long-range electrostatic interactions. Tryptophan is larger than the side chains of either glutamic acid or arginine; however, the van der Waals and hydrogen bonds with which tryptophan can interact with its surroundings are necessarily short-range. Therefore, when only direct interactions of the side chain at position 211 are considered, it is clear that the residue at this position does not play a significant role in the transition state for the folding unit association reaction. If the side chain is sufficiently bulky (Trp), the principal effect of replacements at this position is to alter the relaxation time for unfolding and, therefore, the energy of the native conformation. This conclusion resolves the apparent contradiction between the Y175C mutation in strand 6 (equilibrium mutant) and the G211E mutation in strand 7 (kinetic mutant) which are adjacent in the X-ray structure. If long-range interactions are eliminated, replacements at both sites principally affect the energy of the native conformation.

Examination of the X-ray crystal structure of the wild-type α subunit suggests that repulsive/attractive interactions between the Arg or Glu side chain at position 211 and Glu-49 may selectively alter the relative energy of the transition state for the folding unit association reaction. The carboxylate side chain of Glu-49 is the only formal charge that can be found within 5 Å of a glutamate placed at position 211 by computer graphics manipulation. The distance between carboxylate oxygens ranges from 4 to 10 Å depending on the placement of the glutamate at position 211. Given that folding is slowed by the incorporation of a negative charge at position 211 and accelerated by a positive charge, the simplest interpretation in terms of this environment is that repulsive interactions between Glu-211 and Glu-49 raise the relative energy of the transition state while attractive interactions between Arg-211 and Glu-49 lower it. The differential effects on the relaxation times for unfolding and refolding suggest that the relative energies of the native and intermediate conformations are also perturbed to some extent by these substitutions. The net result is that the Glu-211 protein must scale a higher energy barrier and Arg-211 a lower barrier to access the transition state. Note that the presumption of an effect of a replacement at position 211 on the energy of the intermediate is contradictory to the conclusions from previous studies from our laboratory (Beasty & Matthews, 1985) and others (Miles et al., 1982) that the carboxy folding unit is devoid of structure. This point will be considered below.

Another potential explanation is that attractive/repulsive interactions of the side chain at position 211 with the positive end of the helix 8′ dipole moment could alter the energies of the native and intermediate conformations relative to that of the transition state. A third explanation involves attractive/repulsive interactions of Glu/Arg-211 with arginine(s) at positions 179 and/or 188, located in a nearby mobile loop region. These latter possibilities focus attention on the electrostatic interactions between Glu/Arg-211 and the native and intermediate conformations, not the transition state.

The first explanation is supported by the results of an experiment in which the folding kinetics studies for the G211E mutant protein were repeated in the presence of 0.5 M NaCl. It was found that while the unfolding relaxation times of the wild-type protein increased slightly in salt (1.5-fold), those for the G211E were unaffected by salt (data not shown). The absence of a screening effect by added salt suggests that the electrostatic interactions are inaccessible to solvent, consistent with the observed environment between positions 211 and 49 in the native conformation (hydrophobic interior of the barrel). The latter two possibilities seem less likely since these interactions would occur near the surface of the protein and would be expected to be perturbed by salt. The putative role of Glu-49 can be tested by replacement with a neutral side chain; these experiments are in progress.

The stable folding intermediate for the α subunit is thought to have a folded amino folding unit (residues 1–188) and an unfolded carboxy folding unit (residues 189–268) (Higgins et al., 1979; Crisanti & Matthews, 1981; Miles et al., 1982; Beasty & Matthews, 1985). In terms of this model, one would expect that the I $\leftrightarrow$ U transition for replacements in the carboxy folding unit at position 211 would be coincident to that for the wild-type α subunit. The observation that these transitions are not coincident for the G211W, G211E, and G211D mutants at 25 °C (Figures 1 and 2 and Table I) shows that the folding units are interacting with each other. In a previous study, Hurle (1987) found that the I $\leftrightarrow$ U transition curves for wild-type protein and the G211E mutant are coincident in the presence of 0.5 M NaCl. It was proposed that long-range electrostatic interactions are responsible.

