Stability of Myoglobin: A Model for the Folding of Heme Proteins†

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ABSTRACT: Factors governing the stability of sperm whale, pig, and human metmyoglobin were examined by (1) measuring guanidinium chloride induced unfolding of apoglobins containing 22 replacements at positions 29(B10), 43(CD1), 64(E7), 68(E11), and 107(G8), (2) determining the rates of hemin loss from the recombinant holoproteins, and (3) estimating constitutive expression levels of the corresponding genes in *Escherichia coli* TB-1 cells. The denaturant titrations were analyzed in terms of a two-step unfolding reaction, N(native apoprotein) → I(intermediate) → U(unfolded), in which the intermediate is visualized by an increase in tryptophan fluorescence emission. Two key conclusions were reached. First, high rates of hemin loss are not necessarily correlated with unstable globin structures and *vice versa*. In general, both rates of hemin loss and the equilibrium constants for apoprotein unfolding must be determined in order to understand the overall stability of heme proteins and to predict the efficiency of their expression. Second, polar residues in the distal pocket cause marked decreases in the overall stability of apomyoglobin. Removal of hemin from V68N and L29N sperm whale myoglobins produces the molten globular I state at pH 7, 25 °C, without addition of denaturant. In contrast, the H64L and H64F mutations produce apoproteins which are 10–30 times more stable than wild-type apoglobin. The latter results show that protein stability is sacrificed in order to have the distal histidine (H64) present to increase O_2 affinity and inhibit autooxidation.

Myoglobin has long been the paradigm for understanding structure–function relationships in proteins. The development of recombinant expression systems has allowed the use of site-directed mutagenesis to examine how the globin portion of the molecule regulates oxygen affinity, enhances discrimination against CO, influences rates of ligand binding, and inhibits autooxidation [for a review, see Springer et al. (1994)]. Similar protein engineering approaches have been applied to studies of the stability of both myoglobin and apomyoglobin (Barrick & Baldwin, 1993, 1993a; Pinker et al., 1993). The latter work is of general interest for three reasons. First, apomyoglobin has a well defined globular structure comprising eight tightly packed helical regions which are present in all heme-containing proteins involved in oxygen transport and storage. Second, heme binding to apomyoglobin provides a model system for studying the influence of a prosthetic group on protein stability and individual folding processes. Third, these studies of myoglobin provide valuable background information for increasing the stabilities and expression yields of recombinant hemoglobins which are being developed as potential extracellular blood substitutes (Wagenbach et al., 1991; Looker et al., 1992; Hernan et al., 1992; Shen et al., 1993).

The minimal scheme for myoglobin denaturation is shown in Figure 1 [adapted from Hughson et al. (1990)]. The rate of heme loss from reduced myoglobin is extremely slow ($t_{1/2}$ ≈ months at room temperature, pH 7), whereas that from oxidized myoglobin is at least 50-fold faster ($t_{1/2}$ ≈ 1–3 days; Hargrove et al., 1994). Thus, under physiological conditions autooxidation precedes denaturation (Brantley et al., 1993). After hemin loss, unfolding occurs in two stages: partial unfolding of the native apoprotein (N) to a molten globule intermediate (I) and then complete disruption of all the helical segments (U).

The two-step unfolding scheme for apomyoglobin shown in Figure 1 was first proposed by Balestrieri et al. (1976) on the basis of tryptophan fluorescence changes induced by guanidinium chloride (GdmCl) denaturation, 1-anilino-8-naphthalene sulfonate (ANS) binding studies, and previous pH titrations by Kirby and Steiner (1970). The loss of ANS binding ability correlates with an increase in tryptophan fluorescence as [GdmCl] is raised from 0 to 2 M. Higher concentrations of denaturant cause a significant red-shift of the emission maximum and a decrease in fluorescence intensity (Irace et al., 1981; Colonna et al., 1982). Baldwin, Barrick, Hughson, and Wright have used circular dichroism, site-directed mutagenesis, and hydrogen exchange/2D NMR to define the structural features of N and I during acid and urea denaturation (Hughson & Baldwin, 1989; Hughson et al., 1990; Barrick & Baldwin, 1993; Barrick et al., 1994). Native apomyoglobin (N) is estimated to be ~60% helical with partial unfolding of the C, D, and F helices and the CD corner (Hughson et al., 1991; Griko et al., 1988). The N → I transition is thought to involve unfolding of the B and E helices with complete loss of the heme binding site. This intermediate has been designated a molten globule because although two-thirds of the protein is unfolded, the A, G, and H helices appear intact as measured by circular dichroism and amide proton exchange (Hughson et al., 1990; Figure 1).

In order to examine more systematically the factors governing the overall stability of myoglobin, we have measured rates of hemin loss and equilibrium unfolding parameters for

