

Accelerated Publications

Probing the Stability of a Partly Folded Apomyoglobin Intermediate by Site-Directed Mutagenesis[†]

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ABSTRACT: A partly folded form (I) of apomyoglobin has an α -helix content of about 35%; in an earlier study, hydrogen exchange revealed that the A, G, and H helices are folded, while much of the rest of the protein is not [Hughson, F. M., Wright, P. E., & Baldwin, R. L. (1990) *Science* 249, 1544–1548]. Because A, G, and H form a compact subdomain in native myoglobin, we proposed that nativelike packing interactions among the three helices might be retained in the I form of apomyoglobin. To test this proposal, disruptive mutations were introduced into the A·H and G·H helix packing sites. These mutations destabilize native apomyoglobin relative to I. In contrast, the stability of I is relatively insensitive to mutation; in particular, side-chain volume alone does not appear to be important. These results indicate that the I form is not stabilized by nativelike A·H and G·H packing interactions. In support of this we show that partly helical peptides derived from the G and H helix regions of myoglobin do not pair in solution. Since the isolated G and H peptides are at best only partly helical, some type of interaction must stabilize these helices in the I form. Small increases in the stability of I are seen when mutation introduces a side chain of increased nonpolar surface area. We suggest that I is stabilized by relatively nonspecific hydrophobic interactions that allow it to adapt easily to mutation. In this and other respects, I appears to conform to the “molten globule” model, with the caveat that only part of the polypeptide chain appears to participate in the globule.

Apomyoglobin (apoMb)¹—myoglobin without the heme group—is a small (molecular weight 17 000) monomeric protein. At low temperatures, apoMb unfolds in two successive stages as the pH is lowered from 6 to 2 (Griko et al., 1988). At pH 6, its structure resembles that of holomyoglobin (Watson, 1969; Takano, 1977; Hanson & Schoenborn, 1981) by several criteria (Griko et al., 1988; Cocco & Lecomte, 1990), including the detailed pattern of amide proton protection (Hughson et al., 1990). At pH 4, a partly folded equilibrium intermediate (I) is observed with properties midway between those of the native (N) and fully unfolded (U) states. As judged by intrinsic viscosity measurements, I is fairly compact. Circular dichroism (CD) reveals an α -helix content of about 35% for I, as compared to 55% for N. As the pH is lowered further, I unfolds. The CD spectrum and intrinsic viscosity of the resulting U state are consistent with a random coil chain with only small amounts of α -helix (Griko et al., 1988).

The localization of secondary structure in the I form of apoMb was determined by using hydrogen exchange followed by 2D ¹H NMR (Hughson et al., 1990). Helices A, G, and H are formed in I; little structure is detected in the remainder of the molecule. Since the A, G, and H helices pack together in native myoglobin, we proposed that this region of the protein might retain nativelike helix packing in I while the rest of the molecule unfolds. To test the role of nativelike helix interactions in stabilizing I, we sought to disrupt the putative A·H and G·H interactions by site-directed mutagenesis. We mutagenized three positions, one at the A·H helix interface and two along the G·H helix interface. We expressed and purified single mutants with a variety of side-chain volumes, charges, and hydrophobicities. The stabilities of N and I for the mutant proteins were examined in acid and urea denaturation experiments.

¹ Abbreviations: ApoMb, apomyoglobin; CD, circular dichroism; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, (ethylenedinitrilo)tetraacetic acid; Mb, myoglobin; NMR, nuclear magnetic resonance; N, I, and U, native, acid intermediate, and unfolded forms of apomyoglobin.

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Another test for nativelike helix packing interactions in I was made by using peptide fragments of myoglobin corresponding to the G and H helices. In order to obtain monomeric peptides that showed substantial helicity in solution, we made residue substitutions at positions not involved in the native G-H pairing site. We then asked if these peptide helices interact on mixing, using an expected increase in helix content determined by CD as a measure of helix-helix interaction.

