Folding of *Aplysia limacina* Apomyoglobin Involves an Intermediate in Common with Other Evolutionarily Distant Globins†

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ABSTRACT: In the globin family, similarities in the folding mechanism have been found among different mammalian apomyoglobins (apoMb). The best-characterized intermediate of sperm whale apoMb, called I_{AGH}, is mainly stabilized by nativelike contacts among the A, G, and H helices involving a cluster of hydrophobic residues that includes two conserved tryptophans. To verify the hypothesis of a common intermediate in the folding of all members of the globin family, we have extensively studied a site-directed mutant of the myoglobin from *Aplysia limacina*, distantly related to the mammalian counterpart, in which one of the two tryptophans in the A−G−H cluster [i.e., Trp(H8)130] has been mutated to tyrosine. The results presented here show that this mutation destabilizes both the native state and the acid intermediate I_A but exerts little or no effect on the thermally stable core of an intermediate species (called I_T) peculiar to *Aplysia* apomyoglobin. Dynamic quenching of Trp emission by acrylamide provides information on the accessibility of the chromophores at the native and the intermediate states of wild-type and mutant *Aplysia* apomyoglobin, consistent with the thermodynamics. Our results agree well with those obtained for the corresponding topological position of apomyoglobin from sperm whale and clearly show that the H8 position is involved in the stabilization of the main intermediate in both apoproteins. This residue thus plays a role which is evolutionarily conserved in the globin family from invertebrates to mammals; our results support the contention that the A−G−H cluster is important in the folding pathway of different globins.

Structurally homologous proteins have encoded in their amino acid sequence the information for their common fold. A powerful strategy to single out common structural determinants of folding is therefore to compare proteins belonging to the same family, which share a common fold despite the (sometimes) large difference in sequence (*1*).

Proteins from the globin family have been extensively studied, with attention mainly confined to apomyoglobins from horse and sperm whale (called m-apoMb).[1] The folding mechanism for these two mammalian apomyoglobins is similar, involving more than one intermediate that can be populated under different experimental conditions. The best-characterized intermediate (called I_{AGH}) is a species with an α-helical content ∼ 35% (*2*), stabilized mainly by nativelike contacts among the A, G, and H helices (*3*, *4*); although it was initially obtained at acid pH, it is populated on the folding pathway at pH 7.0 as a rapidly formed transient species (*5*, *6*). Besides I_{AGH}, other intermediates were found to be populated at equilibrium, depending on pH, ionic strength and temperature (*7*−*9*).

Factors governing the overall stability and secondary structure of the apomyoglobin intermediate(s) are still not well understood. Perturbation of the folding pathway and stability by introducing mutations at specific sites has been extensively used to explore the folding landscape of m-apoMb. Mutagenesis of some hydrophobic residues in the A−G−H cluster has shown that these contacts are essential to the stability of the I_{AGH} intermediate (*10*, *11*), leading to the conclusion that this involves a loosened but nativelike set of interhelical nonpolar contacts. The outcome that common folding features can be found for m-apoMbs is not surprising in view of their high sequence identity (>80%). However, it is still a matter of debate whether the I_{AGH} intermediate is populated along the folding pathway of most globins, given that only a few other members of the family from evolutionarily distant organisms (characterized by a low degree of sequence identity to m-apoMbs) were studied in detail.

The observation that a proteolytic fragment from horse apoMb, produced by removal of the helix A and part of the helix H, yields a stable minimyoglobin (mini-Mb) that, similarly to m-apoMb, populates at pH 4.3−5.0 a folding intermediate with an α-helical content of ∼35%, despite the absence of the A−G−H domain, suggested the existence of alternative folding intermediates (*12*). This was shown to be the case in the folding of the evolutionarily distant soybean apoleghemoglobin, which shares only 13% sequence identity

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[1] Abbreviations: I_{AGH}, mammalian acid intermediate; I_A, *Aplysia limacina* acid intermediate; I_T, *Aplysia limacina* thermal intermediate; *m*-apoMb, mammalian apomyoglobins; *Al*-apoMb, apomyoglobin from *Aplysia limacina*; W130Y, Trp130Tyr mutant.

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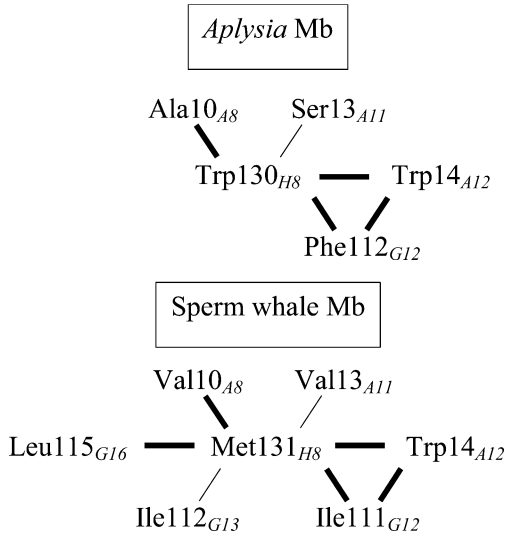

FIGURE 1: Schematic representation of all contacts between the residue at position H8 and residues belonging to the A, G, and H helices. Contacts belonging to the conserved set identified by Ptitsyn and Ting (*19*) as potentially relevant for folding are depicted by bold lines. The topological position of each residue in each helix is indicated in italic type.

with sperm whale apomyoglobin; the prevailing intermediate is stabilized by contacts involving helices G and H and (in part) helix E, while helices A and B become protected against H-exchange only at a later stage (*13*). These results suggest that multiple folding pathways, highly dependent on the local amino acid sequence, may be operative in the globin family.