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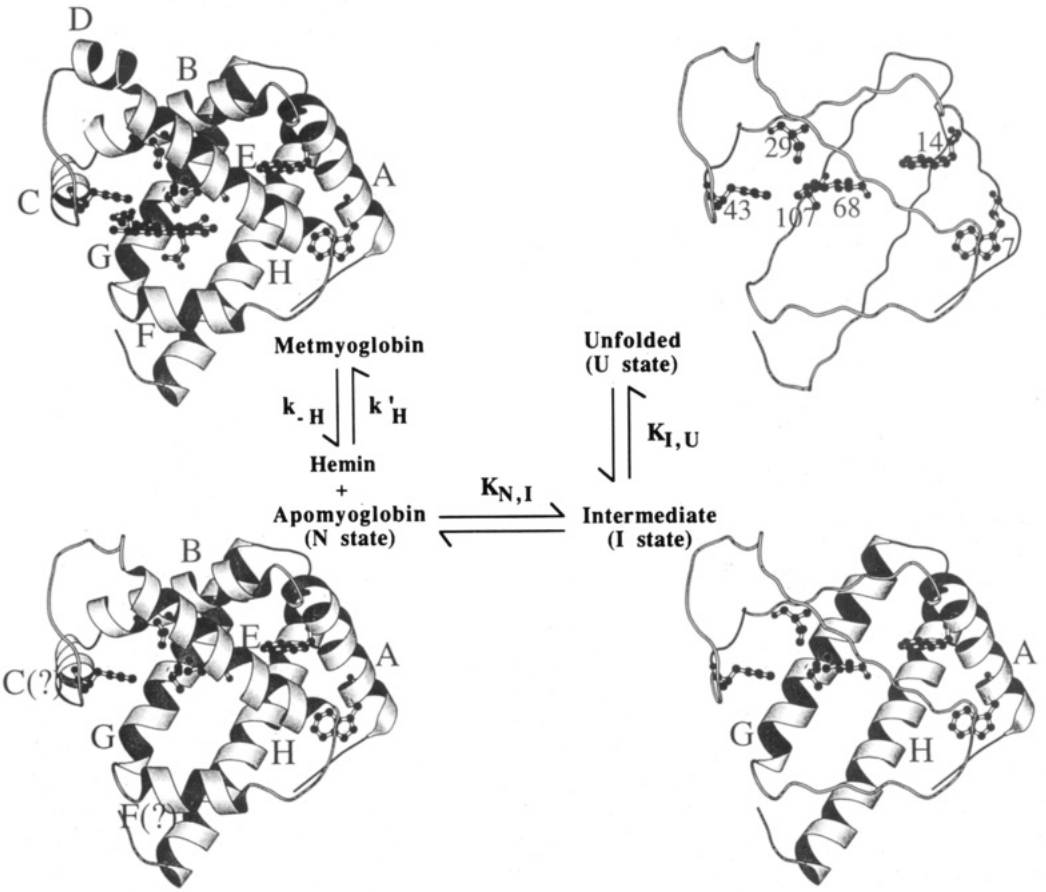

FIGURE 1: Structural model for myoglobin denaturation. The model was adapted from Barrick and Baldwin (1993) and Hughson et al. (1990). Ferric myoglobin oxidizes and loses heme, yielding apomyoglobin in the N state. The B, C, and E helices which make up the heme pocket then unfold to give the molten globule I state in which the A, G, and H helices are still intact. The last step is conversion of the intermediate to the completely unfolded U state. The wide ribbons indicate intact helical secondary structures. The narrow lines indicate unfolded strands that have much more random positions than implied in the MOLSCRIPT (Kraulis, 1991) drawings which keep the atoms in the original locations in the P_6 wild-type metmyoglobin structure (Phillips et al., 1990). Trp7 and Trp14 are shown on the right side of each globin, along the A helix, and residues Leu29, Phe43, Val68, and Ile107 are seen in the hemin binding pocket.

22 mutants with substitutions in the distal portion of the heme pocket. Plots of tryptophan fluorescence emission intensity versus [GdmCl] facilitate quantitative analysis in terms of the N → I → U scheme because the intermediate is observed as a peak in the titration data (Figures 2–4). Our attention was focused on residues located adjacent to the bound ligand and the heme group in an effort to understand the evolutionary pressures which strike a compromise between efficient oxygen binding, resistance to autooxidation, high heme affinity, and apoglobin stability. Spectacular and often compensating changes are observed in resistance to hemin loss and apoglobin stability. These results demonstrate that the stability of heme proteins must be considered in terms of both folding of the apoprotein and binding of the prosthetic group. They also confirm independently that the E and B helices are disrupted in the molten globule intermediate of apomyoglobin and suggest protein engineering strategies for improving both the overall yield and extracellular half-life of recombinant hemoglobins.

EXPERIMENTAL PROCEDURES

Preparation of Myoglobins and Hemin Loss Assay. Native sperm whale myoglobin was obtained by special permit from Sigma. Sperm whale myoglobin mutants at positions 68 and 64 were constructed at Rice University using cassette mutagenesis or had been prepared by Barry A. Springer and Karen D. Egeberg (H64L, H64F, H64Q, V68A, and V68F; Rohlfs et al., 1990; Egeberg et al., 1990). Sperm whale myoglobin mutants at positions 29, 43, and 107 were constructed from a pEMBL19 vector containing the gene for wild-type myoglobin (Carver et al., 1992). All recombinant myoglobins were expressed and purified at Rice University following the procedures described by Springer and Sligar (1987) and Carver et al. (1992). Expression yields of recombinant myoglobins were estimated as milligrams of purified protein produced per 100 g of cell paste. These levels were estimated from routine fermentation runs and protein preparations. For the unstable mutants, protein was kept in the reduced CO form during and after cell lysis in an effort to prevent hemin loss and denaturation during purification. Some losses still occurred during purification of the L29N and V68N mutants, but the lack of color in the cell pastes confirmed that the major cause of the low yield was poor expression. Pig and human myoglobin mutants were constructed and expressed as fusion proteins following the procedures described by Varadarajan et al. (1985), Smerdon et al. (1991), and Ikeda-Saito et al. (1991). Apomyoglobins were prepared using the methyl ethyl ketone method described by Ascoli et al. (1981) and Hargrove et al. (1994).

Hemin dissociation was measured by following the absorbance changes (usually at 409 nm) associated with transfer of hemin from holoprotein to excess H64Y/V68F apomyoglobin (Hargrove et al., 1994). Resultant time courses were fitted to a single-exponential expression to obtain the first-order dissociation rate constant for hemin loss, k_{-H}. These experiments were carried out in 0.15 M buffer/0.45 M sucrose,

37 °C, at either pH 7.0 (potassium phosphate) or pH 5.0 (sodium acetate).

Fluorescence Measurements. Frozen apomyoglobin samples were quickly thawed, centrifuged at 4 °C to remove any precipitate, and stored on ice. Desired GdmCl concentrations were achieved by mixing a stock GdmCl solution [prepared as described in Pace et al. (1990)] with an appropriate volume of 0.2 M potassium phosphate, pH 7.0, to give a total volume of 1 mL in the cuvette. Ten microliters of a stock (100–500 μM) apoprotein solution was added to give a final protein concentration between 1 and 5 μM. Each spectrum in Figure 2 and point in Figures 3 and 4 represents an independently prepared solution, with great care taken to ensure that the same protein concentration was present during a specific titration. Errors due to differences in protein concentration were estimated to be ~±5% based on volumetric accuracy and to have little effect on the observed titrations curves (Figure 2) and fitted unfolding constants (see the legend to Table 2).