EXPERIMENTAL PROCEDURES

Plasmid pMb413a [a derivative of pMb413 (Springer & Sligar, 1987)], bearing a synthetic sperm whale Mb gene, was the kind gift of B. A. Springer and S. G. Sligar. Cassette replacement was used for all sequence substitutions, including the replacement of Asp 122 in pMb413a with Asn, giving the wild-type sequence for sperm whale myoglobin (pMb413b). Oligonucleotides degenerate at positions corresponding to residues 108, 123, and 130 were used to introduce mutations into pMb413b.

Proteins were overexpressed in *Escherichia coli* strain TB-1. Typically a 4-L culture of cells was grown at 37 °C to late log phase, harvested, and frozen at -80 °C. Purification was essentially as described previously (Springer & Sligar, 1987; Springer et al., 1989) with a few modifications. Briefly, cells were resuspended in 50 mM Tris/1 mM EDTA/0.5 mM dithiothreitol/1 mM phenylmethanesulfonyl fluoride, pH 8. DNase I, RNase A, and lysozyme were added to 25 µg/mL, 30 µg/mL, and 2 mg/mL, respectively. Cells were stirred 3 h at 4 °C and centrifuged. The pellets were sonicated in 50 mM Tris/1 mM EDTA, pH 8, and centrifuged; all supernatants were pooled and fractionated by ammonium sulfate precipitation. The 60–95% cut was redissolved in a minimum volume of 50 mM Tris/1 mM EDTA, pH 8, and fractionated on a Sephacryl S-200 column equilibrated in the same buffer. Reddish-brown Mb-containing fractions were pooled and desalted into 20 mM sodium phosphate, pH 6.0, on a Sephadex G-25 column. Final purification was achieved on a Whatman CM-52 column developed with a gradient from 20 mM sodium phosphate, pH 6.0, to 50 mM sodium phosphate (dibasic). Myoglobin-containing fractions were pooled and reconcentrated several times from distilled water prior to storage at -80 °C. This procedure yielded about 1–5 mg of protein/L of cell culture. Final purity was at least 95% as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

ApoMb was prepared from Mb by the method of Teale (1959), except that dialysis was replaced with passage over a PD-10 column (Pharmacia) equilibrated with distilled water. Apoprotein concentration was determined by the method of Edelhoch (1967).

ApoMb folding and unfolding were monitored by measuring the mean residue ellipticity at 222 nm with an Aviv 60DS spectropolarimeter. Quartz cuvettes with a 1.00-cm path length were used. Sample pH was measured at room temperature at the conclusion of the experiment. Urea denaturation curves were fitted to a two-state model as previously described (Hughson & Baldwin, 1989).

Peptides were synthesized by solid-phase synthesis using either *t*-Boc chemistry (G_{wt} and modified H_1 , Table II) or Fmoc chemistry and were purified by reverse-phase chromatography as described (Marqusee et al., 1989). Fast atom bombardment mass spectrometry was used to verify peptide identity. Peptide stocks were prepared by dissolving pure lyophilized peptide in water at concentrations of 1–2 mg/mL. Stock concentrations were measured as described (Marqusee et al., 1989), except that 20 mM sodium phosphate was added and the pH was adjusted to 6.5.

Table I: Denaturation Curve Analysis^a

protein	pH _{mid} ^b		urea denaturation ^c		
	N ↔ I	I ↔ U	C_m ^d	ΔG_{app}° ^e	$\Delta\Delta G_{app}^\circ$ ^f
wild-type	5.0	3.5	3.24 (0.02)	5.8 (0.3)	
A130V	5.2	3.5			
A130L	5.5	3.4	2.50 (0.03)	3.5 (0.2)	-2.3 (0.4)
A130S	5.4	3.6			
A130N	5.6	3.5			
A130K	5.8	3.6	2.03 (0.06)	2.1 (0.1)	-3.7 (0.3)
F123G	5.7	3.6			
F123I	5.5	3.7			
F123T	5.4	3.6	1.96 (0.05)	2.3 (0.1)	-3.5 (0.3)
F123K	5.8	3.9			
F123W	5.7	3.4			
S108T	5.0	3.5			
S108N	4.8	3.7			
S108L	5.1	3.2			
S108F	4.8	3.2			
S108K	5.2	3.8			

^aStandard deviations resulting from nonlinear least-squares analysis are given in parentheses. ^bTransition pH midpoints for acid denaturation of apoMbs at 0 °C obtained by visual examination of data. Values are ± 0.1 pH unit. ^cParameters calculated from data in Figure 1 (urea denaturation at pH 7.5, 0 °C). ^d C_m is the midpoint denaturant concentration given in molarity units. ^e ΔG_{app}° is given in kilocalories per mole in the absence of urea. ^f $\Delta\Delta G_{app}^\circ$ is the apparent free energy change in kilocalories per mole relative to the wild-type protein.