Myoglobin from the sea mollusc *Aplysia limacina* (*Al*-Mb) has a classical globin fold (*14*) but low (24%) sequence identity with sperm whale Mb. It proved to be a valuable protein to study the role of net charge, hydrophobicity, and intrinsic stability of the helical segments on folding. Early studies carried out on the holoprotein had already pointed out some unique features (*15*, *16*). The corresponding globin (*Al*-apoMb) at neutral pH and low salt displays a two-state equilibrium unfolding in urea; at acid pH or in high salt, an intermediate (I_A) with ~35% α-helix, sharing many properties with the acid intermediate observed for the m-apoMb (*17*), was observed. However, at high temperature (70 °C) *Al*-apoMb populates yet another intermediate (I_T) more solvated and with reduced helical content (27% vs 35%) compared with I_A (*18*). Analysis of the properties of I_T lent support to the view that hydrophobic interactions are an important component for the stability of this intermediate, which may still correspond to a core of stable helices in the A−G−H region, with considerable fraying at the extremities.

Since the equilibrium folding intermediate I_A found for *Al*-apoMb shares some features with that fully characterized for m-apoMbs, we decided to establish whether this species is stabilized by contacts in the A−G−H cluster, to test the hypothesis of a prevailing folding intermediate for all members of the globin family. The structure of native *Aplysia* myoglobin in the interhelical A−G−H region shows that the two tryptophan residues, i.e., Trp14 on helix A (topological position A12) and Trp130 on helix H (topological position H8), are both buried in an extended apolar cavity that is filled by the two indolic side chains and is part of the contact area at the A/H helical interface. A scheme representing the main contacts involving Trp(H8)130 in *Al*-Mb is compared in Figure 1 with the corresponding topological position of sperm whale myoglobin [Met(H8)131]. Four of these contacts belong to the cluster of conserved interactions located within the A−G−H nucleus, highlighted by the structural analysis of Ptitsyn and Ting (*19*) as crucial residues in globin folding.

The mutation of Met131 to Ala in sw-apoMb (*10*) was shown to destabilize the native state [$\Delta\Delta G^0_{(mut-wt)} = 2.2$ kcal mol^{-1}) as well as the acid intermediate [$\Delta\Delta G^0_{(mut-wt)} = 0.9$ kcal mol^{-1}]. Therefore, to verify whether the A−G−H domain makes nativelike contacts in the main folding intermediate of *Aplysia* apoMb, we have extensively studied a site-directed mutant in which the key residue Trp(H8)130 in the H helix is substituted with Tyr. The experiments show that in *Al*-apoMb (i) perturbation of the H8 residue affects the stability of the I_A acid intermediate and (ii) Trp(A12)14 is still partially protected from the solvent in the I_A state of both wild type and Trp130Tyr mutant (W130Y). These data support the hypothesis that the A−G−H cluster represents a common and evolutionarily conserved (though not unique) folding intermediate of globins, at least from invertebrates to mammals.

MATERIALS AND METHODS

Purification of Aplysia limacina Wild-Type and Mutant Myoglobins. Wild-type and mutant myoglobins were purified from *Escherichia coli* TB-1 cells as described by Cutruzzolà et al. (*20*). The apoproteins were prepared by the acid−acetone precipitation method described by Rossi-Fanelli et al. (*21*) and were stored at −70 °C in water. The molar extinction coefficients of the wild-type and mutant apoproteins at 280 nm were measured experimentally by the Bradford assay (*22*) to be respectively 13 500 and 8000 cm^{-1} mol^{-1}. All reagents used were of analytical grade.

Equilibrium Unfolding Titrations. The stability of the holoprotein in the ferric form was measured at pH 7.0 and 25 °C by urea denaturation of the cyano-Mb derivative, prepared by adding a large excess of sodium cyanide to the protein. The concentration was determined by using the absorbance at 423 nm ($\epsilon = 116$ mM^{-1} cm^{-1}) characteristic of the cyanomet derivative. The samples were incubated for at least 1 h in urea to allow equilibration prior to measurements; denaturation was followed by both CD and absorbance at 421 nm. All experiments were performed also in renaturation by diluting to different final urea concentrations a protein solution containing either the urea concentration corresponding to the midpoint of the transition (4.8 M for W130Y; 5.9 M for the wild type) or 8 M urea. Denaturation was found to be >90% reversible for both the wild type and W130Y mutant. Given that the denaturation curves are fully cooperative, data were fitted to a two-state model without baseline contribution and are presented as fraction folded, where the native state is 1.

