

How Ala→Gly Mutations in Different Helices Affect the Stability of the Apomyoglobin Molten Globule[†]

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ABSTRACT: The apomyoglobin molten globule has a complex, partly folded structure with a folded A[B]-GH subdomain; the factors determining its stability are not yet known in detail. Ala→Gly mutations, made at solvent-exposed positions, are used to probe the role of helix propensity of individual helices in stabilizing the molten globule. Molten globule stability is measured by reversible urea unfolding, monitored both by circular dichroism and by tryptophan fluorescence. Two-state unfolding is tested by superposition of these two unfolding curves, and stability data are reported only for variants which satisfy the superposition test. Results for sites Q8 in the A helix and E109 in the G helix confirm that the helix propensities of the A and G helices both strongly affect molten globule stability, in contrast to results for the G65A/G73A double mutant which show that changing the helix propensity of the E-helix sequence has no significant stabilizing effect. Changing the helix propensity of the B-helix sequence with the G23A/G25A double mutant affects molten globule stability to an intermediate extent, confirming an earlier report that this mutant has increased stability. These results are consistent with the bipartite structure for the molten globule in which the A, G, and H helices are stably folded, while the long E helix is unfolded and the B helix has intermediate stability. Some differences are found in the shapes of the unfolding curves of different mutants even though they satisfy the superposition test for two-state unfolding, and possible explanations are discussed.

It has been controversial whether molten globules (MGs)¹ are stable, partly native, on-pathway intermediates in a hierarchic folding process, as suggested by some lines of evidence (1, 2), or whether they are off-pathway species caught in kinetic traps during folding, as suggested by some simulations of the folding process (3, 4; see, however, 5). Although the issue remains controversial, recent progress in predicting the folding rates of small, two-state folding proteins supports the hierarchic model in which folding intermediates with partial nativelike structure are formed during the folding process (6). If the corresponding kinetic intermediates are on-pathway (7), then characterizing the structures and folding behavior of the stable molten globules found at acid pH for some proteins (apoMb, cyt *c*, α-LA, RNase H) can give information about properties of short-lived transient intermediates provided NMR H/D exchange has demonstrated that the kinetic and equilibrium intermediates have closely similar structures, as has been found for apoMb (8) and for RNase H (9).

An earlier study of Gly and Pro substitutions in apoMb suggested that the helix propensities of the A- and G-helix sequences strongly affect the stability of the pH 4 MG (10). This tentative conclusion was strengthened by the finding that trifluoroethanol (TFE)–water mixtures stabilize the pH 4 MG, just as TFE addition stabilizes peptide helices although it destabilizes most native proteins (11). To investigate the role of helix propensity in stabilizing different helices of the apoMb MG, we make the same mutation, Ala→Gly, at solvent-exposed sites near the centers of various helix sequences, and then compare the changes in MG stability. The Ala→Gly mutation has two important advantages for analyzing the effect of helix propensity on MG stability: (1) both Ala and Gly do not make any specific side chain interactions which could affect MG stability, and (2) the Ala→Gly mutation produces a particularly large change in helix propensity in peptide helix systems (12). When Ala→Gly mutations are made in the helices of a native protein, account must be taken of the change in buried nonpolar surface area caused by the mutation (13). The helices of a molten globule are thought to be better exposed to solvent than those of native proteins, but only sites which are reasonably solvent-exposed in native apoMb are used here.

Mutation-induced changes in stability of the apoMb MG can be measured with confidence only in conditions that give two-state unfolding. Evidence that urea unfolding is two-state, to a first approximation, comes both from the unfolding/refolding kinetics (14, 15) and from a superposition test (10, 16). In the superposition test, far-UV CD and tryptophan fluorescence (FL) are used as probes of secondary and

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¹ Abbreviations: MG, molten globule; apoMb, apomyoglobin; CD, circular dichroism; FL, fluorescence; TFE, trifluoroethanol; α-LA, α-lactalbumin; RNase H, ribonuclease H; cyt *c*, cytochrome *c*; SNase, staphylococcal nuclease; H/D, hydrogen–deuterium; GdmCl, guanidinium chloride; *C*_m, denaturant molarity at midpoint of unfolding transition; *m*, slope of curve relating unfolding free energy to denaturant molarity; *m*^{*}, apparent value of *m*.

tertiary structure, respectively, to provide unfolding curves measured by two distinctly different properties. Although this type of superposition test has been used successfully with native proteins, its use with molten globules is still preliminary: possible problems are considered under Discussion. Experience has shown that the two-state unfolding of the apoMb MG found for wild type is easily lost by destabilizing mutations (10, 17). Stabilizing anions can restore cooperativity as well as stability in the folding reactions of less stable mutants (10), and 20 mM Na_2SO_4 is used here both to stabilize and to promote two-state folding.