CD samples of peptides contained 10 mM sodium chloride and 1 mM each of sodium citrate, sodium phosphate, and sodium borate; pH was adjusted to 6.0. All measurements were made at 3 °C. Mean molar residue ellipticity at 222 nm ($[\theta]_{222}$) was determined for each peptide by measuring ellipticity of seven or more samples ranging in concentration from 2.5 to 100 µM. Mean molar ellipticity was then determined as the best-fitting slope of ellipticity versus concentration. In this way the independence of ellipticity on concentration was established, and the effect of loss of signal due to adsorption of peptide in sample preparation was mitigated.

Association between peptides was investigated by CD; a tandem mixing CD cell with path lengths of 0.438 cm for each chamber was used. CD spectra were the result of averaging three separate scans, signal averaging each for 1 s every 0.25 nm.

RESULTS

A·H Mutations. In holomyoglobin, the side chain of Ala 130, a residue in the H helix, is completely buried in a hydrophobic pocket formed by A helix residues Glu 6, Leu 9, and Val 10 (Lee & Richards, 1971; Lesk & Chothia, 1980). Mutations that increase the volume, polarity, or charge of residue 130 might be expected to destabilize native apoMb and also I if it retains nativelike A·H pairing. To test this hypothesis, wild-type and five mutant proteins were overexpressed in *E. coli* and purified for stability studies (Table I).

Urea denaturation curves for some of these proteins were generated by monitoring $[\theta]_{222}$ as a function of urea concentration at pH 7.5 (Figure 1). As expected, substitution of Leu or Lys for Ala destabilizes the N state relative to the unfolded state (Table I). Such destabilization can be attributed to steric and electrostatic disruption of helix packing interactions in the N state.

Acid denaturation curves were generated by monitoring $[\theta]_{222}$ as a function of pH (Figure 2); pH midpoints for the N ↔ I and I ↔ U transitions are listed for all mutants in Table I. All mutations at position 130 increase pH_{mid}^{N→I}, indicating that mutation destabilizes N relative to I. Changes are as large as 0.8 pH units. In contrast, the midpoint of the I ↔ U

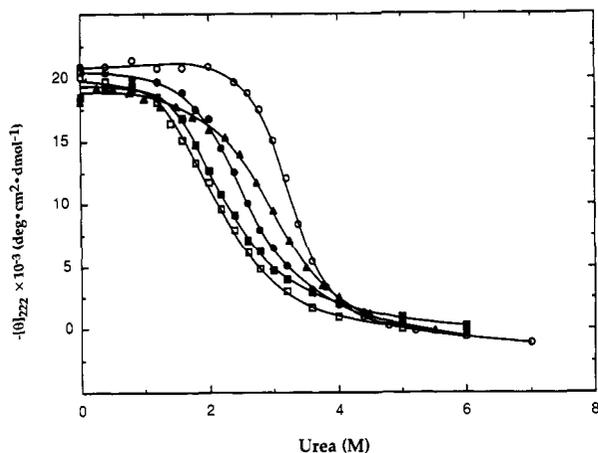


FIGURE 1: Urea denaturation of wild-type (○), A130L (●), A130K (□), F123T (■), and S108K (▲) apoMbs. Mean residue ellipticity at 222 nm, $[\theta]_{222}$, is plotted as a function of urea concentration. Samples were measured at 0 °C and contained, in addition to urea, 1.00 μ M apoMb and 10 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.5.