All denaturation studies were carried out at 25 °C. Fluorescence intensity and emission spectra were measured with an SLM 8100 spectrofluorimeter. One photomultiplier was used to measure emission spectra between 300 and 400 nm. At the same time, a 320-nm cutoff filter was placed in front of another photomultiplier to measure total intensity during the emission scan. Excitation was normally at 285 nm, but the results are independent of excitation wavelength between 285 and 295 nm. To ensure that equilibrium unfolding was achieved, emission spectra were recorded successively until no changes in total intensity were observed during the scan. Most samples (including native and wild-type sperm whale apomyoglobin) reached equilibrium before the first scan. The V68T, V68S, and V107T mutants often required 2–5 min to reach equilibrium.

Estimates of the unfolding equilibrium constants, $K_{N,I}$ and $K_{I,U}$, were obtained by fitting the fluorescence titration data to the three-state scheme described by Barrick and Baldwin (1993). Total fluorescence intensity, F_{obs}, is given by

$$F_{obs} = \frac{F_N + F_I K_{N,I} e^{(m_{N,I}x)} + F_U K_{N,I} e^{(m_{N,I}x)} K_{I,U} e^{(m_{I,U}x)}}{1 + K_{N,I} e^{(m_{N,I}x)} + K_{N,I} e^{(m_{N,I}x)} K_{I,U} e^{(m_{I,U}x)}} \quad (1)$$

where F_N, F_I, and F_U are the fluorescence intensities of pure N state, I state, and U state, respectively; x is the molarity of GdmCl, and $m_{N,I}$ and $m_{I,U}$ are the differential denaturant binding parameters [eq 15 of Barrick and Baldwin (1993)]. The unfolding equilibrium constants are assumed to vary exponentially with denaturant concentration (i.e., a linear dependence of $\Delta G_{transition}$ on [GdmCl]; Pace et al., 1990). Initially, the titration data for all proteins in Table 2 were fitted to eq 1 allowing all seven parameters to vary. Then the average of the m values was calculated and fixed in succeeding analyses to allow more direct comparisons between the mutants. The average values were 4.1 and 2.8 M^{-1} for $m_{N,I}$ and $m_{I,U}$, respectively. Fluorescence intensity was normalized to the maximum observed at intermediate GdmCl concentrations and then fitted to eq 1 allowing F_N, F_I, F_U, $K_{N,I}$, and $K_{I,U}$ to vary. For most mutants, relative values of F_N, F_I, and F_U were 0.6, 1.0, and 0.7, respectively. Because L29N and V68N are almost entirely in the I state under nondenaturing conditions, F_N was fixed at 0.6 for these mutants during fitting. Overall stability of the native apoglobin was estimated as $1/K_{N,I}K_{I,U}$, or $K_{U,N}$. A compilation of the fitted equilibrium constants is given in Table 2 along with rates of hemin loss at pH 5 and 7 for 25 different recombinant myoglobins. Errors in the unfolding constants were estimated to be ± 20–30% based on the standard deviation from the mean of parameters fitted to three completely independent titrations of wild-type sperm whale myoglobin (Table 2).

Table 1: Hemin Loss Rates and Constitutive Expression Yields of Sperm Whale Myoglobin Mutants[a]

myoglobin	k_{-H} at pH 7, 37 °C (h^{-1})	yield of pure protein (mg of MbCO/100 g of cells)
wild-type (D122N)	0.007 ± 0.005[b]	~120
L29N	0.10	~1–5
L29F	<0.01	~100
F43V	2	~6
F43I	2	~50
H64A	0.40	~80
H64F	0.08	~120
V68N	~0.01	~1–5
V68F	≤0.01	~110

[a] k_{-H} is the rate of hemin dissociation at pH 7, 37 °C. Myoglobins were prepared and expression yields estimated as described under Experimental Procedures. [b] Hargrove et al. (1994).

As a control, circular dichroism spectra for sperm whale wild-type, L29N, and V68N holo- and apoproteins were measured between 215 and 250 nm in a Jasco J-500C spectropolarimeter. The samples were prepared in 0.01 M potassium phosphate buffer, pH 7.0, 25 °C, and the fractional amount of helicity was estimated as described by Pace et al. (1990).

RESULTS

Expression Levels of Holomyoglobin and Rates of Hemin Loss. A large number of thermal, pH, urea, and guanidinium chloride unfolding studies have focused on holomyoglobin, primarily because large absorbance changes accompany denaturation and the loss of hemin. However, interpretation of these results is ambiguous because in most cases resistance to hemin loss, rather than stability of the apoprotein structure, is being measured. It is often assumed that the two properties are directly correlated, but, as will be shown, this is generally not true. Constitutive expression of myoglobins and hemoglobins in *Escherichia coli* is related to the efficiency of the promoter, codon usage, the rate of hemin synthesis, and the intrinsic stability of the apo- and holoproteins (Springer & Sligar, 1987). In Table 1, the yield of recombinant sperm whale myoglobin is compared to the rate of hemin dissociation for the set of mutants showing the widest range of behavior. Because the same expression vector and host strain were used in each case, the differences in expression among the mutants must be due to changes in overall stability of the protein. The results show that the yield is not readily predicted from the rate of hemin loss. For example, the rate of hemin dissociation from V68N metmyoglobin at pH 7.0 is approximately equal to that of the wild-type holoprotein, whereas the yield of the mutant protein is 25–100-fold less than that of wild-type myoglobin. The opposite situation occurs for H64A myoglobin; this mutant loses hemin ~40 times more rapidly than native metmyoglobin but is expressed at a level which is only 30% lower than that of the wild-type protein. The simplest explanation is that H64A apoprotein is very stable, compensating for rapid loss of hemin, whereas V68N apoprotein denatures readily in the bacterial cytoplasm, presumably before hemin can bind. In order to test this interpretation, the resistance of the corresponding apoproteins to denaturation in GdmCl was measured.