Urea unfolding experiments were carried out at different temperatures (4 and 60 °C) by adding different volumes of a solution containing 8 M urea to a 5 μM protein solution in the appropriate buffer (50 mM sodium phosphate, pH 7.0, or 2 mM sodium citrate, pH 2.0). The experiments were also carried out in the presence of KCl, 1 or 2 M. Equilibrium was fully reached within 5 min. Each experiment was repeated at least twice; the corresponding errors in the thermodynamic values given in Table 1 have been calculated

Table 1: Thermodynamic Parameters for the Urea Denaturation of Wild-Type and W130Y *Al*-ApoMb at 4 °C[a]

	pH	KCl salt (M)	$-\Delta G^0$ (kcal mol^{-1})	c_m	transition
wt	7.0	0	4.4 ± 0.4	3	N → U
W130Y	7.0	0	3.6 ± 0.3	2.4	N → U
wt	7.0	2	5.3 ± 0.15	5.3	I_A → U
W130Y	7.0	2	4.4 ± 0.2	4.3	I_A → U
wt	2.0	0	2.4 ± 0.5	1.9	I_A → U
W130Y	2.0	0			I_A → U
wt	2.0	1	4.3 ± 0.2	4.4	I_A → U
W130Y	2.0	1	3.8 ± 0.15	3.8	I_A → U

[a] Experimental conditions were 50 mM sodium phosphate buffer, pH 7.0, or 2 mM citrate buffer, pH 2.0.

by fitting a data set containing all the different experimental measurements.

Thermal denaturation was carried out by equilibrating the protein in 50 mM sodium phosphate buffer, pH 7.0, between 4 and 78 °C in a 0.1 cm Peltier cell. The reversibility of thermal denaturation was found to be >95%.

Tryptophan fluorescence emission spectra were recorded in a cuvette (1 cm light path) between 300 and 400 nm on Fluoromax single photon counting spectrofluorometer (Jobin-Yvon). The excitation wavelength was 290 nm. Circular dichroic spectra were measured in a cuvette (0.2 cm light path) on a Jasco J715 spectrometer between 200 and 300 nm in order to determine the α-helical content of the protein. The reversibility was found to be 100% under all conditions for the apoproteins.

The ellipticity measured at 222 nm was used to determine the α-helicity of the protein by use of the simple calculation proposed by Chen et al. (*23*):

$$\%\ \alpha\text{-helicity} = (\theta_{222nm} - \theta_{min}) \times 100/(\theta_{max} - \theta_{min}) \quad (1)$$

where θ_{222nm} is the molar ellipticity of the observed protein, $\theta_{min} = 2340$ is the minimum value of the molar ellipticity at 222 nm calculated for the "unordered" fraction of five proteins, and $\theta_{max} = 30\,300$ is the maximum value for the ellipticity at 222 nm as measured for the helical fraction of these five proteins.

Assuming a standard two-state model, the urea transitions were fitted to (*24*)

$$\Delta G_d = \Delta G_w - m_{D-N}D \quad (2)$$

where ΔG_w and ΔG_d are the free energy of folding in water and at the concentration D of denaturant, respectively, m_{D-N} is the slope of the transition (proportional to the increase in solvent-accessible surface area in going from the native to the denatured state), and D is the denaturant concentration. ΔG_d can be derived directly from experimental data given that

$$\Delta G_d = -RT \ln (N/U) \quad (3)$$

where $N/U = (S_N - S)/(S - S_U)$; S is the observed signal at the concentration of denaturant D, S_U is the signal of the unfolded form, and S_N is the signal of the folded form. In certain cases, it is necessary to correct this expression in order to take into account the sloping baselines for N and U, which change independently of the folding transitions under study (*25*).

Acrylamide Quenching and ANS Binding. Quenching of intrinsic tryptophan fluorescence was performed with a stock solution of 4.2 M acrylamide. Increasing amounts of acrylamide were added to a fixed amount of protein and the samples were incubated for 15 min before the fluorescence measurements. To minimize excitation of the tyrosine residue, the protein samples were excited at 295 nm and emission spectra were recorded between 300 and 400 nm. The experiments were carried out at 20 °C at two different pH values (7.0 and 2.0) in the absence of denaturant and at pH 7.0 in the presence of 5.5 M urea. The data obtained were analyzed according to a two-term Stern−Volmer equation (*26*):

$$I_0/I = (1 + K_{SV}[Q])e^{V[Q]} \quad (4)$$

where I_0 is the fluorescence of the protein in the absence of quenchers and I is the observed fluorescence at the concentration [Q] of the quencher. K_{SV} is the collisional quenching constant while V is the static constant, a parameter that is related to the probability of finding a quencher molecule close enough to a newly formed excited state to quench it immediately, or statically.

