Apoglobin Stability Is the Major Factor Governing both Cell-free and in Vivo Expression of Holomyoglobin*±

Premila P. Samuel1, Lucian P. Smith1, George N. Phillips, Jr.15, and John S. Olson12
From 1BioSciences at Rice and 2Department of Chemistry, Rice University, Houston, Texas 77005

Expression levels in animal muscle tissues and in Escherichia coli vary widely for naturally occurring mammalian myoglobins (Mb). To explore this variation, we developed an in vitro transcription and wheat germ extract-based translation assay to examine quantitatively the factors that govern expression of holoMb. We constructed a library of naturally occurring Mbs from two terrestrial and four deep-diving aquatic mammals and three distal histidine mutants designed to enhance apoglobin stability but decrease hemin affinity. A strong linear correlation is observed between cell-free expression levels of holo-metMb variants and their corresponding apoglobin stabilities, which were measured independently by guanidine HCl-induced unfolding titrations using purified proteins. In contrast, there is little dependence of expression on hemin affinity. Our results confirm quantitatively that deep diving mammals have highly stable Mbs that express to higher levels in animal muscle tissues and in E. coli, and the wheat germ cell-free system than Mbs from terrestrial mammals. Our theoretical analyses show that the rate of aggregation of unfolded apoproteins is very large, and as a result, the key factor for high level expression of holoMb, and presumably other heme proteins, is an ultra high fraction of folded, native apoglobin that is capable of rapidly binding hemin. This fraction is determined by the overall equilibrium folding constant and not hemin affinity. These results also demonstrate that the cell-free transcription/translation system can be used as a high throughput platform to screen for apoglobin stability without the need to generate large amounts of protein for in vitro unfolding measurements.

Myoglobin (Mb) serves as a model system for examining the factors that govern the expression of large amounts of heme proteins for which both protein folding and cofactor binding are required. It is a single domain globin comprised of eight α-helical segments, labeled A to H, that can bind iron protoporphyrin IX either in the ferrous (heme) or ferric state (hemin) through axial coordination of the iron atom with a proximal histidine (His-93 at the F8 helical position). Ligands bind to the iron atom on the distal side of the protoporphyrin ring and include O2 in the ferrous state and H2O in the ferric state. These exogenous ligands are stabilized by hydrogen bonding to the distal histidine (His-64 at the E7 helical position) in almost all wild-type mammalian Mbs (1).

Recently, Culbertson and Olson (2) developed the six-state model shown in Fig. 1 to describe the unfolding of holo-metMb and dissociation of hemin and then used it to analyze quantitatively sets of GdnHCl-induced folding curves for a series of holo- and apoMb variants. This analysis was based on the mechanisms reported by Barrick and co-workers (3, 4) and Wright and co-workers (5, 6) for the unfolding of apoMb and the generation of a molten globule intermediate (I) in which the heme pocket was melted but the A, G, and H helices remained mostly intact. Culbertson and Olson (2) showed that hemin can bind to the intermediate to generate a hemichrome structure but that the affinity of the I state for hemin is much less than that of the native (N) state. In the absence of GdnHCl, unfolding leads to aggregation of the unfolded globin states (UH, U), making the process irreversible. Any released free hemin is highly toxic in vivo where it partitions into membranes and promotes lipid oxidation and generation of reactive oxygen species (7–10).

Most studies of gene expression examine the regulation of mRNA synthesis by promoter sequences and transcription factors, mRNA structure and stability, and rates of translation on ribosomes. Our focus is on the final steps of expression involving the folding and assembly of fully functional holomyoglobin,
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![Six-state mechanism for holoMb unfolding modified from Culbertson and Olson (2).](Image)

The three states of apoMb are native (N) with most of the helices folded, intermediate (I) with the heme pocket mostly unfolded, and completely unfolded (U). HoloMb states containing bound heme (H) are native (NH), intermediate (IH) with the heme pocket melted and a hemichrome structure, and unfolded (UH) with heme bound nonspecifically. which include the reverse of the processes shown in Fig. 1 and depend on the amino acid sequence of the polypeptide chain. Unfolded (U) polypeptide comes off the ribosomes, folds into the N state, and binds heme to produce the ferric NH state, which in turn can be reduced, bind O₂ or CO, and be further stabilized in the holoprotein form. However, as shown in Fig. 1, net synthesis of holo-metMb and its reduction competes with aggregation and precipitation of both unfolded apoprotein and free hemin. Thus, multiple factors should contribute to expression yields of holoMb, including apoprotein stability, hemin affinity, and reduction and ligand binding.