Further information on this issue was obtained in the present study, where at 4 °C the I $\leftrightarrow$ U transitions for the wild-type, G211E, and G211R proteins are coincident (Figure 3). The different effects of these replacements on the I $\leftrightarrow$ U transition at 4 and 25 °C may be understood in terms of the concept of cold denaturation (Brandts, 1964; Privalov et al., 1986; Chen & Schellman, 1988; Privalov & Gill, 1988). If the sequence from 189–268 in the equilibrium intermediate has residual structure at 25 °C, position 211 could be sufficiently close to the amino folding unit that electrostatic or steric interactions are possible. Lowering the temperature to 4 °C may disrupt this residual structure by the cold denaturation process and, thereby, eliminate these interactions. This explanation presumes that the residual structure involves hydrophobic interactions which are especially susceptible to cold denaturation. A hydrophobic cluster could have escaped detection in the previous studies.

The proposal that the α subunit of tryptophan synthase folds in stages and that the folding units correspond to recognizable elements of secondary structure must be subjected to further tests. It is not clear at this point whether the actual folding units correspond precisely to the proteolytic fragments or whether the boundaries are upstream or downstream from Arg-188. As noted previously, the segment from 178–191 was not located in the X-ray crystal structure (Hyde et al., 1988), suggesting significant mobility and a potential role in the division of the two folding units. It also appears that the carboxy folding unit may have some residual structure at intermediate urea concentrations. Nevertheless, the data available strongly support a multistate folding model and independent folding of the first six and last two β strands in the protein.

It is interesting to speculate on whether the 6 + 2 folding pattern observed for the α subunit also is applicable to other proteins which have the β barrel motif [14 such structures have now been elucidated (Chothia, 1988)]. The successful transposition of the last two strands of the phosphoribosyl-anthranilate isomerase barrel from positions 7 and 8 to positions 1 and 2 by genetic engineering (Luger et al., 1989) may be another manifestation of the same 6 + 2 folding mechanism. It would be interesting to learn if other strand permutations, e.g., 5 + 3, could also result in folded α/β barrels.

Acknowledgments

We thank Drs. Charles Yanofsky and Brian Nichols for the gifts of the mutant strains and wild-type plasmid, Dr. Edward Garvey for a critical review of the manuscript, and Gail Feldman for assistance in manuscript preparation.

References

Alber, T., Dao-pin, S., Wilson, K., Wozniak, J. A., Cook, S. P., & Matthews, B. W. (1987) *Nature 330*, 41.

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.

Brandts, J. (1964) *J. Am. Chem. Soc. 86*, 4291.

Chen, B., & Schellman, J. A. (1989) *Biochemistry 28*, 685.

Chothia, C. (1988) *Nature 333*, 598.

Crisanti, M. M., & Matthews, C. R. (1981) *Biochemistry 20*, 2700.

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

Herskovits, T. T. (1967) *Methods Enzymol. 11*, 748.

Hibler, D. W., Stolowich, N. J., Reynolds, M. A., Gerlt, J. A., Welde, J. A., & Bolton, P. H. (1987) *Biochemistry 26*, 6278.
Higgins, W., Fairwell, T., & Miles, E. W. (1979) *Biochemistry 18*, 4827.
Hurle, M. R. (1987) Ph.D. Thesis, The Pennsylvania State University.
Hurle, M. R., & Matthews, C. R. (1987) *Biochim. Biophys. Acta 913*, 179.
Hurle, M. R., Michelotti, G. A., Crisanti, M. M., & Matthews, C. R. (1987) *Proteins: Struct., Funct., Genet. 2*, 54.
Hyde, C. C., Ahmed, S. A., Padlan, E. A., Miles, E. W., & Davies, D. R. (1988) *J. Biol. Chem. 263*, 17857.
Kirschner, K., Wiskocil, R. L., Foehn, M., & Rezeau, L. (1975) *Eur. J. Biochem. 60*, 513.
Luger, K., Hommel, U., Herold, Hofsteenge, J., & Kirschner, K. 1989) *Science 243*, 206.
Marqusee, S., & Baldwin, R. L. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 8998.
Matthews, C. R. (1987) *Methods Enzymol. 154*, 498.
Matthews, C. R., & Crisanti, M. M. (1981) *Biochemistry 20*, 784.
Matthews, C. R., Cristanti, M. M., Manz, J. T., & Gepner, G. L. (1983) *Biochemistry 22*, 1445.
Miles, E. W., Yutani, K., & Ogasahara, K. (1982) *Biochemistry 21*, 2586.
Murgola, E. J., & Yanofsky, C. (1974) *J. Mol. Biol. 86*, 775.
Oas, T. G., & Kim, P. S. (1988) *Nature 336*, 42.
Perry, K. M., Onuffer, J. J., Touchette, N. A., Herndon, C. S., Gittelman, M. S., Matthews, C. R., Chen, J. T., Mayer, R. J., Taira, K., Benkovic, S. J., Howell, E. E., & Kraut, J. (1987) *Biochemistry 26*, 2674.
Privalov, P. L. (1986) *J. Mol. Biol. 190*, 487.
Privalov, P. L., & Gill, S. (1988) *Adv. Protein Chem. 39*, 191.
Roder, H., Elove, G. A., & Englander, S. W. (1988) *Nature 335*, 700.
Schellman, J. A. (1978) *Biopolymers 17*, 1305.
Udgaonkar, J. B., & Baldwin, R. L. 1988) *Nature 335*, 694.
Yanofsky, C., Berger, H., & Brammar, W. J. (1969) *Proc. Int. Congr. Genet., 12th 3*, 155.