EXPERIMENTAL PROCEDURES

Construction of Mutants and Protein Preparation. A synthetic gene for sperm whale myoglobin, originally the gift of Dr. S. G. Sligar and modified slightly by Hughson et al. (18), was inserted in the pMbT7 plasmid by Loh et al. (19), and myoglobin was expressed and purified as described by Loh et al. (19) and Luo et al. (10). Heme was removed by the methyl ethyl ketone method, and protein concentration was determined by the absorbance in 6 M GdmCl, as described by Luo et al. (10). Mutants were made by the polymerase chain reaction, and DNA sequences were verified by standard dideoxy methods in the PAN facility of Stanford University. Certain mutants (H12A and H12G in the A helix, D27A and D27G in the B helix, and K140G in the H helix) failed to give sigmoidal unfolding curves, and no stability data are reported for these mutants.

CD and Fluorescence Measurements. CD results were obtained with an Aviv 62DS circular dichroism spectropolarimeter at 222 nm and at 4 °C, using a 1 cm \times 1 cm quartz cell, with 2 μM protein concentration. The data were averaged over 300 s acquisition time. Reversibility of unfolding was tested as described by Luo et al. (10), and all unfolding data reported here pertain to reversible unfolding. Fluorescence results were acquired with an SLM-Aminco-Bowman Series 2 spectrofluorometer, using a 1 cm light path and 1 μM protein concentration, at 4 °C. Excitation was at 280 nm, with a band-pass of 4 nm, and emission was monitored at 330 nm, with a band-pass of 16 nm.

Data Analysis. Curve fitting was performed by using KaleidaGraph software for the Macintosh. Urea unfolding curves were fitted using the procedure of Santoro and Bolen (20), in which the native and unfolded baselines are represented by straight lines, the unfolding curve is fitted to the two-state model, and the baselines are determined using data inside as well as outside the transition zone. The baselines were first determined for the Q8G mutant (which has a particularly sharp unfolding curve), and the Q8G baseline slopes were then used for all variants, to reduce the effect of scatter in the baselines. Before superimposing the normalized unfolding curves monitored by CD and FL, the unfolding curves given by the raw data were first checked for superimposability in the transition zone, as illustrated in Figures 2A and 3A.

RESULTS

Superposition Test for Two-State Folding. Before making the superposition test, each unfolding curve (given by the raw data; see Figure 2A) is checked to see if it has a cooperative (sigmoid) shape, because the baseline subtraction

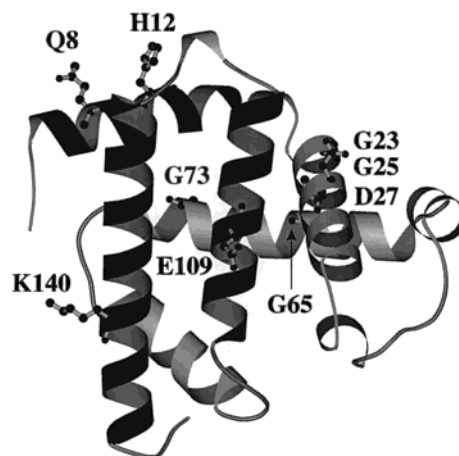


FIGURE 1: Molscript diagram (42) of the structure of sperm whale myoglobin, showing the locations of the Ala→Gly mutations.

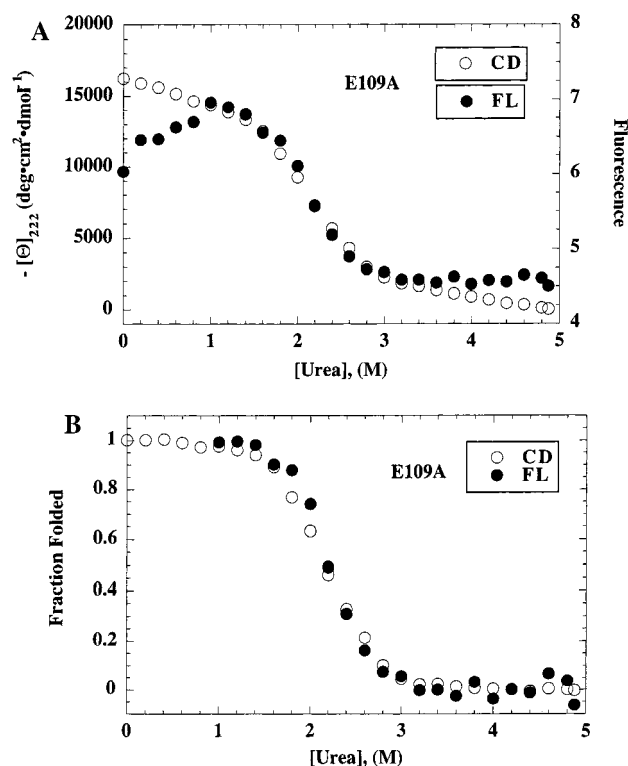


FIGURE 2: (A) Urea unfolding of the E109A (G helix) mutant in 20 mM Na_2SO_4 and 2 mM sodium acetate, pH 4.2, monitored by circular dichroism and by fluorescence. The raw data are shown to illustrate problems in fitting the baselines (see text) and to check whether the unfolding transitions indicated by the raw data are superimposable when monitored by CD and FL. The unfolding curves are reversible. (B) The two unfolding curves of (A) are normalized by fitting each one separately to the two-state model, using the procedure of Santoro and Bolen (20), and then superimposed.