Initial semi-quantitative studies by Hargrove et al. (11) suggested that heterologous expression of mammalian Mbs in *Escherichia coli* is governed more by globin stability than by heme affinity. Scott et al. (12) showed that apomyoglobins from deep diving whales are 10–500 times more resistant to unfolding than apoglobins from terrestrial animals. They suggested that the increased resistance to denaturation was required due to acidosis that occurred in whale muscle during prolonged divers. Scott et al. (12) also noted that the enhanced apoglobin stability could explain the high expression yields of sperm whale holoMb in *E. coli* but not pig or human holoMbs. This issue had been puzzling because most mammalian holoMbs appear to have similar stabilities (11, 13). However, Scott et al. (12) did not examine the differences in expression of the mammalian Mbs in *E. coli* quantitatively.

More recently, in a bioinformatics study, Mirceta et al. (14) suggested that the enhanced apoprotein stabilities of deep diving mammal Mbs evolved to allow much higher expression of the protein in the muscles of these animals. For example, the level of Mb in the skeletal muscles of sperm whale is roughly 70 mg/g of wet tissue, whereas the amount in pig muscle is only 2–4 mg/g of wet tissue, a greater than 10-fold difference (14). They noted that MbO₂ is the primary source of oxygen for swimming during deep dives when blood circulation is diverted from the skeletal muscles to keep the heart and brain of the diving animal well oxygenated. Mirceta et al. (14) also discovered that the higher expressing Mbs in aquatic mammals had, on average, a larger positive surface charge (Zₘₐₜ = +2.5 to +4.8 at neutral pH) that may have contributed to their higher apoglobin stability.

Between 2001 and 2003, Smith (15) surveyed the expression levels in *E. coli* of a large library of ~250 site-directed and randomly generated heme pocket mutants of sperm whale Mb. All the variant genes were cloned into the same expression vectors, and the goal was to examine compromises between apoglobin expression, stability, and functional O₂ binding. Smith (15) observed a correlation between the overall folding constant (1/K₅₉₅ or K₅₉₅) and the relative expression yield in the *E. coli*, but the scatter in the data were substantial and there were clear outliers, which suggested enhanced susceptibility to proteolysis (Fig. 10A) (15).

In this work, we have adapted the CellFree Sciences (ENDEXT® technology) *in vitro* protein synthesis system (16, 17) to test and examine quantitatively the various ideas about holoMb expression that were derived from our previous more qualitative studies. The decoupled *in vitro* transcription and wheat germ-based translation system allows more control over the amounts of DNA, mRNA, amino acids, ATP, and hemin present (16). Most proteases have also been eliminated (17). In addition, the soluble holoMb product can be quickly separated from the translation mixture and partially purified, and its spectral properties in the ferric state can be measured quantitatively.

In *E. coli* most of these variables cannot be as easily controlled. Because the availability of cofactor can also be limiting, hemin is often added exogenously, with or without co-expression of heme transporter genes, or δ-aminolevulonic acid is added to enhance bacterial heme synthesis (18, 19). In the expression studies performed by Smith (15) in *E. coli*, no external hemin was added; Mb was not purified from lysates; and absolute spectra were not measured. Another complicating factor in *E. coli* is that the expressed holoMb is kept reduced in the bacterial cytoplasm, and in some cases, it binds endogenously produced CO, which could greatly stabilize the protein, particularly those variants in which the distal histidine is replaced with Phe or Leu (1, 20).