Titrations with ANS (1-anilino-8-naphthalenesulfonate) were made by adding small aliquots of concentrated ANS to protein solution (5.0 μM) (*27*). Excitation was at 295 nm. Bandwidths for excitation/emission light were 1.0/1.0 nm. The temperature of sample solution was maintained at 20 °C by a thermostatically controlled water bath.

RESULTS

Stability of the Native State of Wild-Type and W130Y Mutant Al-Mb. It was previously observed (*17*, *18*) that the denaturation profile of wild-type *Al*-apoMb is complicated by the presence of intermediate states, which can be populated to a different extent depending on experimental conditions, yielding profiles that cannot be simply analyzed. Therefore, to estimate the effect of the W130Y mutation on the total stability of the native state, we chose to compare the unfolding of the holoproteins under conditions where two-state behavior is expected (*11*, *28*). The denaturation profile of the cyanomet derivative in urea was followed by two different probes, namely, absorbance in the Soret region (421 nm) and CD at 222 nm (Figure 2). The good agreement between the two data sets demonstrates that the change in the secondary and tertiary structure report on the same transition. The reversibility of unfolding under these conditions is good (>90%) if adequate time is allowed to reach equilibrium: the slow equilibration is caused by heme dissociation upon unfolding. The single cooperative transition observed at 25 °C and pH 7, fitted to a two-state model, shows that wild type and mutant have c_m values of 5.9 and 4.8, respectively. The $\Delta G°$ for the wild type (−10.3 ± 0.2 kcal mol^{-1}) is reduced for the mutant W130Y ($\Delta G° = -8.4$ ± 0.2 kcal mol^{-1}); on the other hand the m values for the two Mbs are very similar ($m = 1.7$ kcal mol^{-1} M^{-1}).

Stability of the Intermediates of Al-ApoMb: (*A*) *Intermediate State* I_A. Inspection of the spectroscopic properties

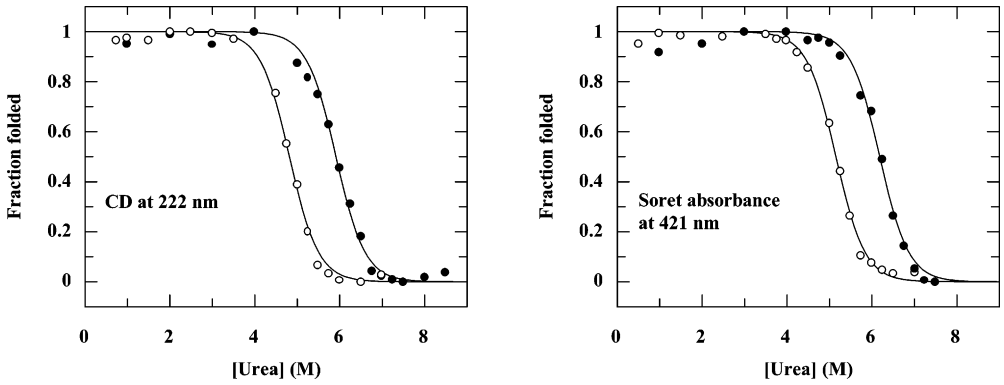

FIGURE 2: Urea-induced unfolding at pH 7 and 25 °C for wild-type (●) and W130Y (○) cyanomet *Al*-Mb. The experimental data show the dependence on urea concentration of the fraction of native state; the continuous lines are fits to the data, according to eq 3.