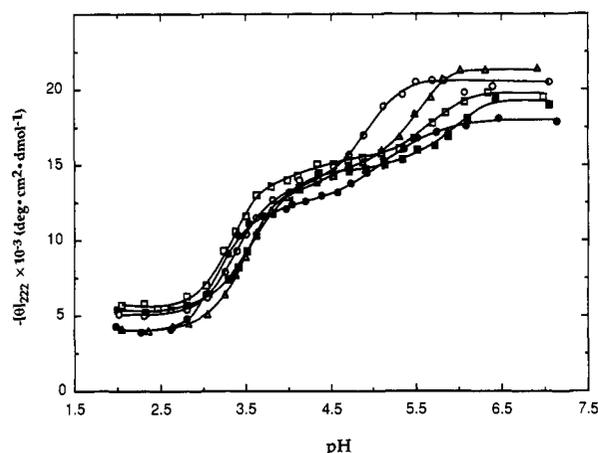


FIGURE 2: Acid denaturation of wild-type (○), S108L (●), F123W (□), F123G (■), and A130S (Δ) apoMbs. $[\theta]_{222}$ at 0 °C is plotted as a function of pH. ApoMb concentration was 1.00 μ M in 10 mM sodium acetate buffer. Lines through the data points were drawn by inspection.

transition ($\text{pH}_{\text{mid}}^{\text{I} \leftrightarrow \text{U}}$) is largely unaffected by mutation at position 130; pH midpoints are both increased and decreased but in no case by more than 0.1 pH unit.

G-H Mutations. In native myoglobin, the G and H helices are nearly antiparallel. Their extensive interaction involves more than four helical turns from each of the two helices. In order to probe the G-H interface, we mutagenized two positions (Phe 123 and Ser 108) in separate regions of the G-H helix pair. Phe 123, a residue highly conserved within the globin family, is in the turn connecting the G and H helices; its side chain projects into the G-H interface (Hanson & Schoenborn, 1981) at one end. Ser 108, in the G helix, is in the center of the G-H interface (Lesk & Chothia, 1980). Its side chain makes close contacts with H helix residues Leu 135, Phe 138, and Arg 139. However, the side chain of Ser 108 also borders a large, nonpolar cavity (Lee & Richards, 1971). Mutations that increase side-chain volume might be accommodated by the cavity, diminishing the destabilizing effect of mutation.

A number of substitutions for Phe 123 were made by site-directed mutagenesis (Table I), expressed, and purified. Acid and urea denaturation experiments (Figures 1 and 2, Table I) were performed to assess the stabilities of the mutant proteins. As in the case of Ala 130 substitutions, replacement of Phe 123 resulted in substantial increases in $\text{pH}_{\text{mid}}^{\text{N} \leftrightarrow \text{I}}$, in-