procedure used in fitting the curve to the two-state equation tends to induce a sigmoid shape and some mutants have been found to cause a complete loss of sigmoidal unfolding, for unexplained reasons. If the unfolding curve given by the raw data is not sigmoidal, the mutant is not used for further study. In the Santoro–Bolen (20) procedure for fitting data to the two-state equation, both the native and the unfolded baselines are represented by straight lines, and data taken inside as well as outside the unfolding transition zone are used to determine the native and unfolded baselines. For the apoMb

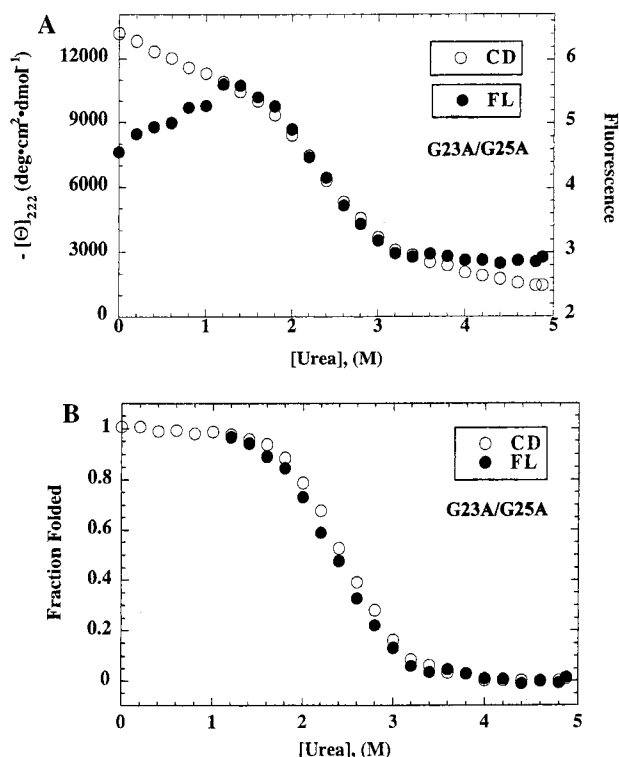


FIGURE 3: Urea unfolding curves of the G23A/G25A (B helix) double mutant are shown for comparison with the curves for a different mutant in Figure 2. (A) Raw data; (B) normalized unfolding curves. See legend to Figure 2 for the unfolding conditions.

MG, the FL data below 1 M urea are not used to obtain the native baseline because there are two forms of the folded apoMb MG (Ia, Ib), and the Ib form is converted to Ia between 0 and 1 M urea, giving an increase in fluorescence which is not part of the Ia folding reaction (21). Instead, it is necessary to obtain the native FL baseline by the Santoro-Bolen procedure from data inside the unfolding transition zone. In contrast, the native CD baseline can be obtained from data taken below 1 M urea because the Ib and Ia forms have similar specific CD values (Jamin and Baldwin, unpublished data). Both the native and unfolded CD baselines slope downward with increasing urea concentration, indicating that some helix unfolding occurs outside the transition zone (cf. 22). The backbone structure of the acid-unfolded form of apoMb at pH 2 has been characterized by chemical shift data (23) and found to contain residual helix; thus, it is not surprising that some urea-induced helix unfolding occurs in the post-transition zone at pH 4.2. In contrast, the FL baseline in the post-transition zone is nearly horizontal. The FL change on unfolding Ia results from partial burial of the two Trp residues in the molten globule (21). Figure 2B shows that, after fitting both the CD and FL curves to the two-state equation, the two normalized unfolding curves of E109A monitored by CD and FL are superimposable within error. A further example of using the superposition test is given in Figure 3 for a second mutant, G23A/G25A. The unfolding curves given by the raw data for unfolding monitored by CD and FL are compared in Figure 3A, and the superposition test made with the two normalized curves is shown in Figure 3B. The results for the two variants in Figures 2 and 3 are similar to each other and typical of the other variants whose stabilities are reported here.