We have used the cell-free protein synthesis system to examine holo-metMb expression for a library of naturally occurring Mb variants, including pig, human, sperm whale, gray seal, goosebeak whale, and dwarf sperm whale, which span the range of *in vitro* apoglobin stabilities reported by Scott et al. (12) and the levels of myoglobin found in skeletal muscle as reported by Mirceta et al. (14). Gray seal Mb was chosen at the suggestion of Berenbrink and co-workers (14) based on their sequence comparisons and preliminary unfolding studies, both of which indicate strongly that deep diving seals also have large apoMb stability constants. Berenbrink and co-workers are systematically testing this idea of a convergent evolution of high Mb stability in various families of diving animals. Then, three distal pocket mutants were constructed to significantly enhance globin sta-

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4 M. Berenbrink, personal communication.
bility but reduce hemin affinity based on the results described by Smith (15) and include: H64F/V68F sperm whale Mb and H64L and H64F/V68F dwarf sperm whale Mb.

Our study verifies unambiguously that apoMb stability determines holoprotein expression levels, regardless of whether translation occurs in vitro, in E. coli, or in mammalian myocytes (14). This work also lays the groundwork for high throughput screening of apoprotein stability for large libraries of globin variants without having to purify milligram quantities of pure protein.

**Experimental Procedures**

**Construction and Expression of Mb Genes—**Three of the recombinant whale and seal Mb genes were synthetically constructed by Integrated DNA Technologies based on the protein sequences obtained from the UniProtKB/Swiss-Prot database (dwarf sperm whale (Kogia simus, Q0KIV5.3), goosebeak whale (Ziphus cavirostris, P02182.2), and gray seal (Halichoerus grypus, P68081)).

The original wild-type recombinant sperm whale (Sw) (Physeter catodon) Mb protein contains Asn-122 instead of the native Asp-122 residue, based on the gene originally synthesized by Springer and Silgar (21). This original synthetic Sw Mb gene has served as the genetic background for all of Sw Mb mutants used in our cell-free assays, E. coli expression assays by Smith (15), most other in vitro unfolding studies, kinetic measurements, and structural determinations done by the Olson and Phillips groups. These previous studies showed that the differences between the Asp-122 and Asn-122 variants, when they occur, are very small (2, 11, 12, 15, 22, 23). However, as a control we did construct the Asp-122 Sw Mb variant (see Fig. 2, mRNA gel) and saw little difference between the in vitro expression yields of the Asp-122 Sw Mb (data not shown) and that of wild-type Sw Mb containing Asn-122, which was then kept as the control Sw Mb gene. During the large scale expression of all the whale and seal Mbs in E. coli, no removal of the N-Met occurs, whereas the initiator methionine is removed in native muscle Mbs. However, Scott et al. (12) showed that presence or absence of the N-Met had little or no effect on the measured overall folding constant \(K_{\text{UN}}\) for Sw apoMb. Finally, because the N-Met is removed in native mammalian Mbs, the numbering of amino acids starts with N-Val or N-Gly as residue 1, and N-Met in the recombinant Mbs is listed as 0 (1).

The pig Mb gene was a gift from Dr. Anthony Wilkinson (University of York, York, UK (11)). Both the human Mb gene and protein were kind gifts from Dr. Masao Ikeda-Saito. As described in Hargrove et al. (11), these recombinant myoglobins were expressed in E. coli as denatured fusion proteins in inclusion bodies, reconstituted with hemin, and the N-terminal sequence was cleaved with trypsin to generate holoprotein without the N-terminal Met. As a control, we managed to express a small amount of pig Mb in E. coli with the N-Met present. The measured value of \(K_{\text{UN}}\) in 0.01 M potassium phosphate, pH 7, at 25 °C was \(\sim 100\) and almost identical to the value for the native pig apoprotein (\(K_{\text{UN}} = 83\), see Table 1).

For the cell-free expression system, all nine Mb genes were cloned into pEU vectors (Fig. 2) modified by the Center of Eukaryotic Structural Genomics (24–26). For expression in E. coli, the dwarf sperm whale, gray seal, goosebeak whale, and sperm whale Mb genes were cloned into the pVP80K vector, and sequence-specific ribosome-binding sites were designed utilizing the Salis Lab Calculator to optimize yields (27, 28).