Solution Structural Characterization of Cyanometmyoglobin: Resonance Assignment of Heme Cavity Residues by Two-Dimensional NMR†

S. Donald Emerson and Gerd N. La Mar*
Department of Chemistry, University of California, Davis, California 95616
Received June 6, 1989; Revised Manuscript Received September 11, 1989

ABSTRACT: Steady-state nuclear Overhauser effects (NOE), two-dimensional (2D) nuclear Overhauser effect spectroscopy (NOESY), and 2D spin correlation spectroscopy (COSY) have been applied to the fully paramagnetic low-spin, cyanide-ligated complex of sperm whale ferric myoglobin to assign the majority of the heme pocket side-chain proton signals and the remainder of the heme signals. It is shown that the 2D NOESY map reveals essentially all dipolar connectivities observed in ordinary 1D NOE experiments and expected on the basis of crystal coordinates, albeit often more weakly than in a diamagnetic analogue. For extremely broad (~600-Hz) and rapidly relaxing ($T_1 \sim 3$ ms) signals which show no NOESY peaks, we demonstrate that conventional steady-state NOEs obtained under very rapid pulsing conditions still allow detection of the critical dipolar connectivities that allow unambiguous assignments. The COSY map was found to be generally less useful for the hyperfine-shifted residues, with cross peaks detected only for protons >6 Å from the iron. Nevertheless, numerous critical COSY cross peaks between strongly hyperfine-shifted peaks were resolved and assigned. In all, 95% (53 of 56 signals) of the total proton sets within ~7.5 Å of the iron, the region experiencing the strongest hyperfine shifts and paramagnetic relaxation, are now unambiguously assigned. Hence it is clear that the 2D methods can be profitably applied to paramagnetic proteins. The scope and limitations of such application are discussed. The resulting hyperfine shift pattern for the heme confirmed expectations based on model compounds. In contrast, while exhibiting fortuitous ^{1}H NMR spectral similarities, a major discrepancy was uncovered between the hyperfine shift pattern of the axially bound (F8 histidyl) imidazole in the protein and that of the imidazole in a relevant model compound [Chacko, V. P., & La Mar, G. N. (1982) *J. Am. Chem. Soc. 104*, 7002–7007], providing direct evidence for a protein-based deformation of axial bonding in the protein.

Myoglobin (Mb)[1] is a small (~16-kDa) hemoprotein whose function in tissue is to reversibly bind molecular oxygen, which it carries out with remarkable variability with genetic origin of the polypeptide chain. Mb can exist in both the reduced iron(II) state as well as oxidized iron(III) state, and both the diamagnetic and paramagnetic derivatives have been the subject of intense physicochemical studies to elucidate the mechanism of control of ligand binding (Antonini & Brunori, 1971). The wide study of hemoproteins in general is due, in large part, to the ability to compare properties of not only diverse genetic variants but also the intact protein with the extracted prosthetic group (i.e., model compounds), thereby

†This research has been supported by a grant from the National Institutes of Health (HL16087).

[1] Abbreviations: Mb, myoglobin; metMbCN, cyanide-ligated ferric myoglobin; NOE, nuclear Overhauser effect; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; NOESY, two-dimensional nuclear Overhauser effect spectroscopy; COSY, two-dimensional correlation spectroscopy; DQF-COSY, double-quantum-filtered COSY.

0006-2960/90/0429-1545$02.50/0