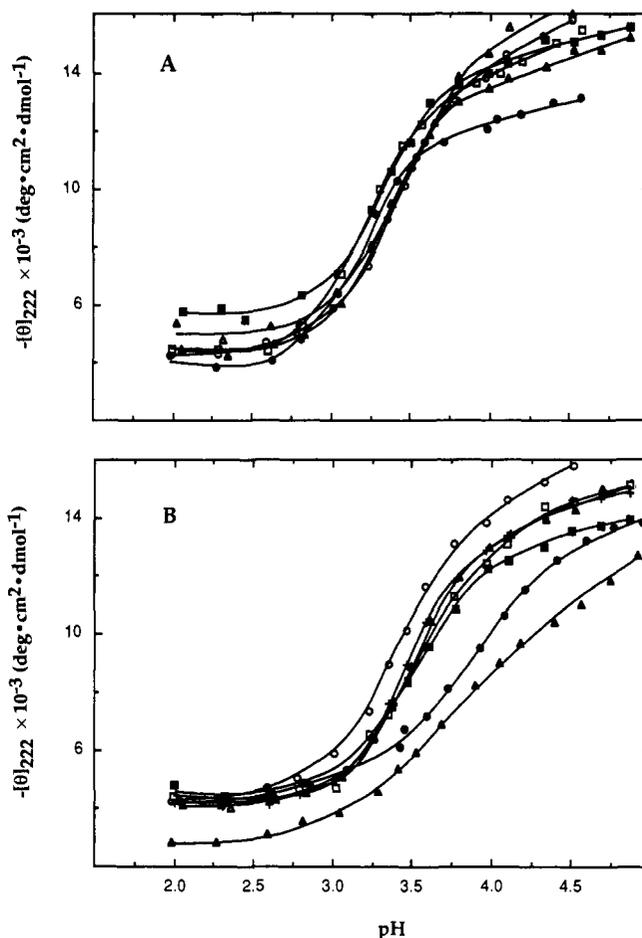


FIGURE 3: Acid denaturation of wild-type (○) and mutant apoMbs. $[\theta]_{222}$ at 0 °C is plotted as a function of pH; only I \leftrightarrow U transitions are shown. Samples were prepared as in Figure 2. Lines through the data points were drawn by inspection. (A) Mutants with increased side-chain nonpolar surface area: S108L (●), S108F (□), F123W (■), A130V (Δ), and A130L (▲) apoMbs. (B) Mutants with increased side-chain polarity: S108K (▲), F123T (■), F123K (●), A130S (Δ), A130K (□), and A130N (+) apoMbs.

dicating that these mutations destabilize N relative to I. They also result in significant perturbations of $\text{pH}_{\text{mid}}^{\text{I} \leftrightarrow \text{U}}$. Most Phe 123 replacements increased $\text{pH}_{\text{mid}}^{\text{I} \leftrightarrow \text{U}}$ (Figure 3). One mutant (F123W) showed a decrease in $\text{pH}_{\text{mid}}^{\text{I} \leftrightarrow \text{U}}$, indicating that in this case I is stabilized relative to U.

Five Ser 108 replacements were also selected for purification and analysis (Table I). Acid denaturation revealed changes in both $\text{pH}_{\text{mid}}^{\text{N} \leftrightarrow \text{I}}$ and $\text{pH}_{\text{mid}}^{\text{I} \leftrightarrow \text{U}}$ (Table I). Unlike substitutions for Phe 123 and Ala 130, mutations at Ser 108 lowered as well as raised $\text{pH}_{\text{mid}}^{\text{N} \leftrightarrow \text{I}}$. Ser 108 mutations also resulted in $\text{pH}_{\text{mid}}^{\text{I} \leftrightarrow \text{U}}$ displacements to both higher and lower values, showing that, relative to U, the free energy of the I form can be both increased and decreased by side-chain replacement at this position.

We note that, for some mutants, ellipticity is significantly and reproducibly decreased for the N, I and U forms. This is most pronounced for F123I, S108L, and S108K and can amount to as much as a 3000 $\text{deg}\cdot\text{cm}^2\cdot\text{dmol}^{-1}$ decrease from wild type. At present the origin of this decrease is not known; it is possible that it is caused by local unfolding or other structural rearrangements.

Peptide Models. Solid-phase synthesis was used to make peptides corresponding in sequence to the G and H helices (Table II). Peptide helix formation was measured by CD. The wild-type H-helix peptide (H_w) shows considerable helix formation, judged by its large negative ellipticity at 222 nm.

Table II: Sequences and $[\theta]_{222}$ Values of G- and H-Helix Peptides

peptide ^a	sequence ^b	$[\theta]_{222}$ ^c
G _{wt}	(Ac) PIKYLEFISEAIIHVLHSR (NH ₂)	nd
G ₂	(Ac) PIKYLEELSEAIIKELHAK (NH ₂)	-9400
H _{wt}	(Ac) ADAQGAMNKALELFRKDIAAKYKE (NH ₂)	-9600
H ₁	(Ac) ADAQEAMNKALELFRKDIAAKYKA (NH ₂)	-15 500

^a Peptides are named as follows: G_{wt} has sequence identical with residues 100–118 of sperm whale myoglobin. H_{wt} is identical with residues 124–148. H₁ and G₂ have sequence substitutions (bold) designed to increase helicity and, in the case of G₂, increase solubility. ^b Peptides were acetylated on their N-termini and amidated on their C-termini. ^c $[\theta]_{222}$ values were determined at 3 °C, pH 6; values are in deg·cm²·dmol⁻¹.