Fluorescence Titrations and Analyses. The fluorescence changes associated with apomyoglobin unfolding are shown

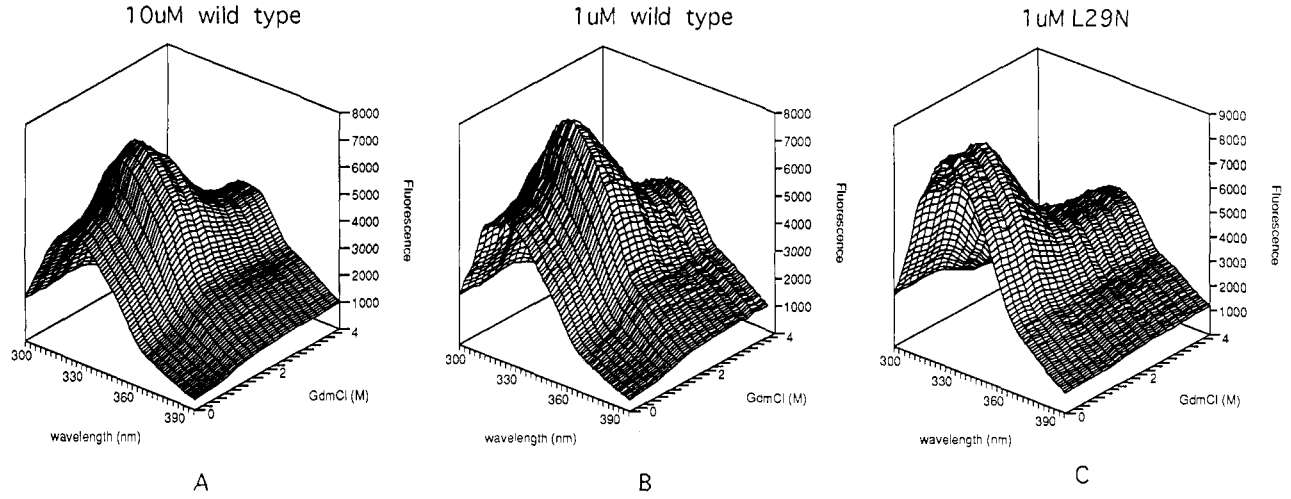

FIGURE 2: Fluorescence measurements. (A and B) 3D plots of fluorescence intensity, wavelength, and [GdmCl] for titrations of wild-type apomyoglobin at 1 and 10 μM, respectively. (C) The same titration carried out with L29N sperm whale apomyoglobin at 1 μM.

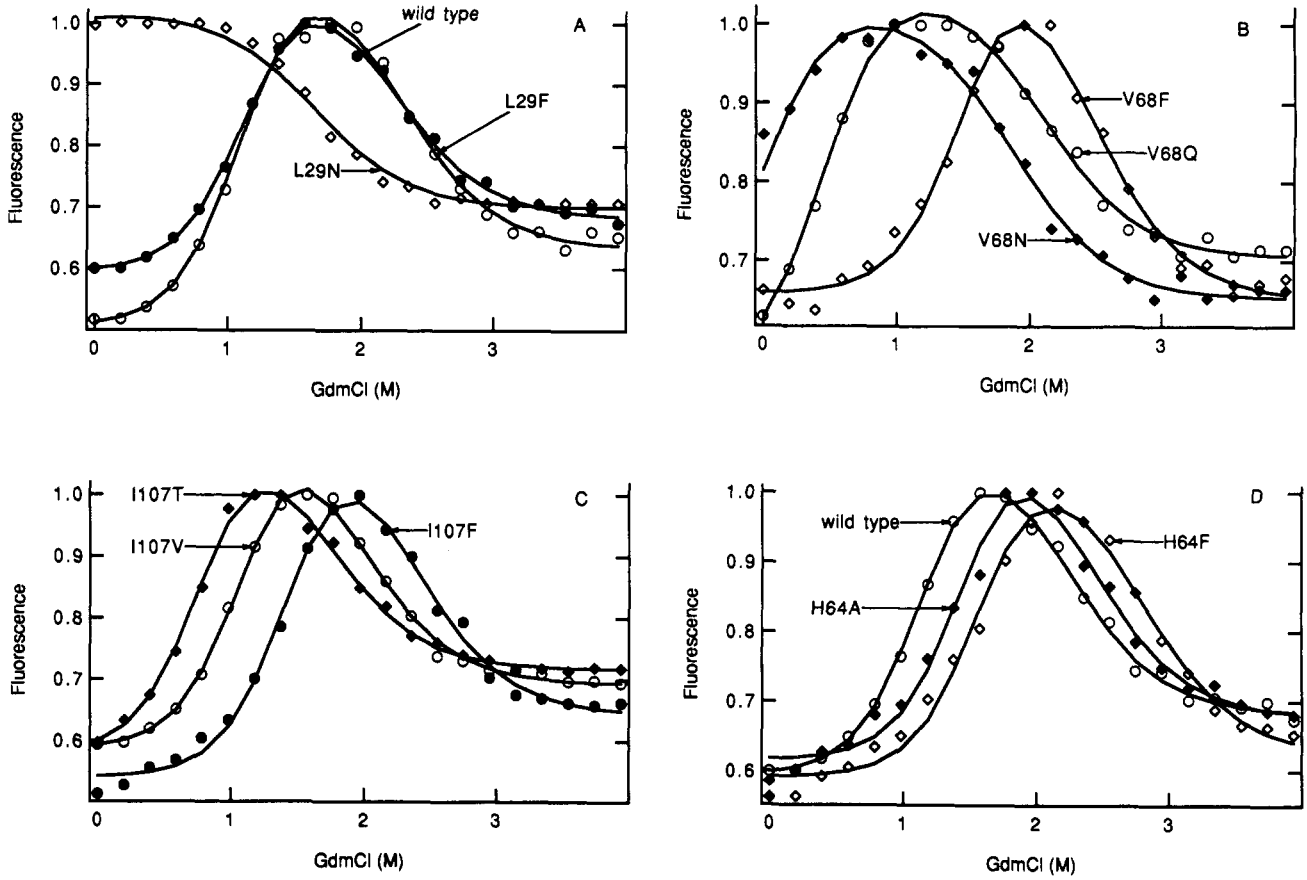

FIGURE 3: Analysis of fluorescence titrations. (A) Total fluorescence intensity of Leu29 mutants is plotted as a function of [GdmCl]. (B, C, and D) The same plots for Val68, Ile107, and His64 mutants, respectively. The symbols represent observed data points and the solid lines fits to eq 1 using the equilibrium parameters listed in Table 2. Total fluorescence intensity was measured directly using a 320-nm cutoff filter.

in Figures 2–5. Holomyoglobin has little tryptophan fluorescence due to quenching by the heme group. Fluorescence from the N state of apomyoglobin is due to Trp7, which is significantly quenched by Lys79(EF2), and Trp14, which emits strongly because it is buried in an apolar region between the A, G, and H helices (Irace et al., 1981; Figure 1). There is an increase in fluorescence and a small red-shift in the emission maximum as [GdmCl] is increased from 0 to 2 M. These changes appear to be due to movement of Lys79 away from Trp7 when the E-helix unfolds during formation of the molten globule intermediate. The increase in fluorescence is not due to aggregation because the shapes of the titration curves are independent of apoprotein concentration, at least in the range 1–10 μM (Figure 2A,B). Complete denaturation at [GdmCl] > 2 M exposes both tryptophans to solvent causing quenching and a red-shift in the emission maximum to ~350 nm (Irace et al., 1981; Figures 2 and 5).