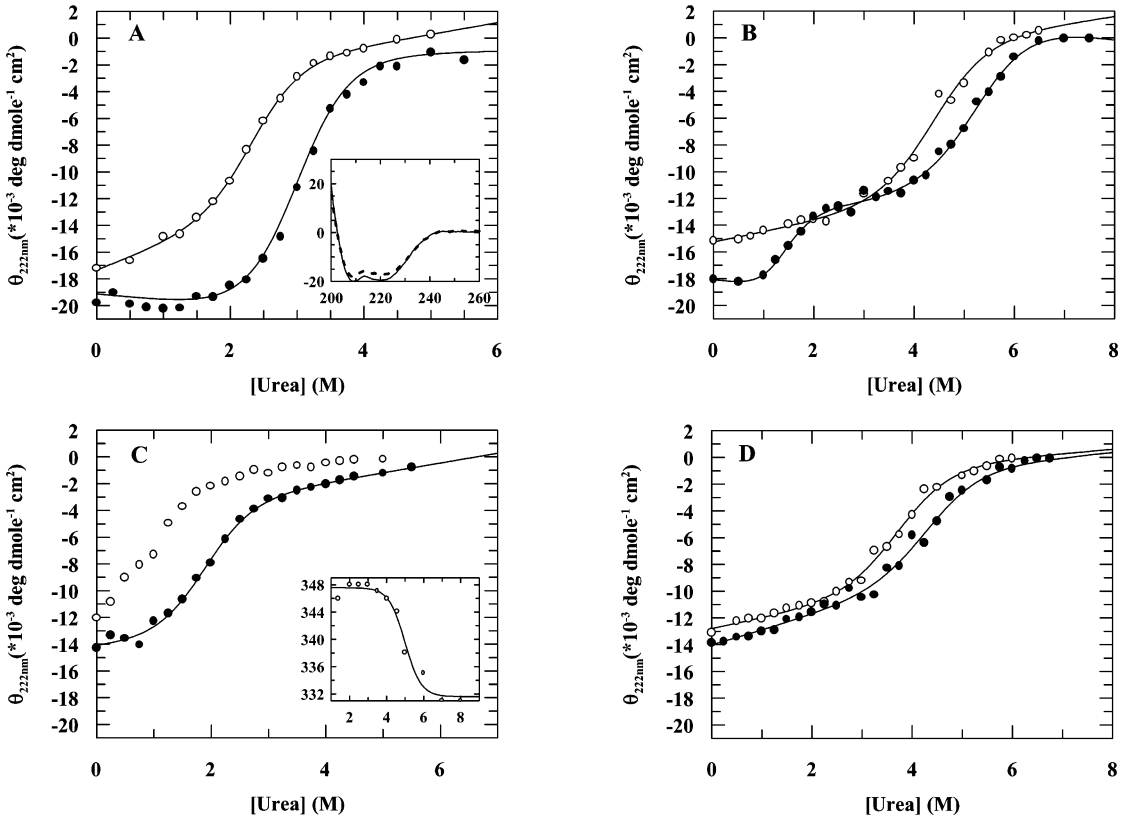

FIGURE 3: Equilibrium unfolding transition induced by urea for wild-type (●) and W130Y (○) *Al*-apoMb, as followed by far-UV CD at 222 nm. (Top panels) Experiments carried out at pH 7 and 4 °C, in the absence (A) or in the presence (B) of KCl (2 M). The inset of panel A shows the far-UV CD spectra of wild-type (solid line) and mutant (dashed line) *Al*-apoMb. The molar ellipticity as a function of wavelength is reported. (Bottom panels) Experiments at pH 2 in the absence (C) or in the presence (D) of KCl (1 M). The inset of panel C shows the pH titration of W130Y monitored by tryptophan fluorescence at 4 °C; the shift in peak wavelength is recorded as a function of urea concentration. The continuous lines are fits to the data according to eq 3 taking into account the baseline contribution.

suggests that the mutation of Trp(H8)130 into Tyr decreases the secondary structure content of the native state and increases exposure to the solvent of the remaining Trp(A12)-14, although comparison of the fluorescence data with the wild-type protein is not straightforward. At pH 7.0 and 4 °C, the ellipticities at 222 nm for the W130Y and wild-type proteins (Figure 3A, inset) are −17 000 and −20 000 deg dmol^{-1} cm^2, respectively (α-helical content 55% and 65%); the maximum of emission of the mutant (λ_{max} = 332 nm) is red-shifted compared to the wild type (λ_{max} = 326 nm). The denaturation transitions followed by CD and fluorescence are in most cases superimposable, showing not only that the two optical techniques follow the same species but also that the two Trp of wild-type *Al*-apoMb report on the same unfolding events.

The urea unfolding curves of the mutant W130Y and the wild type at pH 7.0 and 4 °C (Figure 3A) can be described by a single two-state transition, which can be fitted to eq 3 (see Materials and Methods). The folding parameters, reported in Table 1, show a destabilization of the mutant that can be estimated to be about 0.8 kcal mol^{-1}.

In an attempt to evaluate the properties of the intermediate state I_A of the mutant at pH 7.0 and 4 °C, urea denaturation was also followed in the presence of 2 M KCl. In the presence of high salt, the wild-type protein unfolds via a two-transition curve since I_A is stabilized (Figure 3B);

however in the W130Y mutant the denaturation profile is significantly different. First of all, the ellipticity of the species populated in the absence of urea is −15 300 deg dmol^{-1} cm^2, which resembles the value calculated for the *Al*-apoMb acid intermediate (∼−14 000 deg dmol^{-1} cm^2) (*17*) rather than that for the wild-type native species. Moreover, only one transition is evident over the urea concentration range where both the N ↔ I_A and the I_A ↔ U transitions are observed for the wild type (Figure 3B). Comparison of the energetics of the I_A ↔ U transition in both proteins yields a $\Delta\Delta G^0_{(mut-wt)}$ = 0.9 kcal mol^{-1} (Table 1), which confirms the destabilization seen in the absence of salt (see above).

A property typical of wild-type *Al*-apoMb acid intermediate is its stability at very acidic pH, even in low salt. This has been confirmed also for W130Y by acid denaturation experiments. The pH-induced unfolding profile of the mutant displays typical two-state behavior with a pH midpoint of 5.1 (±0.3) (Figure 3C, inset), a value higher than that of the wild type (pH midpoint of 4.0 ± 0.2) (*17*). It should be recalled that the acid denaturation of sperm whale apoMb involves protonation of two salt bridges as reported by others (*29*); only one of these is conserved in *Aplysia* apoMb, i.e., that between Glu(A4)6 and Lys(H10)132. This contact is located in the same A/H interface containing Trp(H8)130, the residue changed into Tyr in the present study. We believe that in the mutant the loosening of the nativelike interactions between the residue at position H8 and those located in the A helix (see Figure 1) may also weaken the Glu6−Lys132 salt bridge and increase the pK_A of Glu6, resulting in a destabilization of both the native state and acid intermediate I_A. At pH 2.0 and low salt conditions, however, the mutant clearly retains some secondary structure, since its ellipticity at 222 nm is −12 000 deg dmol^{-1} cm^2 (Figure 3C).