**Expression of Hologlobins**

![Image](media/fig2.jpg)

**FIGURE 2. Cell-free screening assay for holomMb incorporating in vitro transcription and cell-free expression (ENDEXT® technology) and small scale protein purification.** DNase-RNase free pEU-Mb DNA template was used to transcribe mRNA in vitro. The gel electrophoresis image at the upper right corner shows Dw, pig, Sw Asn-122, and Sw Asp-122 Mb mRNAs transcribed without any degradation, where Dw and Sw refer to dwarf sperm whale and sperm whale Mbs, respectively. The Mb mRNA transcript was then incubated with the wheat germ extract in a bilayer cell-free translation reaction for 20 h at 15 °C (17, 24, 26, 31, 32). The cell-free expressed holomMb was purified on a Zn2⁺-chelating resin mini-column using a well filter plate (see “Experimental Procedures”), and 6 μl of the purified sample was loaded on an SDS-polyacrylamide gel. The SDS-polyacrylamide gel image at the bottom left corner shows the successful cell-free expression of Dw, pig, and Sw holomMbs, confirmed by the sharp bands at 17 kDa, with the less stable pig Mb showing a less intense band. The 4th and 5th lanes in the gel represent reactions in which no mRNA was added for the translation incubation. Hemin was added to the wheat germ extract loaded in the 4th lane, whereas none was added for the sample in the 5th lane. The supernatants for the control extracts were also run through the Zn²⁺-chelating resin mini-column filter plates following translation incubation. The SDS-polyacrylamide gels verify that no significant amount of protein with a mass of 17 kDa was expressed and purified when Mb mRNA was omitted.
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with other translation cofactors into the wheat germ extract where the Mb mRNA is being translated into polypeptides off the ribosomes. Preparations of required materials and the cell-free expressions were done in a DNase-RNase-free room at Rice University. All glassware was baked at 180 °C for 3 h before use, and solutions were prepared with DNase-RNase-free distilled water (Invitrogen) (25).

Free hemin can dimerize, aggregate, and adsorb to glass and plastic surfaces at high hemin concentrations and low pH values (33). Glassware and pH probes used for hemin preparation were rinsed with 1 M NaOH to reduce hemin adsorption. A stock solution of fresh hemin in 1 M NaOH was prepared 1–2 h before initiating the cell-free translation reaction because irreversible hemin aggregation increases slowly with time (34). The pH value of the stock hemin solution was then lowered to pH 8.4 by gradual titration with 50% acetic acid. Acetic acid is a weak ligand for hemin, can reduce hemin self-aggregation, and has been shown to increase hemin solubility at neutral and low pH values (35). Next, the hemin stock solution was spun down to separate out any insoluble hemin before the solution concentration was measured spectrophotometrically, using ε598 = 122 mm−1 cm−1 (36, 37). Finally, a total of 5.65 nmol of hemin was added to the upper bilayer of the cell-free translation reaction in 3 aliquots every hour beginning an hour after initiation of translation to decrease hemin aggregation. The pH of the hemin added had been optimized to ensure that both hemin solubility and the conditions for the cell-free protein expression system were not compromised.

Small Scale Purification of Cell-free Expressed Mb—Hemoglobinins and Mbs are rich with natural Zn2+-binding sites on their surfaces (38, 39). A small scale holoMb purification protocol was modified from the protocol for His6-tagged proteins (S. Makino, University of Wisconsin, Center for Eukaryotic Structural Genomics) (40). Zn2+-chelating resin (GBiosciences) was added into the wells of a MultiScreenHTS HV 0.45-µm filter plate (Millipore) to purify 10 µl of each resuspended translation reaction mixture containing the Zn2+ resin for collection of wash buffers and then the eluted holoMb, following centrifugation of the plates at 3640 rpm for 1 min (40). The pH and salt concentration of the purification wash and elution buffers were optimized as described below to reduce nonspecific binding of eukaryotic proteins on the Zn2+ resin (41).

Forty µl of Zn2+ resin was loaded in each well of the filter plate and washed with 200 µl of distilled, deionized water. Next, 100 µl of each resuspended translation reaction mixture was pipetted on top of the wells containing resin, along with 140 µl of 0.1 M sodium phosphate equilibration buffer, pH 7, and incubated on a plate shaker for 20 min before the plate was centrifuged. Then, the protein bound on the resin was subjected to decreasing salt washes of 150-µl volumes as follows: 1st wash buffer (0.5 M sodium chloride, 0.1 M sodium phosphate, pH 7), 2nd wash buffer (0.3 M sodium chloride, 0.1 M sodium phosphate, pH 7), and 3rd wash buffer (0.1 M sodium chloride, 0.1 sodium phosphate, pH 7). Finally, the resin was incubated with 60 µl of elution buffer (0.1 M sodium phosphate, 15 mM EDTA, pH 7.4) for 10 min on a plate shaker. The plates were centrifuged again to elute the bound holoMb protein.