When the same GdmCl titration is carried out with V68N or L29N sperm whale apomyoglobin, the initial fluorescence is much greater than that of the native or wild-type protein (Figures 2C, 3A, and 3B). These mutant apoproteins are partially or completely in the I state even before denaturant is added. As shown in Figures 2C and 3A, the L29N apoprotein shows only a single transition associated with

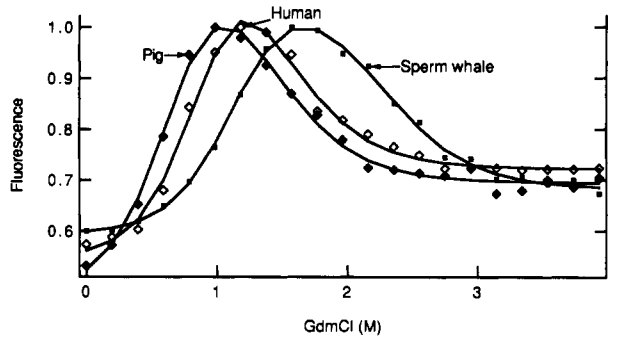

FIGURE 4: Comparisons of sperm whale, pig, and human apomyoglobins. The total fluorescence intensities of sperm whale, pig, and human apomyoglobins are plotted versus [GdmCl], and the lines represent fits to eq 1.

conversion of I to U. To confirm that the increased fluorescence intensities of V68N and L29N apoglobins are due to the changes in helicity associated with the N → I transition, circular dichroism spectra of the mutant apoproteins and holoproteins were measured from 215 to 250 nm. Under nondenaturing conditions, V68N and L29N apoproteins were found to have approximately half the helicity of wild-type apomyoglobin, whereas the helicities of the mutant and wild-type holoproteins were identical (data not shown).

Effects of Mutagenesis at Positions 29(B10) and 68(E11). Replacing aliphatic side chains with amides or alcohols produces a more polar heme pocket and, in general, destabilizes the native apoglobin conformation (N) with respect to the molten globule intermediate (I) and the completely unfolded state (U). These changes are observed as increases in the unfolding equilibrium constants, $K_{N,I}$ and $K_{I,U}$, and a decrease in overall stability, $1/K_{N,I}K_{I,U}$ (Table 2). Replacing smaller residues with larger aliphatic or aromatic amino acids creates a more hydrophobic distal heme pocket and stabilizes the N state, with the major effect being a decrease in $K_{N,I}$.

The L29N mutation causes complete loss of the native apoprotein conformation, and, as a result, the mutant apoglobin exists in the molten globular I state in the absence of GdmCl at pH 7, 25 °C (Figures 2 and 3A; Table 2). The instability of L29N apomyoglobin results in extremely poor constitutive expression in *E. coli* TB-1 cells (Table 1). Presumably the molten globule intermediate is incapable of binding heme efficiently and is readily degraded by intracellular proteases. However, once the prosthetic group is bound, the L29N mutant folds into a relatively stable holoprotein whose rate of hemin loss is only 8–10 times greater than that of wild-type myoglobin at both pH 5.0 and 7.0. Val68 was replaced by six different residues of varying size and polarity (Figure 3B; Table 2). Again, increasing polarity increases $K_{N,I}$ but does not have a large effect on $K_{I,U}$. The asparagine substitution produces the most unstable apoprotein, explaining the poor expression yield of the V68N mutant (Table 1). The V68T and V68S mutations also produce large increases in $K_{N,I}$, whereas the V68F substitution causes an ~5-fold decrease in $K_{N,I}$ and a 2-fold decrease in $K_{I,U}$.

The generality of these results for sperm whale myoglobin was tested by measuring unfolding of V68N, V68S, and V68T mutants of pig myoglobin. Surprisingly, wild-type pig apomyoglobin is ~100-fold less stable than sperm whale apoprotein (Figure 4). As a result, it is difficult to quantitate the effects of mutagenesis in the pig system. However, qualitatively similar effects are observed when Val68 is replaced with Asn, Ser, and Thr. All three mutations cause extensive unfolding. The fluorescence emission spectrum of the pig V68T apoprotein at 0 M GdmCl appears to be a mixture