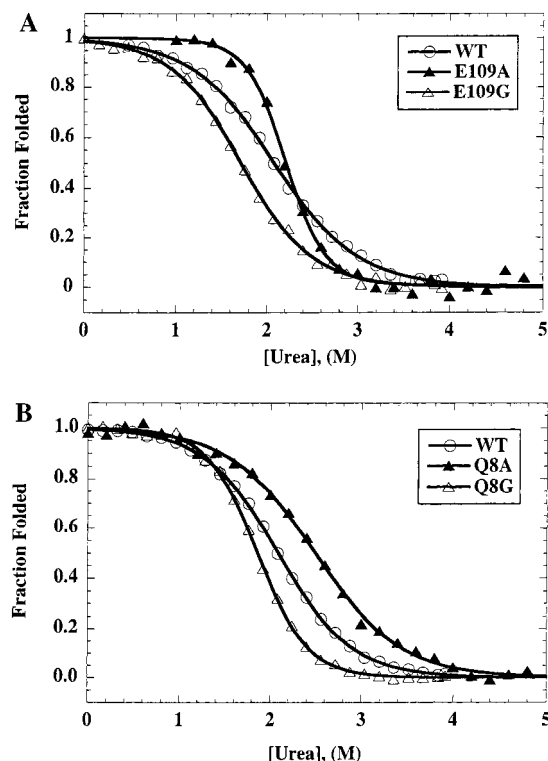


FIGURE 4: (A) Normalized unfolding curves of E109A and E109G (G helix), monitored by FL, are compared with each other and with wild type. (B) The normalized unfolding curves of Q8A and Q8G (A helix), monitored by CD, are compared with each other and with wild type.

Changes in Stability Produced by Mutation. Changes in stability produced by Ala→Gly mutations are reported here only when both the Ala and Gly variants show two-state unfolding by the superposition test, as illustrated in Figures 2 and 3. In most cases, both the Ala and Gly variants are made by mutation, but for G23A/G25A and G65A/G73A, the wild-type protein (which has two glycine residues in both the B and E helices) serves as the Gly variant. Figures 4 and 5 show representative changes in stability produced by Ala→Gly mutations.

Surprisingly, the sharpness of the unfolding curve is obviously different for some variants, and this observation casts doubt on the assumption of two-state folding (see Discussion). The unfolding curve for E109A is much sharper than those of wild type and E109G (Figure 4A). Likewise, the unfolding curve for Q8G is sharper than those of Q8A and wild type (Figure 4B). In the Discussion, we consider possible explanations for this phenomenon and propose that, in studies of molten globules showing this behavior, the change in stability produced by a mutation can be monitored by the difference between C_m -values of the mutant and wild type, where C_m is the urea molarity at the midpoint of the unfolding transition curve. The C_m -values of all mutants and wild type are given in Table 1, as measured both by CD and by FL-monitored unfolding, together with values of the minimum free energy differences estimated from the C_m -values and the largest apparent m -value (see Discussion).

A large change in stability produced by a single Ala→Gly mutation is observed at the A-helix site, Q8, and also at the G-helix site, E109. There is a smaller average change per Ala→Gly mutation in the B-helix double mutant G23A/G25A (Figure 5A). The stabilizing effect of the two

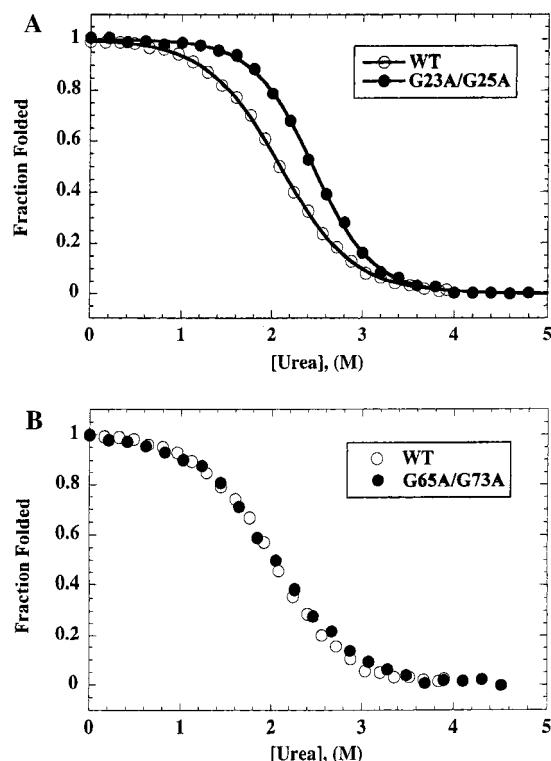


FIGURE 5: (A) Normalized unfolding curve of the double mutant G23A/G25A (B helix), monitored by CD, is compared with wild type. (B) The normalized unfolding curve of the double mutant G65A/G73A (E helix), monitored by CD, is compared with wild type.