Quantitative Measurement of HoloMb Expression—At the end of the cell-free translation incubation, the expression of holoMb was examined qualitatively by the appearance of brownish metMb color in the wheat germ extract. The cell-free expression of holoMb was confirmed after partial purification of the soluble protein by measurement of Soret absorbance (A) peaks (see Fig. 3) and protein bands at ~17 kDa (molecular mass of Mbs) in SDS-polyacrylamide gels (see Fig. 2, bottom left). Note that protein precipitates do not pass through the Zn2+ resin mini-columns, which also act as filters to remove particulates.

The cell-free expression level of the semi-purified, soluble holoMb was quantified as the concentration of Mb determined from the Soret peak absorbance normalized to an estimate of the total protein concentration present, which was quantified by the observed absorbance at 280 nm (Equation 1).

\[
\text{holoMb expression yield} = \frac{A_{\text{Soret}}}{A_{280}} \times \frac{\epsilon_{\text{Soret}}}{\epsilon_{280}} \quad \text{(Eq. 1)}
\]

This ratio takes into account differences in amounts of total protein present in the partially purified sample due to soluble enzymes from the wheat germ ribosomal system, which would affect translational efficiency, and any residual folded apomyoglobin. The latter should be minimal in the soluble fraction because excess hemin was present. For the native ferric holoMb, the Soret peak is at 409.5 nm and has an ε409.5 ≈ 157 mm−1 cm−1, whereas the 280-nm peak has an ε280 ≈ 31.2 mm−1 cm−1, which was used in Equation 1 as an average ε280 for holoMb (42). For the ferric holoMb heme pocket mutants H64L and H64F/V68F, the Soret absorbance peak is at 395 nm with ε395 = 103 mm−1 cm−1 (43). The variance in triplicate assays was reduced significantly when Equation 1 was used to normalize the holoMb yield by the amount of total protein present.

Measurement of mRNA Levels Transcribed in Vitro for Cell-free Translation—After transcription, the mRNA was treated with DNase I (RNase-free) to digest any template DNA left
untranscribed. Then, the transcription solution was purified with a MEGAclear™ kit (Life Technologies, Inc.) to minimize the presence of free nucleotides and digested DNA template. To measure the amount of mRNA present, a cDNA reverse transcription reaction was performed using a sequence-specific primer for each Mb mRNA species. The amount of cDNA generated was measured through quantitative PCR, utilizing the SYBR® Green JumpStart™ Taq ReadyMix™ (Sigma) for high throughput quantitative PCR.

Indirect ELISA—After translation of dwarf sperm whale and sperm whale Mbs, unpurified cell-free reaction mixtures were spun down to separate the pellet containing precipitated protein from the lysate-containing soluble protein. Wells in a 96-well clear flat bottom plate (Corning® Costar®) were then individually coated with 50 μl of lysate and pellet dilutions in 50 mM carbonate buffer, pH 9.6 (Sigma). The plates were incubated overnight at 4 °C. Each well was then blocked at room temperature for 1 h with a 1% bovine serum albumin (BSA), bated overnight at 4 °C. Each well was then blocked at room temperature for 1 h with a 1% bovine serum albumin (BSA), 0.02% azide solution in phosphate-buffered saline (PBS). Next, each well was incubated at room temperature with 50 μl of FL-154 rabbit polyclonal IgG Mb antibody (Santa Cruz Biotechnology) at 1:50 dilution in the blocking buffer for 2 h and then incubated with 100 μl of goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology) at 1:2000 dilution in 1% BSA in PBS for 1 h. Wells were washed with 100 μl of PBS with 0.05% Tween 20 between different steps. Finally, each well was incubated with 50 μl of Thermo Scientific Pierce 1-Step™ Ultra TMB-ELISA substrate for 15 min before the peroxidase reaction was stopped with 50% sulfuric acid. Absorbance was read at 450 nm using a TECAN Infinite M1000 Pro microplate reader (44).