This $[\theta]_{222}$ value corresponds to roughly 30% helix formation (Padmanabhan et al., 1990) and is unusually high for a naturally derived, monomeric peptide. A similar peptide was shown to populate helical conformations by 2D ¹H NMR and by CD spectroscopy (Waltho et al., 1989). Although molar ellipticity in H_{wt} showed no concentration dependence, ellipticity for the wild-type G-helix peptide (G_{wt}) showed a strong concentration dependence from 2.5 to 100 μM, suggesting intermolecular association. In support of this, substantial light scattering resulting from turbidity was observed between 290 and 350 nm and showed a similar, nonlinear concentration dependence (data not shown).

Because self-association would hinder the formation of native helix pairs and obscure analysis, we made sequence changes to enhance solubility of the G-helix peptide (F106E, V114E). Other changes were designed to increase helix content [I107L and S117A; see Padmanabhan et al. (1990)] in order to stabilize a potential helix pair. Substitutions were made at positions in the sequence that do not contact the H helix in the native structure, so that the putative G-H pairing surfaces remain unchanged.

Both these goals were achieved in the substituted G-helix peptide (G₂): helix content was increased to around 30% and was independent of concentration over the range studied. Helix content was also increased by making substitutions in the H-helix peptide. The substituted peptide (H₁) has approximately 50% helix by CD. Again, substitutions were made at positions that do not make interhelical contacts with the G helix in the native structure.

Peptides listed in Table II were used to test for helix-helix pairing in solution. Ellipticity was measured before and after mixing of G₂ and H₁ in equimolar ratios. An example of such an experiment is shown in Figure 4. CD samples (100 μM) of equal volume were partitioned in a tandem cell, and the composite CD spectrum was recorded. The two CD samples were then mixed, and another spectrum was recorded. The two spectra (partitioned and mixed) were superimposable, indicating that helix content did not increase and therefore no detectable helix pair formation had occurred. The same result was obtained at concentrations of 0.75 mM peptide by using a 1-mm cell and monitoring ellipticity at 222 nm: no increase in helix content was observed upon mixing (data not shown).

DISCUSSION

Here we have tested a model for tertiary interactions in the I form of apoMb. The model, based on a map of secondary structure in I (Hughson et al., 1990), has the A, G, and H helices packed in a natively like conformation. Our tests of this consist of examining the effect of mutation in these helix pairing sites and attempting to demonstrate the proposed helix

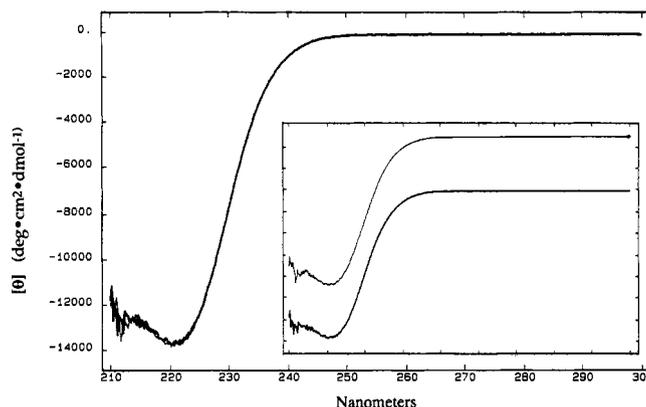


FIGURE 4: Mixing of G₂ and H₁ peptides. Peptides were partitioned in a tandem CD cell at 100 μM peptide, and the composite CD spectrum was acquired. Peptides were then mixed, and another spectrum was collected. Spectra of composite unmixed (light trace) and of mixed (heavy trace) peptides are shown; the two spectra superimpose so closely that they can only be distinguished at low wavelength, where errors in the CD signal are large. The inset shows the two spectra offset by 5000 deg·cm²·dmol⁻¹ so that individual spectra can be resolved. Sample preparation was described under Experimental Procedures.

packing with helical peptides.