At pH 2.0 and low salt, the urea-induced denaturation for the wild-type protein corresponds to a single transition (referred to I_A ↔ U) (Figure 3C and Table 1); under the same conditions the mutant is partially folded, but it already unfolds at a very low urea concentrations (with a $c_m \cong 1$ M), which prevented a reliable estimate of the $\Delta G°$. The same experiment carried out in the presence of 1 M KCl (Figure 3D) shows that the acid intermediate is more stable and the unfolding curve can be followed more accurately. One of the most significant effects of KCl on the wild-type protein at pH 2.0 is a broadening of the I_A ↔ U transition, with loss of cooperativity; this effect was also seen for W130Y (*m* value is about 1 for both proteins). The $\Delta\Delta G^0_{(mut-wt)}$ calculated for this transition (assigned to the I_A ↔ U process) is 0.5 kcal mol^{-1} (Table 1), which indicates that at pH 2.0 addition of salt is associated with partial recovery of stability even for the W130Y mutant (see Figure 3D).

(*B*) *Intermediate State* I_T. Previous studies on *Al*-apoMb (*18*) have shown that an additional intermediate species (I_T), displaying considerable residual secondary structure, becomes evident in the denaturation profile at high temperature (≥60 °C).

To evaluate the effect of the W130Y mutation on the properties of this intermediate (I_T), the thermal unfolding profile of W130Y was monitored by far-UV CD spectroscopy (Figure 4, inset). A straightforward observation is that at high temperatures (above 70 °C) both the mutant and the wild type retain some secondary structure ($\theta_{222nm} \sim -9000$ deg dmol^{-1} cm^2). The thermal denaturation of holo-*Al*-Mb (*15*, *16*) and of the apoprotein (*18*) was already described to be fully reversible, and this is the case also for the W130Y mutant (see Materials and Methods).

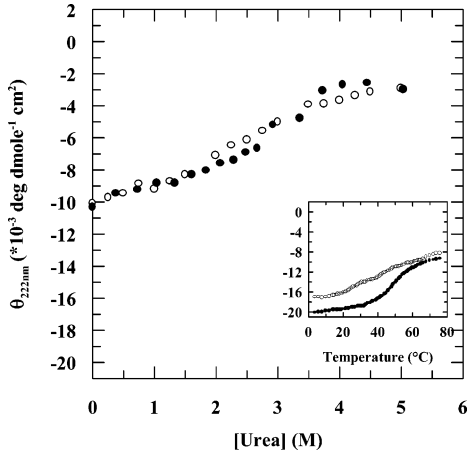

FIGURE 4: Equilibrium unfolding transition of wild-type (●) and W130Y (○) *Al*-apoMb induced by urea at 60 °C and pH 7, as followed by far-UV CD at 222 nm. The inset shows the unfolding profile as a function of temperature at 0 M urea; data were recorded between 4 and 78 °C, at 1 °C intervals.

The urea denaturation of the mutant and wild type was also followed at 60 °C and pH 7 (Figure 4), a condition where the I_T ↔ U transition is the only discernible process for the wild type. Despite the significant noise, due to intrinsic experimental difficulties, the two denaturation curves superimpose, suggesting that the stability of I_T is not significantly affected by the mutation W130Y.

Tryptophan Exposure and Heme Pocket Conformation. Measurements of the intrinsic fluorescence of proteins in the presence of a quencher can yield useful information about dynamics and accessibility of the Trp residue(s). Among other quenchers, acrylamide is often used because, being uncharged, it can diffuse in the protein matrix and quench tryptophan residues located not only on the surface but also in the apolar interior. It is accepted that acrylamide can penetrate into proteins through a diffusion process facilitated by conformational fluctuations (*30*).

To investigate the effect of acrylamide on the native, intermediate, and denatured states of *Al*-apoMb, we have carried out experiments under different conditions chosen to populate the species of interest. The experimental conditions employed were (i) 20 °C and pH 7.0 to study the native state, (ii) 20 °C at pH 2.0 to characterize the acid intermediate, and (iii) pH 7.0 in the presence of 5.5 M urea for the unfolded state.

The Stern−Volmer plot for the quenching of intrinsic emission by acrylamide in the different states of the protein, either wild type or W130Y, is shown in Figure 5. Some complexities in the interpretation of the Stern−Volmer plot may arise from the different content of tryptophanyl residues, two in the wild type and only one in the mutant. Thus, the upward curvature seen for the wild-type *Al*-apoMb may be attributed to the presence of two Trp residues: however, since the upward curvature is seen also in the single tryptophan-containing W130Y mutant, the acrylamide quenching results will be discussed, albeit qualitatively.