Large Scale Preparation of Proteins—for the apo- and holo-protein unfolding and the heme loss assays, Mbs were generated by large scale expression in E. coli to obtain milligram quantities of highly purified samples. For the pVP80K-Mb gene constructs, the holoproteins were expressed in E. coli BL21-DE3 cells (Agilent) and grown at 25–28 °C post-lag phase following induction with 0.8 mM isopropyl β-D-1-thiogalactopyranoside in Terrific Broth medium with 50 μg/ml kanamycin. For the sperm whale Mb H64F/V68F mutant gene in the pEMBL 19+ vector, the Mb had been expressed in TB1 cells (15) and purified according to the method of Springer and Sligar (21) modified by Carver et al. (45).

MBs expressed in E. coli were purified and stored in the carbon monoxide-bound ferrous form. The concentration of the CO-bound form was determined spectrophotometrically using ε_{424} = 187 mm^{-1} cm^{-1} (42). For holoMb unfolding and heme loss assays, the samples were oxidized to the ferric form using a TECAN Infinite M1000 Pro microplate reader (44). ApoMb was obtained by heme extraction into 2-butanol after decreasing the pH of the ferric sample to ~2.5–2.8 (47). ApoMb remained in the aqueous phase and was dialyzed overnight in cold 10 mM potassium phosphate, pH 7, and then centrifuged to remove the precipitates. ApoMb concentration was determined spectrophotometrically using ε_{280} = 15.2 mm^{-1} cm^{-1} (48).

Measurement and Analysis of Apo- and HoloMb Unfolding—Measurements of GdnHCl-induced unfolding of apo- and holoMbs were performed in 10 mM potassium phosphate, pH 7, at 20 °C. The individual samples were incubated in a water bath at different concentrations of GdnHCl (Sigma) ranging from 0 to 4 M at 20 °C for 2 h to achieve equilibrium (2). The fractional decrease in helical content was measured as the loss of negative ellipticity at 222 nm during unfolding using a Varian Cary Eclipse spectropolarimeter. Changes in Mb fluorescence emission originating from Trp residues were measured at 341 nm for apoMb unfolding and at 355 nm for holoMb unfolding using a Cary 100 Bio UV-visible spectrophotometer.

The CD signal for the native (N) state of all apo Mbs and the fluorescence signal for the unfolded (U) state of all the apo Mbs were normalized to 1. The CD and fluorescence equilibrium unfolding data for apoMb were fit simultaneously to the 3-state, 2-step mechanism shown in the right-hand column in Fig. 1 using Equation 2 (2). The dependence of the CD or fluorescence signal (S) change on [GdnHCl] is defined in Equation 2 with the signals at the different folding states defined by their accompanying subscripts. Data fitting was done with the Solver program in MS Excel 2011 to obtain the unfolding equilibrium constants, K[N], and K[U], extrapolated at [GdnHCl] = 0. The I state signals, S_I, the CD signal for U, and fluorescence signal for N were allowed to vary during fitting. The differential GdnHCl binding parameters n[N] and m[U] were set as 9.85 and 5.68 kJ mol^{-1} M^{-1} based on the work of Culbertson and Olson (2) and define the linear dependence of free energies on GdnHCl concentration for the N to I and I to U transitions respectively.

\[
S = \frac{S_N + S_{U}\exp(m[GdnHCl]/RT)}{1 + K_{U}\exp(m[GdnHCl]/RT)}
\]  

(Eq. 2)

CD, absorbance, and fluorescence equilibrium unfolding data for holoMb were fitted simultaneously to the complete six-state mechanism (Fig. 1) using Equation 3 and the Gnuplot program (50).

\[
S = Y_{SN}S_{N} + Y_{SH}S_{H} + Y_{IH}S_{IH} + Y_{I}S_{I} + Y_{U}S_{U} + Y_{UH}S_{UH} + Y_{H}S_{H}
\]  

(Eq. 3)