Peptide Models. The proposed model for I suggests that the G-H and A-H helix pairs may be stable, isolable structures. This prediction was tested with peptide models. The above work shows that helical, monomeric peptides that have sequences closely resembling the G and H peptides do not pair in solution, even at concentrations of 0.75 mM. These results place a limit on the hypothetical dissociation constant for helix pair formation of millimolar or greater. This demonstrates that the native G-H helix pair is not a stable structure in isolation and brings its role in the partly unfolded I form of apomyoglobin into question. Since the wild-type peptides corresponding to the A, G, and H helices show no more than 30% helix in isolation, these helices appear to be stabilized by nonnative interactions in the intermediate. It remains possible that the A helix plays an important role in stabilizing the G-H interaction. Studies of the A-H interaction have not been completed, since a peptide corresponding to the A helix seems to aggregate in a nonhelical conformation (data not shown).

A-H and G-H Mutations. If the proposed model for I is correct, mutations at buried positions in the AGH subdomain should perturb the close side-chain packing and destabilize I relative to U. Furthermore, such mutations should have little effect on the stability of N relative to I since, according to this model, the AGH subdomain is similarly structured in N and I. A representative set of urea-induced unfolding curves (Figure 1, Table I), which allow the free energy of N to be compared directly with that of U, demonstrates that mutations at any of these positions destabilize N. This destabilization is presumably a consequence of steric and/or electrostatic disruption of the close-packed, predominantly hydrophobic A-H and G-H interfaces.

We use these mutants to test for natively like structure in I. pH titrations, which allow I to be resolved from N and U, were performed for all 15 mutants (Table I). In the absence of high-resolution structural data, we cannot interpret the observed stability changes in detail; indeed, loss of ellipticity for some mutants indicates that minor structural rearrangements may have occurred. Nevertheless, several generalizations emerge.

First, mutations nearly always (in 12 out of 15 cases; see Table I) decrease the stability of N relative to I. This is true

for mutations that both increase (A130V, L, S, N, K; F123W; S108L, K) and decrease (F123G, I, T) side-chain size and for mutations that both increase (A130V, L; F123W; S108L) and decrease (A130S, N, K; F123T, K; S108K) side-chain hydrophobicity. The fact that such a wide variety of substitutions all lead to destabilization of N relative to I suggests that the N \rightarrow I transition involves disruption of tight packing interactions in the vicinity of the mutations, i.e., in the AGH subdomain. This interpretation is inconsistent with the above model for the structure of I: retention of proposed nativelylike packing in I is not seen. The mutations exceptional to this rule, those that either increase or do not affect the stability of N relative to I, all occur at position 108. In the crystal structure of holomyoglobin, the Ser 108 side chain borders a large internal cavity (see Results). It is possible that this cavity can accommodate packing changes and even allow for energetically favorable rearrangements. Without high-resolution structural data, however, such rearrangements remain speculative.

Second, mutations give rise to larger perturbations in $\text{pH}_{\text{mid}}^{\text{N} \leftrightarrow \text{I}}$ than in $\text{pH}_{\text{mid}}^{\text{I} \leftrightarrow \text{U}}$ (see Figure 2 and Table 1). The root-mean-square deviation from the wild-type $\text{pH}_{\text{mid}}^{\text{N} \leftrightarrow \text{I}}$ is 0.5 pH unit, with values ranging over 1.1 pH units (4.8 to 5.8). For $\text{pH}_{\text{mid}}^{\text{I} \leftrightarrow \text{U}}$ the root-mean-square deviation from wild-type is 0.2 pH unit, with values ranging over 0.8 pH unit (3.2–3.9). This is opposite to what is expected if the proposed model is correct: if nativelylike packing interactions are maintained in I, the I \leftrightarrow U transition should be highly sensitive to mutation while the N \leftrightarrow I transition should remain unchanged. We conclude that nativelylike helix interactions are not present in I.

Third, mutations increase the stability of I as well as decrease it. Examination of the effects of specific types of substitutions on $\text{pH}_{\text{mid}}^{\text{I} \leftrightarrow \text{U}}$ shows that the stability of I is increased by mutations that introduce nonpolar surface area (A130L; F123W; S108F, L; Figure 3A). Conversely, stability of I is decreased by substitutions that either decrease nonpolar surface area (F123G, I) or introduce polar side chains (A130K; F123T, K; S108K; Figure 3B). This behavior is not seen in the N \leftrightarrow I transition: substitutions that introduce large nonpolar side chains destabilize N relative to I.