As expected, the solvent exposure as monitored by acrylamide quenching increases with the degree of denatur-

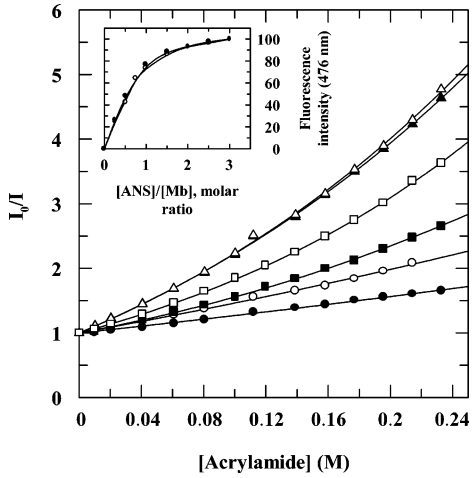

FIGURE 5: Quenching of tryptophan fluorescence by acrylamide. Wild-type (solid symbols) and W130Y (open symbols) *Al*-apoMb were assayed at 20 °C under different experimental conditions: pH 7 (circles), pH 2 (squares), and pH 7 in the presence of 5.5 M urea (triangles). I_0 is the emission of the protein in the absence of acrylamide, and I is the emission at any given concentration of acrylamide. The continuous lines represent a fit to the data with eq 4. The inset shows the effect of ANS increasing concentrations on normalized fluorescence emission intensity at 476 nm (20 °C, pH 7.0) on wild-type (●) and W130Y (○).

ation, for both the wild type and W130Y. The quenching profile of the two proteins is identical under denaturing conditions, as expected. In the native state at pH 7.0, Trp emission is more easily quenched in the mutant than in the wild type, suggesting that the mutation W130Y is associated with a greater solvent accessibility of the remaining Trp(A12)14; this behavior agrees with the significant destabilization [$\Delta\Delta G^0_{(mut-wt)} \sim 0.8$ kcal mol^{-1}] observed under these conditions for the native state of W130Y. ANS binding experiments carried out under these same conditions (pH 7.0, 20 °C; inset of Figure 5), however, indicate that, as far as the heme pocket is considered, the native state of the two proteins is essentially the same. At pH 2.0 (where the acid intermediate is populated), accessibility to acrylamide is again more marked for the mutant (Figure 5). This is a significant result which supports the view that the acid intermediate of *Al*-apoMb is stabilized by contacts involving Trp(H8)130 in the A−G−H cluster.

DISCUSSION

We have produced and characterized a site-directed mutant of *Aplysia limacina* myoglobin, a protein distantly related to the extensively investigated mammalian apomyoglobins (m-apoMb). The mutation was introduced in a key position of the A−G−H cluster of helices (Figure 1), previously shown as a region of major importance in the folding pathway of m-apoMb (*3*−*6*). We believe that the results presented here have some relevance as to the nature of the *Al*-apoMb equilibrium intermediate populated in acid (I_A) and may shed light on the folding of evolutionarily distant globins.

The mutation of Trp(H8)130 into Tyr affects the stability of *Aplysia* Mb, both for the native state and for the acid intermediate I_A, but exerts little or no effect on the thermally stable core of the intermediate I_T. Native *Aplysia* Mb is destabilized by ∼2 kcal mol^{-1} (Figure 2), and the corresponding globin by ∼0.8 kcal mol^{-1} (at pH 7.0 in low salt); moreover, the mutation W130Y considerably destabilizes I_A with respect to the wild type under all conditions. At pH 2.0, where the wild type populates the I_A species, the transition is shifted to lower denaturant concentrations ($\Delta c_m \sim 1$ M) such that a reliable measure of the stability was only possible in the presence of 1 M KCl [$\Delta\Delta G^0_{(mut-wt)} \sim 0.5$ kcal mol^{-1}, Figure 3D]. Comparison of our results on the *Al*-apoMb mutant W130Y with those obtained for the sperm whale apoMb mutant M131A (*10*) in the corresponding topological position H8 (Figure 1) clearly shows that mutation of these residues exerts a similar effect in both apoproteins.

Dynamic fluorescence quenching experiments carried out with acrylamide provide information on the accessibility of tryptophan residues and thus on the structural properties of the protein domain(s) carrying the chromophore(s). The results obtained with the *Aplysia* apoproteins (both wild type and W130Y) are in good agreement with the stability measurements and show that mutation of Trp(H8)130 to Tyr increases solvent accessibility of the remaining Trp(A12)14 in both the native and I_A intermediate states (Figure 5). It is important to note, however, that in the latter case the chromophore Trp(A12)14 is still partially buried from the solvent; at pH 2.0 the acrylamide concentration dependence of Trp quenching is indeed clearly different from what is observed in the unfolded state. This result indicates that the intermediate I_A, despite being more accessible to the quencher compared to the native state, is still a fairly compact species. The observed partial protection to quenching of Trp(A12)-14 in the intermediate state I_A of W130Y, together with the effect of the mutation on the free energy of I_A, suggests that stabilization of this species involves interactions between the A and H helices.