Table 1: Stability^a to Urea Unfolding of Apomyoglobin Mutants

apoMb	C_m	$\Delta\Delta G^b$
WT-FL	2.08	0.00
WT-CD	2.09	0.00
Q8A-FL	2.47	+1.07
Q8A-CD	2.50	+0.89
Q8G-FL	1.85	-0.63
Q8G-CD	1.86	-0.50
E109A-FL	2.21	+0.36
E109A-CD	2.17	+0.17
E109G-FL	1.73	-0.96
E109G-CD	1.68	-0.89
K140A-FL	2.30	+0.60
K140A-CD	2.25	+0.35
G23A/G25A-FL	2.35	+0.74
G23A/G25A-CD	2.45	+0.78
G65A/G73A-FL	2.03	-0.14
G65A/G73A-CD	2.04	-0.11

^a Stability is monitored by C_m , the urea molarity at the transition midpoint. The conditions are 2 mM NaOAc, 20 mM Na₂SO₄ at pH 4.2, 4 °C. FL: Tryptophan fluorescence emission at 330 nm (with excitation at 280 nm). CD: circular dichroism at 222 nm. ^b $\Delta\Delta G = m^*\Delta C_m = m^*(C_{m,mutant} - C_{m,WT})$ (the difference in apparent free energy of unfolding), where m^* (the largest apparent m -value in this set of variants) is 2.74 kcal mol⁻¹ M⁻¹ for FL-monitored unfolding (taken from E109A), and 2.17 kcal mol⁻¹ M⁻¹ for CD-monitored unfolding (also taken from E109A). The units of $\Delta\Delta G$ in Table 2 are kcal mol⁻¹.

Gly→Ala mutations in the B helix was reported earlier by Kiefhaber and Baldwin (24), on the basis of a different procedure for measuring MG stability. On the other hand, there is no change in the stability of the MG when two G→A mutations are made in the E-helix sequence (Figure 5B),

Table 2: Comparative Stability^a in Three Sets of Anion Conditions of Wild Type and G23A/G25A Mutant

apoMb	30 mM NaCl	20 mM Na ₂ SO ₄	50 mM NaClO ₄
WT-FL	1.42	2.08	3.14
WT-CD	1.45	2.09	3.24
G23A/G25A-FL	1.68	2.35	3.48
G23A/G25A-CD	1.73	2.45	3.52

^a Stability is monitored by C_m , the urea molarity at the transition midpoint. The conditions are 2 mM NaOAc with different anions, as indicated, at pH 4.2, 4 °C. FL: Tryptophan fluorescence emission at 330 nm (with excitation at 280 nm). CD: circular dichroism at 222 nm.

which is consistent with the conclusion that the E helix is unfolded in the molten globule.

Results with Different Stabilizing Anions. Goto et al. (25) demonstrated that various anions induce the formation of molten globules from the acid-unfolded forms of cyt *c* and apoMb at pH 2. Moreover, 20 mM sodium trichloroacetate strongly stabilizes the pH 4 MG of apoMb (19): the C_m for urea-induced unfolding increases remarkably from 1.5 M in the absence of trichloroacetate to 3.9 M in its presence. Three different anion conditions (30 mM NaCl, 20 mM Na₂SO₄, and 50 mM NaClO₄) were used by Luo et al. (10) to analyze the effects of proline and glycine substitutions on the cooperativity of urea-induced unfolding. The same order of effectiveness of the three anions ($Cl^- < SO_4^{2-} < ClO_4^-$) in stabilizing the MG was found with wild type and with the mutants: 20 mM Na₂SO₄ was chosen as a standard condition because ClO_4^- causes mild photobleaching in fluorescence experiments. Table 2, which compares the stability of wild type and of the G23A/G25A double mutant in these three anion conditions, shows the same pattern of anion stabilization found earlier (10).

DISCUSSION

Use of Ala→Gly Mutations To Probe the Effect of Helix Propensity. The Ala→Gly mutation is a standard tool for probing the effect of helix propensity on stability, in the study of both native proteins (13) and folding transition states (26, 27), but it has not yet been used in any systematic study of molten globules. In peptide helices, the substitution of proline produces a much larger decrease in helix propensity than glycine (28), and Schulman and Kim (29) found the surprising result that proline substitutions in helices are readily tolerated in an α -LA molten globule construct, suggesting that its structure is much more flexible than that of a native protein and its unfolding transition (which was not analyzed in this study) should be relatively noncooperative. To find out if the apoMb molten globule responds in a similar way to proline substitutions, a comparative study of substituting Gly versus Pro was made at two sites in apoMb (Gln 8 and E109), and the urea unfolding curves were analyzed (10). Of the two proline mutants, only the E109P mutant could be expressed in an amount sufficient to study, and application of the superposition test to its unfolding transition showed a drastic loss of two-state unfolding behavior. In contrast, both the Q8G and E109G mutants retained nearly two-state unfolding if 20 mM Na₂SO₄ or 50 mM NaClO₄ was present to give increased MG stability (10), while both glycine mutants showed substantial losses of MG stability compared to wild type, indicating that helix propensity is important

for MG stability in apoMb. Our present results confirm this suggestion and show in addition that the effect of an Ala→Gly mutation in a MG helix depends on whether the helix is stably folded in the molten globule: Ala→Gly mutations have big effects on MG stability when made in the A or G helices (Table 1), but not in the E helix (which is unfolded in the MG) (23, 31), and only an intermediate effect in the partly stable B helix. Our observation that helix propensity is important for stability in the apoMb molten globule [see also (10, 11)] is parallel to the recent finding of Lorch et al. (30) that mutations affecting β -sheet propensity are important for the stability of a transient folding intermediate of CD2.d1, a small β -sheet protein.