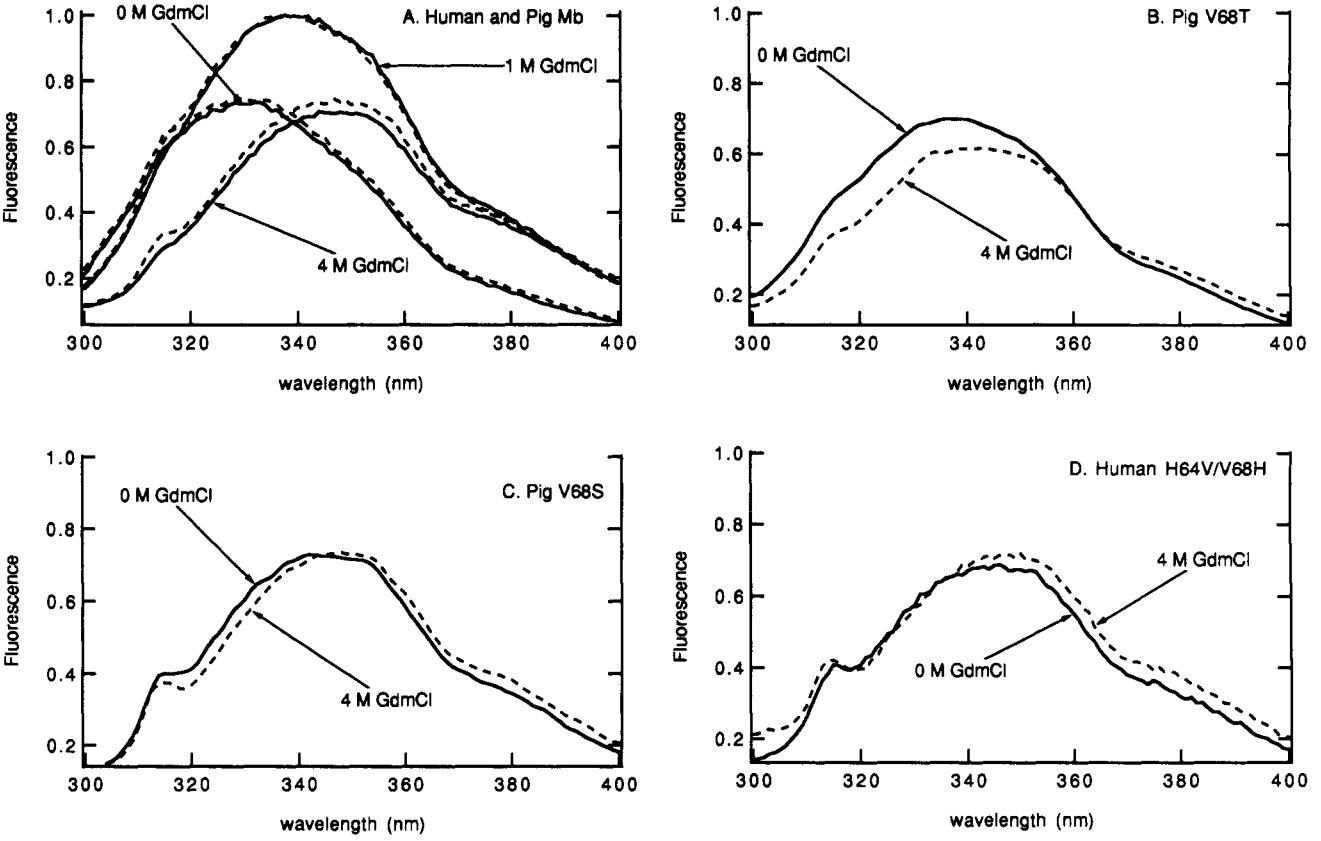

FIGURE 5: Stabilities of pig and human apomyoglobin mutants. (A) Fluorescence emission spectra of pig (solid lines) and human (dashed lines) apomyoglobins at 0, 1, and 4 M GdmCl. (B, C, and D) Spectra of pig V68T, pig V68S, and human H64V/V68H myoglobins, respectively, at 0 (solid lines) and 4 M GdmCl (dashed lines). Spectra were recorded as described under Experimental Procedures.

Table 2: Hemin Loss, Folding, and Stability Parameters for apoMb[a]

myoglobin	k_{-H} at pH 5.0 (h^{-1})	k_{-H} at pH 7.0 (h^{-1})	$K_{N,I}$ at pH 7.0	$K_{I,U}$ at pH 7.0	$1/(K_{N,I}K_{I,U})$ (stability)
(A) sperm whale proteins					
native	1.0	~0.01	0.002	0.004	125 000
wild-type(N122)	1.0	~0.01	0.011 (±0.003)	0.002 (±0.0002)	45 000 (±18 000)
wild-type(D122)	1.0	~0.01	0.010	0.0015	70 000
L29F	2.5	<0.01	0.01	0.001	100 000
L29N	8.3	0.10	~50	0.01	<10
F43V	54	2	0.7	0.001	1400
F43I	38	2	0.018	0.002	28 000
H64Q	3.5	0.12	0.01	0.002	50 000
H64A	17	0.40	0.003	0.001	330 000
H64L	11	0.20	0.0027	0.0005	740 000
H64F	4.8	0.08	0.0016	0.0004	1 600 000
V68F	2.0	~0.01	0.002	0.001	500 000
V68A	5.8	0.10	0.019	0.002	26 000
V68Q	2.4	0.03	0.16	0.003	2100
V68N	5.2	~0.01	1	0.005	200
V68S	0.9	0.10	0.60	0.002	800
V68T	~0.1	<0.01	0.068	0.002	7400
I107F	1.1	~0.01	0.003	0.001	330 000
I107V	1.3	~0.01	0.01	0.005	20 000
I107T	1.4	0.15	0.038	0.009	2900
(B) pig myoglobin					
wild-type	1.0	<0.01	0.057	0.029	600
V68N	1.6	0.07			≪1
V68S	8.7	≤0.01			≪1
V68T	0.5	≤0.01			<1
(C) human myoglobin					
wild-type	3	~0.01	0.019	0.026	2000
H64V/V68H	<0.1	≪0.01			≪1

[a] Hemin dissociation experiments were carried out at 37 °C. Unfolding equilibrium constants were measured at 25 °C. The errors (~±20–30%) estimated for the wild-type sperm whale apoglobin (N122) equilibrium constants represent the standard deviation from the mean of three completely independent titrations and analyses carried out for this purpose. Normally, wild-type sperm whale myoglobin is expressed with an Asn122 residue to facilitate crystallization in the P_6 form (Phillips et al., 1990). The unfolding of "corrected" wild-type (D122) was measured to examine the effect of the Asn substitution. All the recombinant sperm whale proteins have an extra N-terminal Met residue which is designated as residue 0. Wild-type human myoglobin has a C110A mutation to facilitate expression (Varadarajan et al., 1985).

of contributions from the molten globular intermediate and the completely unfolded state with no evidence for the presence of the N state (Figure 5B). V68N and V68S pig apoproteins have almost identical spectra at 0 and 4 M GdmCl, implying that these proteins are completely unfolded under nondenaturing conditions (Figure 5C).