In summary, the data reported above highlight the role of the H8 position in the stability of the main intermediate in the folding pathway of globins and suggest that different apoMbs may fold via compact helical intermediates whose structure is tuned by the local propensities to form elements of secondary structures and by the network of tertiary interactions established by the helices. A notable case is the folding pathway of apoLb (*13*) and that of some site-directed mutants of m-apoMb with altered helical stability (*31*, *32*) in which alternative helices may be recruited in the main intermediate.

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REFERENCES

1. Gunasekaran, K., Eyles, S. J., Hagler, A. T., and Gierasch, L. M. (2001) *Curr. Opin. Struct. Biol. 11(1)*, 83−93.
2. Griko, Y. V., Privalov, P. L., Venyaminos, S. Y., and Kutyshenko, V. P. (1988) *J. Mol. Biol. 202*, 127−138.
3. Jennings, P. A., and Wright, P. E. (1993) *Science 262*, 892−896.
4. Hughson, F. M., Wright, P. E., and Baldwin, R. L. (1990) *Science 249*, 1544−1548.
5. Jamin, M., Syun-Ru, Y., Rousseau, D. L., and Baldwin, R. L. (1999) *J. Mol. Biol. 292*, 731−740.
6. Nishimura, C., Dyson, H. J., and Wright, P. (2002) *J. Mol. Biol. 322*, 483−489.

7. Loh, S. N., Kay, M. S., and Baldwin, R. L. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 5446−5450.
8. Gilmanshin, R., Williams, S., Callender, R. H., Woodruff, W. H., and Dyer, R. B. (1998) *Nat. Struct. Biol. 5*, 363−365.
9. Jamin, M., and Baldwin, R. L. (1998) *J. Mol. Biol. 276*, 491−504.
10. Kay, M. S., and Baldwin, R. L. (1996) *Nat. Struct. Biol. 3*, 439−445.
11. Kay, M. S., Ramos, C. H. I., and Baldwin, R. L. (1999) *Proc. Natl. Acad. Sci. U.S.A. 96*, 2007−2012.
12. De Sanctis, G., Ascoli, F., and Brunori, M. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 11507−11511.
13. Nishimura, C., Prytulla, S., Dyson, H. J., and Wright, P. E. (2000) *Nat. Struct. Biol. 7*, 679−686.
14. Bolognesi, M., Onesti, S., Gatti, G., Coda, A., Ascenzi, P., and Brunori, M. (1989) *J. Mol. Biol. 205*, 529−544.
15. Brunori, M., Giacometti, M., Antonini, E., and Wyman, J. (1972) *J. Mol. Biol. 63*, 139−152.
16. Giacometti, G. M., Antonini, E., and Brunori, M. (1979) *Biophys. Chem. 10*, 119−127.
17. Staniforth, R. A., Bigotti, M. G., Cutruzzolà, F., Travaglini-Allocatelli, C., and Brunori, M. (1998) *J. Mol. Biol. 275*, 133−148.
18. Staniforth, R. A., Giannini, S., Bigotti, M. G., Cutruzzolà, F., Travaglini-Allocatelli, C., and Brunori, M. (2000) *J. Mol. Biol. 297*, 1231−1244.
19. Ptitsyn, O. B., and Ting, K. H. (1999) *J. Mol. Biol. 291*, 671−682.
20. Cutruzzolà, F., Travaglini-Allocatelli, C., Brancaccio, A., and Brunori, M. (1996) *Biochem. J. 314*, 83−90.
21. Rossi-Fanelli, A., Antonini, E., and Caputo, A. (1958) *Biochim. Biophys. Acta 30*, 608−615.
22. Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.
23. Chen, Y.-H., Yang, J. T., and Martinez, H. M. (1972) *Biochemistry 11*, 4120−4131.
24. Pace, C. N. (1990) *Trends Biotechnol. 8*, 93−98.
25. Santoro, M. M., and Bolen, D. W. (1988) *Biochemistry 27*, 8063−8068.
26. Eftink, M. R., and Ghiron, C. A. (1976) *Biochemistry 15*, 672−680.
27. Stryer, L. (1965) *J. Mol. Biol. 13*, 482−495.
28. Ramos, C. H., Kay, M. S., and Baldwin, R. L. (1999) *Biochemistry 38*, 9783−9790.
29. Yang, A. S., and Honig, B. (1994) *J. Mol. Biol. 237*, 602−614.
30. Eftink, M. R., and Ghiron, C. A. (1981). *Anal. Biochem 114(2)*, 199−227.
31. Cavagnero, S., Nishimura, C., Schwarzinger, S., Dyson, H. J., and Wright, P. E. (2001) *Biochemistry 40*, 14459−14467.
32. Garcia, C., Nishimura, C., Cavagnero, S., Dyson, H. J., and Wright, P. E. (2000) *Biochemistry 39*, 11227−11237.

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