Two-State Unfolding of Molten Globules. Expectations concerning the likelihood of two-state unfolding are influenced by current models for molten globules, which are still quite preliminary. The side chains of the apoMb MG are not locked firmly in place, unlike those of native apoMb, because the proton NMR spectrum of the pH 4 MG shows little chemical shift dispersion of side chain protons (19, 23) and the protection factors of peptide NH protons in the A, G, and H helices are quite low (10–100) compared to those of native apoMb (10^5) (31). Thus, the side chains of the pH 4 MG are fairly mobile, suggesting that the helices are not likely to interact with each other specifically. Nevertheless, there is evidence for specific interactions among the A, G, and H helices of apoMb. Helical structure in the A, G, and H helices is stable compared to that found in peptides with the sequences of these helices, and mutual stabilization of the A, G, and H helices in the molten globule is difficult to explain by any mechanism except specific interaction, because the A, G, and H helices come from opposite ends of the polypeptide chain (31). Moreover, mutational experiments demonstrate that there are natively like side chain interactions between helices in the molten globules both of cyt *c* (32–34) and of apoMb (10, 16, 17). Furthermore, both molten globules show highly cooperative unfolding transitions, as expected if the helices interact specifically with each other.

The unfolding transition of human α -LA MG at pH 2 monitored by NMR chemical shifts shows, however, that its unfolding transition is not two-state (35). The apparent contradiction between non-two-state folding of the α -LA MG versus two-state folding of the apoMb and cyt *c* MGs may be reconciled by the effect of the disulfide bonds present in α -LA but absent in apoMb and cyt *c*, because the 28–111 S–S bond is known to weaken the cooperativity of folding of an α -LA MG (36; see also 37).

A direct test for cooperative unfolding is that the entire unfolding curve should shift down in response to a destabilizing point mutation: this test is satisfied here for the Ala→Gly mutations in the A, B, and G helices. The downward shift in stability is observed when unfolding is monitored either with a probe of secondary structure (far-UV CD) or with a probe of tertiary structure (Trp fluorescence), and the two unfolding curves are coincident. Although NMR monitoring of the unfolding transition of α -LA (35) provides a more sensitive test for two-state unfolding, the superposition test used here is sufficient to demonstrate that unfolding is cooperative and that a destabilizing mutation made in one helix affects the stability of the entire molten globule.

Changes in Shape of the Unfolding Curve. As noted under Results, there are evident differences in the shape of the unfolding curve between wild type and some variants (E109A, Figure 4A; Q8G, Figure 4B). For two-state unfolding, the shape of the unfolding curve is determined by the *m*-value (see below). Provided that the *m*-value depends only on the difference between the number of sites for interaction with the denaturant in the native and denatured forms, as suggested by the analysis of Schellman (38), point mutants are not expected to change the *m*-value significantly if unfolding is truly two-state. Consequently, differences in the apparent *m*-value among the variants raise the question of whether unfolding of the molten globule is truly two-state.

There are various possible explanations for mutation-induced changes in the apparent *m*-values. Extensive experiments by Shortle and Meeker on the unfolding of native staphylococcal nuclease (SNase) reveal large differences in the apparent *m*-values of point mutants (39), with results indicating that the residual structure in the denatured protein is responsible for the mutation-induced differences. SNase is not very stable, and its unfolding curves are measured at low denaturant concentrations where its residual structure is not unfolded, so that the differences in the apparent *m*-values among some SNase mutants are probably the result of using the two-state model to analyze three-state unfolding, as discussed by Pace and Shaw (40).

A different explanation, incomplete folding of the molten globule, is plausible here as the major cause of variation in apparent *m*-values among the variants. Although most native proteins have unique structures and incomplete folding to the native form is rarely observed [an exception is native apoMb, whose F helix is not folded (23)], this is not necessarily true of molten globules. Direct kinetic evidence for incomplete folding of a transient molten globule intermediate has been given (41) for some H-helix mutants of apoMb.