Stabilization of I by mutations that increase side-chain volume confirms the absence of closely packed structure in the proximity of these substitutions. The observation that I can be stabilized by increasing nonpolar surface suggests, furthermore, that I is stabilized by hydrophobic interactions. Finally, these hydrophobic interactions seem to be flexible and nonspecific, on the basis of decreased sensitivity of the I \leftrightarrow U transition to mutation.

Molten Globule Model. The mutagenesis results obtained here, together with evidence that I retains substantial secondary structure (Griko et al., 1988; Hughson et al., 1990) and is relatively compact (Griko et al., 1988) and that its enthalpy is close to that of the unfolded state (Griko et al., 1988; Privalov et al., 1989), strongly suggest that the AGH subdomain of I resembles a molten globule (Ohgushi & Wada, 1983; Dolgikh et al., 1985; Ptitsyn, 1987; Kuwajima, 1989). Our results suggest that I is flexible, accommodating mutation more readily than the native state. This is consistent with near-UV, CD, and NMR studies of several proteins, which suggest that side chains in the molten globule are flexible (Kuwajima, 1989; Ptitsyn et al., 1987; Dolgikh et al., 1985; Baum et al., 1989).

The question remains, what stabilizes the A, G, and H helices in I if not nativelylike packing interactions? The sta-

bilization of I by increasing nonpolar surface area suggests that it is stabilized by hydrophobic forces, implying that hydrophobic side chains can interact favorably in I. Stabilization of the molten globule by nonspecific hydrophobic interactions has been proposed (Kuwajima, 1989). Isothermal titration calorimetry of a thermally modified form of apoMb, exhibiting properties similar to the intermediate form studied here, suggests that unfolding of I is accompanied by an increase in unfolding enthalpy change with temperature (Griko et al., 1988), consistent with a positive ΔC_p . The latter is also indicated in Figure 9 of Privalov et al. (1989): the heat capacity difference between I and U is approximately 20% of the ΔC_p found for complete unfolding of native apoMb. Therefore, although much of the polypeptide appears to be solvated, it is likely that a limited number of hydrophobic groups remain desolvated, consistent with a limited set of hydrophobic interactions.

CONCLUSIONS

Our results show that the acid-unfolded intermediate of apoMb is not stabilized by native-like helix packing interactions as previously proposed (Hughson et al., 1990). Instead, the A, G, and H helices appear to be stabilized by loose hydrophobic interactions. It is plausible that this type of interaction is also characteristic of the molten globule forms of other proteins (Goto et al., 1990). Recently, it has been proposed that molten globules are universal intermediates on kinetic folding pathways (Ptitsyn et al., 1990). In the case of apoMb, however, there is as yet no direct evidence that I is a kinetic folding intermediate.

Application of site-directed mutagenesis to the acid-induced unfolding of apoMb yields results that bear on both the structure of the molten globule intermediate and the forces that stabilize it. First, the results show that the intermediate lacks a well-defined tertiary structure, in accord with the view that side-chain packing occurs cooperatively. For apoMb, tertiary structure seems to be lost in an all-or-none fashion, while secondary structure and hydrophobic side-chain interactions can persist in localized regions of the polypeptide chain. Second, the lack of nativelylike helix packing interactions, both in the intermediate and between the partly helical peptides, suggests that helix packing interactions found in globular proteins (which have been discussed extensively, e.g., Richmond and Richards (1978) and Chothia et al. (1981)), are not stable as individual structural elements. Third, the increase in stability of the apoMb intermediate, caused by increasing side-chain nonpolar surface area, provides experimental evidence that hydrophobic interactions can help to keep the polypeptide chain compact and helical in the absence of specific tertiary interactions. This finding is in agreement with theoretical studies which show that, for a self-avoiding polymer, attractive forces between monomers that cause compactness result in a high degree of secondary structure (Chan & Dill, 1990). The role of α -helix formation in molten globule stability is currently being tested by using site-directed mutagenesis.

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