The hemo dissociation constants at [GdnHCl] = 0, K[NH] and K[UH] were used to derive the population fractions (Y) of the different unfolding states as defined by Culbertson and Olson (2). The K[UH] value at [GdnHCl] = 0 was fixed at 1 × 10^{-6} M from previous studies that showed that the nonspecific binding of heme to unfolded globins is roughly independent of amino acid sequence (49, 51). The dependence of the free energies on [GdnHCl] for heme dissociation (K[0]) from the heme-bound native (NH), intermediate (IH), and unfolded (UH) states are defined as m[NH], m[IH], and m[UH] was fixed to 10 kJ mol^{-1} M^{-1}, and for wild-type Mbs, the m[NH] and m[UH] values were initially set at 18.42 and 16.75 kJ mol^{-1} M^{-1}, respectively, based on analyses by Culbertson and Olson (2). For the heme pocket mutants, the m[NH] and m[UH] values were initially set at 14.65 and 13.82 kJ mol^{-1} M^{-1}, respectively, again based on previous work (2). When allowed to vary, the m constants did not change.
much. The $K_{NH}$ and $K_{NU}$ values were fixed to the values obtained for unfolding of the corresponding apoMb species. The CD signal for the native state of the holoMb ($CD_{NH}$) was renormalized and fixed to 1, and the CD signals for the apoMb N, I, and U states were fixed at values from the apoMb unfolding studies with respect to the native state of holoMb. The Soret absorbance values for the native state of the holoMb was assigned a value of 0.06. This value is only slightly greater than any of the holoMbs from deep diving mammals (Table 1).

### Measurement of Rates of Hemin Dissociation—Rates of hemin dissociation from metMb variants were measured by reacting the ferrous holoprotein with excess H64Y/V68F apoMb, which serves as a hemin-scavenging agent and turns “green” when it takes up hemin (46). The decrease of Soret absorbance for the holo-metMb variants was monitored over time at 37°C. For each reaction, 60 μM sperm whale H64Y/V68F apoMb was added to 6 μM of metMb variant. Reaction conditions were 0.45 mM sucrose in 0.15 M buffer that was either sodium acetate at pH 5 or potassium phosphate at pH 7 (46, 49). Sucrose was added as a cosmolute to inhibit apoglobin precipitation after hemin dissociation, as described in Hargrove et al. (46).

### Results

#### Quantitation of HoloMb Expression in the Cell-free System—
A summary of the cell-free expression data for our library of nine recombinant myoglobin variants is shown in Table 1. In all cases, holoMb samples were first semi-purified on a Zn2+ -chelating resin filter plate, and then expression was measured as the ratio of the heme protein concentration calculated at the Soret absorbance peak to an estimate of total protein present measured by absorbance at 280 nm (Equation 1 and Fig. 3). For highly expressing Mbs, the Soret absorbance peaks were sharply defined (Fig. 3).

The cell-free expression yields for the H64F/V68F and H64L dwarf sperm whale, and H64F/V68F sperm whale holoMb mutants were significantly higher than any of the other naturally occurring variants examined. The cell-free expression yields of human and pig holoMbs were dramatically lower than any of the holoMbs from deep diving mammals (Table 1). In fact, the pig Mb expression yield measured by the absorbance ratio defined in Equation 1 was comparable with the “blank” expression yield for the wheat germ extract with added hemin but without mRNA addition during translation incubation (Fig. 3). The Soret absorbance peaks for the semi-purified lysates containing pig and human Mbs and the blank wheat germ extract were not sharply defined (Fig. 3). When the semi-purified Mbs were analyzed on SDS gels, however, a small amount of pig Mb was clearly being expressed in the system, albeit with a much less intense protein band compared with those of the other Mbs expressed in the cell-free system (Fig. 2, bottom left).

As shown in Fig. 3, there is variability in the $A_{280}$ values for the translation products, and thus normalization of total protein (both expressed product and soluble enzymes from the translation reaction mixture) in the partially purified sample is needed. Similarly, it is clear from Fig. 3 that the H64F/V68F dwarf sperm whale metMb has a different Soret maximum (395 nm) from that of the native whale metMbs (409.5 nm), and thus the extinction coefficient difference needs to be taken into account as prescribed in Equation 1. The broader and less intense Soret band for the H64L and H64F/V68F mutants of Mb is due to the loss of coordinated water and reflects the spectral properties of a five-coordinate hemin (1).