In both pig and sperm whale myoglobin, the isosteric V68T replacement uncouples the rate of hemin loss from apoglobin stability. Sperm whale Thr68 apoprotein is 8 times less stable than wild-type apoglobin but loses hemin at least 10 times more slowly at both pH 5 and 7. The extremely slow rate of hemin dissociation is due to the "extra" hydrogen bond between the coordinated water molecule and the β-hydroxyl group of the Thr68 side chain (Smerdon et al., 1991). This interaction increases hemin affinity, compensates for apoglobin instability, and facilitates higher yields of holoprotein in both the pig and sperm whale myoglobin expression systems. The most extreme example of compensation for low globin stability was observed for the H64V/V68H double mutation in human myoglobin. Removal of hemin produces a completely unfolded apoglobin (Figure 5D). The reason for success in producing holoprotein is that His68 coordinates the heme iron, creating a cytochrome b-like structure (Qin et al., 1994). As a result, hemin binding is effectively irreversible under physiological conditions, and hemin loss from the H64V/V68H mutant could not be detected at either pH 5 or 7 (Table 2).

Mutagenesis at Positions 107(G8) and 43(CD1). Unfolding of I107V, I107T, and I107F sperm whale apoglobins was examined to test whether the effects of mutagenesis at positions 29 and 68 are due specifically to changes in polarity of residues in the B and E helices or a result of more global changes in polarity of the distal portion of the heme pocket. The latter interpretation appears valid since the overall stabilities of the G8 mutants are Phe107 > Ile107(wild-type) ≥ Val107 > Thr107 apoglobin (Table 2; Figure 3C). Again, the greatest effect was on the N → I equilibrium constant. Only the I107T mutation had a significant effect on the rate of hemin loss, causing k_{-H} to increase from ~0.01 to 0.15 h^{-1} at pH 7.

Phe43(CD1) and the proximal histidine, His93(F8), are conserved in all globins (Dickerson & Geis, 1983). Naturally occurring point mutations at the CD1 positions in the α and β subunits of human hemoglobin produce unstable proteins which readily lose heme and cause the formation of Heinz bodies inside red cells (Bunn & Forget, 1986). These observations led Honig et al. (1990) and others to suggest that Phe-CD1 is required for heme binding. This interpretation is verified quantitatively in Table 2. Both the F43V and F43I mutations cause 200-fold increases in the rate of hemin dissociation at pH 7. Phe43(CD1) also plays an important role in stabilizing the native apoglobin structure because there is a 40-fold decrease in apoprotein stability when Val replaces this residue (Table 2). As with the other substitutions in the distal pocket, this appears to be a general hydrophobic effect since the F43I mutation produces only a small, ~2-fold decrease in the stability of the N state and in expression yield (Tables 1 and 2).

Replacement of the Distal Histidine. Mutations at position 64 exert the same general effects on apomyoglobin stability as those at other positions in the heme pocket. Replacing the polar distal histidine with Leu or Phe stabilizes the apoprotein 20–30-fold (Figure 3D and Table 2) whereas the Gln substitution has little effect. Even the H64A mutation stabilizes the apoprotein 6-fold with respect to wild-type apoprotein. As in the case of the position 68 mutations, there

is little correlation between rates of hemin loss and apoglobin stability. His64 (wild-type) metmyoglobin has a low rate of hemin loss because the imidazole side chain stabilizes coordinated water by hydrogen-bonding interactions which are lost or weakened in the aliphatic mutants at this position (Quillin et al., 1993).

DISCUSSION

Relative Importance of Hemin Affinity and Apoglobin Stability. The resistance of holomyoglobin to denaturation is a function of both the intrinsic stability of the apoprotein tertiary structure and the strength of interactions with the prosthetic group. Because the affinity of apomyoglobin for hemin is extremely high ($\approx 3 \times 10^{14}$ M^{-1}), the holoprotein is much more stable to denaturation than the apoprotein (Griko et al., 1988; Hughson & Baldwin, 1989; Hargrove et al., 1994). Thermal, urea, and GdmCl induced "melting" of intact myoglobin is determined by the extent of hemin dissociation, which is followed immediately by rapid globin unfolding at the elevated temperatures and denaturant concentrations required to induce loss of the prosthetic group. The bimolecular rate of hemin binding appears to be approximately the same for all apohemoglobins and apomyoglobins, $k'_H \approx 2$–8×10^7 M^{-1} s^{-1} (Gibson & Antonini, 1960; Rose & Olson, 1983; Light, 1986). Even in preliminary heme binding experiments with those mutants showing molten globular behavior, the initial bimolecular rate constant is reduced only 3–4-fold (M. S. Hargrove and J. S. Olson, unpublished observations). Thus, hemin affinity is governed primarily by the rate of hemin dissociation, k_{-H}, which varies 100–1000-fold when comparing hemoglobins and myoglobins (Hargrove et al., 1994). A comprehensive survey of the factors governing the interaction of heme and globin is currently underway [see Smerdon et al. (1993) and Hargrove et al. (1994)].

Polar residues adjacent to the iron atom can inhibit hemin loss by forming hydrogen bonds with coordinated water but, after hemin dissociation, destabilize the native apoglobin structure by causing solvation of the heme pocket and subsequent unfolding of the E and B helices. Aliphatic and aromatic substitutions at the B10, E11, and G8 positions have a greater favorable effect on $K_{N,I}$ than on k_{-H} (Table 2). Thus, the apolar residues found naturally at these locations are more important for stabilizing the native apoglobin structure than for hemin binding. These results also support the two-step unfolding model shown in Figure 1, which predicts that the introduction of polar side chains in the middle of the E and B helices should induce unfolding of these secondary structures and formation of the molten globule intermediate. Hydrogen exchange studies of some of these mutants are underway to test these structural interpretations more directly.

His 64(E7): Functionality at the Expense of Stability. Hydrogen-bonding interactions with the distal histidine are required for high O_2 affinity, discrimination against CO binding, and inhibition of autooxidation (Brantley et al., 1993; Springer et al., 1994). Many of these functions can also be carried out by the amide side chain of Gln. Consequently, His or Gln are at the E7 helical position in almost all myoglobins and hemoglobins, and high rates of autooxidation and poor discrimination against CO binding are observed for the exceptions which have Val or Leu at the E7 position (Dickerson & Geis, 1983; Rohlfs et al., 1990; Brantley et al., 1993). As shown in Table 2, conservation of the distal histidine is at the expense of globin stability. All aliphatic or aromatic substitutions at position 64 produce apoglobins that are more stable than wild-type apoprotein. A similar sacrifice of protein folding stability for functionality has been noted for key catalytic residues in barnase (Meiering et al., 1992).