Incomplete folding to the equilibrium molten globule form, resulting in a heterogeneous distribution of forms with differing stabilities, is the likely explanation for the pH 4.2 unfolding curves of the proline mutant studied by Luo et al. (10). The unfolding curve of E109P monitored by CD in 20 mM Na₂SO₄ is nearly linear, suggesting a broad distribution of stabilities among partly folded molecules, while the FL-monitored unfolding curve is sigmoidal but shows a drastic decrease compared to wild type in the amplitude of the FL change upon unfolding, suggesting that only a small fraction of the mutant MG molecules contain tryptophan residues that are partially buried in the molten globule interior. [For evidence that tryptophan burial accounts for the fluorescence change on molten globule unfolding, see (21).] Similar, but less severe, changes in unfolding behavior were observed for glycine mutants in certain conditions (10). Kay et al. (17) also obtained similar results for apoMb mutants in which substitution of a hydrophobic side chain causes the loss of two-state unfolding.

Although the differences in the apparent *m*-values reported here represent a small effect compared to the gross effects discussed in these earlier papers, the underlying cause may nevertheless be the same: incomplete folding to the molten globule form, leading to a distribution of stabilities and broadening of the unfolding transition curve.

Measurement of Average Stability in a Heterogeneous System. The discussion above of incomplete folding to the molten globule form focuses interest on the question of how best to measure average stability for a system containing a distribution of incompletely folded forms with differing stabilities. Consider a system of closely related folded molecules which differ among themselves in stability to unfolding. Suppose that the linear extrapolation model applies to each species i , with

$$\Delta G_i = \Delta G_i(0) - mC \quad (1)$$

where C is the denaturant molarity and ΔG is the Gibbs free energy of unfolding at concentration C , and all species have the same value of m but differing values of ΔG at $C = 0$. At the midpoint of the unfolding transition curve of each species, $\Delta G_i = 0$ and

$$mC_{mi} = \Delta G_i(0) \quad (2)$$

The fractional contribution, α_i , that each species makes to the mean value, $\langle C_m \rangle$

$$\langle C_m \rangle = \sum \alpha_i C_{mi} \quad (3)$$

is determined by its weight fraction w_i and by the property Y used to measure the unfolding transition curve:

$$\Delta Y = Y_N - Y_U = \sum w_i Y_{Ni} - \sum w_i Y_{Ui} \quad (4)$$

where $\Delta Y_i = w_i(Y_{Ni} - Y_{Ui})$ and $\alpha_i = \Delta Y_i / \Delta Y$, with N = native, U = unfolded, and baseline corrections of the kind discussed by Santoro and Bolen (20) must be applied in determining Y_N and Y_U .

If all species have the same values of Y_{Ni} and Y_{Ui} , which might be the case here, then the mean value $\langle C_m \rangle$ is a weight-average measure of stability and multiplying it by m gives the weight-average free energy of unfolding. Provided the difference in stability between wild type and mutant is large compared to the standard deviation of the distribution of stabilities for each of them, then m times the difference between their $\langle C_m \rangle$ values gives a well-defined difference between their free energies of unfolding. For a set of variants whose unfolding curves satisfy the superposition test but whose apparent m -values differ for the reason discussed here, the highest apparent m -value gives the best approximation to the true m -value. However, in the case of the FL-monitored unfolding curves of the proline mutant studied by Luo et al. (10) and the nonpolar side chain mutants of Kay et al. (17), only a small fraction of the protein apparently is folded well enough to contribute to the fluorescence change on unfolding, and then the mean value $\langle C_m \rangle$ refers only to the average stability of these exceptional species.

CONCLUSIONS

Ala→Gly mutations confirm that helix propensity of individual helices is a major determinant of stability for the pH 4 molten globule of apomyoglobin, provided that the known bipartite helical structure of the molten globule, found by measurement of protection factors for H/D exchange and confirmed by chemical shift data, is taken into account. Changing helix propensity in two stably folded helices (A, G) strongly affects molten globule stability, while a change

in the unfolded E helix has little effect and a change in the partly folded B helix has an intermediate effect. Superposition of the urea unfolding curves measured by circular dichroism and Trp fluorescence confirms that unfolding of the pH 4 molten globule is two-state to a first approximation. Differences among the variants in apparent m -values suggest, however, that subtle deviations from two-state unfolding are present. These deviations are tentatively attributed to variations in the extent of folding of the molten globule, and a procedure is given for taking this effect into account when measuring stability.