The results in Table 2 suggest that it will be difficult to engineer myoglobin to carry out peroxidase, oxidase, and other reactions which require multiple polar or charged residues in the heme pocket. Introduction of polar groups at the B10, E11, or G8 positions inhibits globin folding and expression markedly, particularly in pig and human myoglobin, where complete denaturation occurs when hemin is removed from these mutants (Figure 5). This unfavorable effect on holoprotein expression can be overcome by putting His at the E11 position to facilitate direct coordination to the iron atom (Table 2). However, the resultant protein is hexacoordinate in both the ferrous and ferric states and relatively unreactive toward exogenous ligands (Qin et al., 1994). Thus, the myoglobin fold appears to be a highly specialized tertiary structure that is not readily readapted for other heme protein functions.

Species Differences. As shown in Figure 4, both pig and human apomyoglobins are much more readily denatured than the sperm whale apoprotein. Native horse apomyoglobin is also significantly less stable than sperm whale apoglobin (data not shown). Both $K_{N,I}$ and $K_{I,U}$ are larger for the pig and human apoproteins, and their overall stabilities are decreased 30 and 100-fold, respectively, compared to sperm whale apomyoglobin. In contrast, the rates of hemin loss from all three holoproteins are the same at pH 7.0. Because most previous comparative studies involved denaturation of the holoproteins, the marked differences between apoglobins have gone relatively unnoticed (Flanagan et al., 1983; Puett, 1973). However, Baldwin and co-workers have reported differences between the pH denaturation profiles of sperm whale and human apoglobins. The human protein forms the I state at somewhat higher pH values than the whale globin (Hughson & Baldwin, 1989; Hughson et al., 1991).

The physiological significance of enhanced stability of sperm whale apomyoglobin is probably related to the harsh conditions that occur in whale muscle tissue during diving. Prolonged exposure to anaerobic conditions causes acidification of muscle cell cytoplasm, and denaturation may also be enhanced by the high pressures associated with deep dives (Schmidt-Nielsen, 1990). A molecular explanation of the differences in stability of pig, human, and sperm whale proteins is less clear. Sequence differences among the three proteins are listed in Table 3. Our working hypothesis is that the Val/Ala vs Gly differences at positions 1, 15, and 74 and the Ser vs Gly difference at position 35 cause the differences in stability to GdmCl denaturation. The V1G and A15G replacements may serve to destabilize the A helix causing an increase in $K_{I,U}$, and the A74G and S35G substitutions could destabilize the E and C helices, respectively, causing an increase in $K_{N,I}$ (Matthews et al., 1987). This interpretation is being tested by additional mutagenesis studies and species comparisons. Finally, native sperm whale myoglobin is ~ 3 times more stable than the recombinant protein which contains an "extra" N-terminal Met residue. This is the first large difference that has been noted between the properties of native and wild-type myoglobin and also indicates that the N-terminal region of the A helix plays a role in stabilizing the native globin structure relative to the molten globule intermediate.

Protein Engineering Strategies. The results in Tables 1 and 2 provide background data for rationally designing more efficient expression systems and more stable, functional proteins. For example, large quantities of V68N and L29N myoglobin cannot be made using the simple constitutive expression system developed by Springer and Sligar (1987)

Table 3: Comparison of the Sequences of Native Sperm Whale, Human, and Pig Myoglobins[a]

	residue number (helical region)																										
myoglobin	1 (NA)	4 (A)	12 (A)	15 (A)	21 (B)	22 (B)	27 (B)	28 (B)	35 (C)	45 (CD)	51 (D)	53 (D)	66 (E)	74 (E)	86 (F)	87 (F)	101 (G)	110 (G)	113 (G)	116 (G)	118 (GH)	132 (H)	140 (H)	142 (H)	144 (H)	145 (H)	151 (H)
sperm whale	V	E	H	A	V	A	D	I	S	R	T	A	V	A	L	K	I	A	H	H	R	N	K	I	A	K	Y
human	G	D	N	G	I	P	E	V	G	K	S	D	A	G	I	K	V	C	Q	Q	K	N	K	M	S	N	F
pig	G	D	N	G	V	A	E	V	G	K	S	D	N	G	L	T	V	A	Q	Q	K	S	N	M	A	K	F

[a] A listing of known mammalian myoglobin sequences was obtained using the EuGene and SAM software package developed by the Molecular Biology Information Resource, Department of Cell Biology, Baylor College of Medicine, Houston, TX, and the Protein Sequence Data Bank from the National Biomedical Research Foundation, Washington, D.C. Wild-type human myoglobin has a C110A mutation to prevent intermolecular disulfide formation so there is no difference at 110 between the pig, sperm whale, and human myoglobins listed in Table 2. The wild-type sperm whale myoglobin used in these studies has an extra N-terminal methionine and a D122N substitution (Table 2) compared to the native sperm whale protein.

for sperm whale myoglobin. The apoproteins are too unstable and hemin synthesis is too slow to prevent denaturation. However, holoproteins containing these mutations can be made in fairly large quantities with the fusion-protein system used to express pig and human myoglobins as inclusion bodies (Nagai & Thørgensen, 1987). Refolding of the denatured apoglobin is induced *in vitro* with excess hemin, and, once formed, the mutant holoproteins are almost as resistant to hemin loss as wild-type myoglobin.

The results in Tables 1 and 2 indicate that expression yields can be enhanced by stabilizing the native apoglobin structure and provide examples of distal pocket mutations which decrease $K_{N,I}$. The V68F substitution was combined with the distinctive spectral properties of the Tyr64 mutant to engineer a more stable reagent for measuring hemin loss (i.e., apoH64Y/V68F; Hargrove et al., 1994). We have also incorporated this substitution into recombinant hemoglobin in an attempt to increase apohemoglobin stability without increasing rates of autooxidation or hemin loss. The differences between pig, human, and sperm whale myoglobin suggest other approaches to stabilizing apohemoglobin subunits. Increasing resistance to hemin loss and globin denaturation is of great clinical importance for maximizing the safety, effectiveness, and retention time of extracellular hemoglobin-based blood substitutes.

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