REFERENCES

- Baldwin, R. L., and Rose, G. D. (1999) *Trends Biochem. Sci.* 24, 26–33.
- Baldwin, R. L., and Rose, G. D. (1999) *Trends Biochem. Sci.* 24, 77–83.
- Chan, H. S., and Dill, K. A. (1998) *Proteins: Struct., Funct., Genet.* 30, 2–33.
- Wolynes, P. G., Onuchic, J. N., and Thirumalai, D. (1995) *Science* 267, 1619–1620.
- Pande, V. S., and Rokhsar, D. S. (1999) *Proc. Natl. Acad. Sci. U.S.A.* 96, 1273–1278.
- Baker, D. (2000) *Nature* 405, 39–42.
- Capaldi, A. P., Shastri, M. C. R., Kleanthous, C., Roder, H., and Radford, S. E. (2001) *Nat. Struct. Biol.* 8, 68–72.
- Jennings, P. A., and Wright, P. E. (1993) *Science* 262, 892–896.
- Dabora, J. M., Pelton, J. G., and Marqusee, S. (1996) *Biochemistry* 35, 11951–11958.
- Luo, Y., Kay, M. S., and Baldwin, R. L. (1997) *Nat. Struct. Biol.* 4, 925–930.
- Luo, Y., and Baldwin, R. L. (1998) *J. Mol. Biol.* 279, 49–57.
- Chakrabarty, A., Schellman, J. A., and Baldwin, R. L. (1991) *Nature* 351, 586–588.
- Serrano, L., Neira, J.-L., Sancho, J., and Fersht, A. R. (1992) *Nature* 356, 453–455.
- Jamin, M., and Baldwin, R. L. (1996) *Nat. Struct. Biol.* 3, 613–618.
- Jamin, M., Yeh, S.-R., Rousseau, D. L., and Baldwin, R. L. (1999) *J. Mol. Biol.* 292, 731–740.
- Kay, M. S., and Baldwin, R. L. (1996) *Nat. Struct. Biol.* 3, 439–445.
- Kay, M. S., Ramos, C. H. I., and Baldwin, R. L. (1999) *Proc. Natl. Acad. Sci. U.S.A.* 96, 2007–2012.
- Hughson, F. M., Barrick, D., and Baldwin, R. L. (1991) *Biochemistry* 30, 4113–4118.
- Loh, S. N., Kay, M. S., and Baldwin, R. L. (1995) *Proc. Natl. Acad. Sci. U.S.A.* 92, 5446–5450.
- Santoro, M. M., and Bolen, D. W. (1988) *Biochemistry* 27, 8063–8068.
- Jamin, M., and Baldwin, R. L. (1998) *J. Mol. Biol.* 276, 491–504.
- Nishii, I., Kataoka, M., and Goto, Y. (1995) *J. Mol. Biol.* 250, 223–238.
- Eliez, D., Yao, J., Dyson, H. J., and Wright, P. E. (1998) *Nat. Struct. Biol.* 5, 148–155.
- Kiefhaber, T., and Baldwin, R. L. (1995) *J. Mol. Biol.* 252, 122–132.
- Goto, Y., Takahashi, N., and Fink, A. L. (1990) *Biochemistry* 29, 3480–3488.
- Matthews, J. M., and Fersht, A. R. (1995) *Biochemistry* 34, 6805–6814.
- Goldberg, J. M., and Baldwin, R. L. (1999) *Proc. Natl. Acad. Sci. U.S.A.* 96, 2019–2024.
- Rohl, C. A., Chakrabarty, A., and Baldwin, R. L. (1996) *Protein Sci.* 5, 2623–2637.
- Schulman, B. A., and Kim, P. S. (1996) *Nat. Struct. Biol.* 3, 682–687.
- Lorch, M., Mason, J. M., Sessions, R. B., and Clarke, A. R. (2000) *Biochemistry* 39, 3480–3485.

31. Hughson, F. M., Wright, P. E., and Baldwin, R. L. (1990) *Science* 249, 1544–1548.
32. Marmorino, J. L., and Pielak, G. J. (1995) *Biochemistry* 34, 3140–3143.
33. Colon, W. A., Elöve, G. A., Wakem, L. P., Sherman, F., and Roder, H. (1996) *Biochemistry* 35, 5538–5549.
34. Marmorino, J. L., Lehti, M., and Pielak, G. J. (1996) *J. Mol. Biol.* 275, 379–388.
35. Schulman, B. A., Kim, P. S., Dobson, C. M., and Redfield, C. (1997) *Nat. Struct. Biol.* 4, 630–634.
36. Luo, Y., and Baldwin, R. L. (1999) *Proc. Natl. Acad. Sci. U.S.A.* 96, 11283–11287.
37. Redfield, C., Schulman, B. A., Milhollen, M. A., Kim, P. S., and Dobson, C. M. (1999) *Nat. Struct. Biol.* 6, 948–952.
38. Schellman, J. A. (1994) *Biopolymers* 24, 1015–1026.
39. Shortle, D., and Meeker, A. K. (1986) *Proteins: Struct., Funct., Genet.* 1, 81–89.
40. Pace, C. N., and Shaw, K. L. (2000) *Proteins: Struct., Funct., Genet., Suppl.* 4, 1–7.
41. Cavagnero, S., Dyson, H. J., and Wright, P. E. (1999) *J. Mol. Biol.* 285, 269–282.
42. Kraulis, P. J. (1991) *Acta Crystallogr.* 24, 946–950.

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