The $A_{Soret}/A_{280}$ (Equation 1) ratio for a cell-free translation reaction mixture that contains heme but no holoMb was assigned a value of 0.06. This value is only slightly below the measured expression yields observed for pig and human Mbs and equal to or just slightly less than values computed for a variety of blank reactions (no mRNA) using Equation 1. The spectrum of the blank in Fig. 3 clearly shows that there is some heme present after purification with the Zn2+ resin as judged by the broad Soret absorbance. This heme is probably bound nonspecifically to proteins that are a part of the translation system and elute from the Zn2+ resin with holoMb in the presence of EDTA. This interpretation is supported by the presence of a 280 nm absorbance peak for the blank control when no mRNA was added and no holoMb was made. In Figs. 4, 8, and 9, the observed data are presented with no subtraction of the blank value (Equation 1) to provide a clearer indication of the original sample spectra (i.e. Fig. 3).

To compare our cell-free results to those reported previously by Smith and others for expression in E. coli, we subtracted the blank value from all the observed yields (Equation 1) and then

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**Table 1**

Summary of Mb cell-free expression yields, globin stabilities, and heme dissociation rate constants

| Mb variant       | Expression yield | Relative expression yield | $K_{NH}$ | $K_{NU}$ | $1/K_{NH}$ ($K_{NU}$) | $k_{-H}$ (h⁻¹), pH 7 | $k_{-H}$ (h⁻¹), pH 5 |
|------------------|------------------|---------------------------|----------|----------|-----------------------|----------------------|----------------------|
| Dwarf Sw (Dw)    | 0.171 ± 0.011    | 1.62 ± 0.24               | 0.00047  | 0.043    | 495000                | 1.2 ± 0.3 (24 ± 1)   |                     |
| Gray seal        | 0.154 ± 0.016    | 1.38 ± 0.23               | 0.00089  | 0.021    | 53500                 | 0.62 ± 0.05 (15.4 ± 1.7) |                     |
| Goosebeak whale  | 0.151 ± 0.005    | 1.34 ± 0.18               | 0.0012   | 0.015    | 55600                 | 0.8 ± 0.1 (21 ± 1)   |                     |
| Sperm whale (Sw) | 0.128 ± 0.017    | 1.00 ± 0.18               | 0.0052   | 0.021    | 9160                  | 1.7 ± 0.01 (2.2 ± 0.1) |                     |
| Human            | 0.079 ± 0.009    | 0.28 ± 0.05               | 0.017    | 0.014    | 4200                  | 0.044 ± 0.004 (2.7 ± 0.3) |                     |
| Pig              | 0.071 ± 0.008    | 0.16 ± 0.03               | 0.021    | 0.019    | 2500                  | 0.049 ± 0.012 (1.0³) |                     |

* Relative expression was computed as described in Equation 4 in the text.

**Notes**

1. The unfolding experiments were done under the experimental conditions of 10 μM Mb, 10 mM potassium phosphate at 20°C as done by Cubellets and Olson (2), and we successfully replicated their results for the equilibrium unfolding constants of Sw apoMb.
2. Hemolysis was done at 37°C in 0.45% sucrose in 0.15 M buffer that was either sodium phosphate, pH 7, or sodium acetate, pH 5.
3. The $K_{-H}$ at pH 5 and pH 7 was taken from Hargrove et al. (22).
4. We assume a 0.5 decrease in both log(1/$K_{NH}$) and log(1/$K_{NU}$) or a 1.0 decrease in log(1/$K_{NH}$) for the low salt condition for pig Mb (high salt $K_{NH}$ = 0.039, $K_{NU}$ = 0.032). These decreases are close to the average for all the variants that Smith (15) examined at high and low phosphate.
The expression of Mb variants to other values in the literature (Table 1, 3rd column (11, 14, 15, 20)).

The amount of mRNA transcribed was engineered to include UTRs at 5′ and 3′ ends derived from tobacco mosaic virus-positive sense RNA, but without the 5′ end cap and poly(A) tail for in vitro translation efficiency and to bypass the issues of decapping and shortening of the poly(A) tail of the mRNAs during cell-free expression (Fig. 2) (16, 24, 26).

Therefore, a dependence of in vitro expression yield on mRNA transcript concentration is less likely, above a certain threshold, due to high mRNA half-life and consistent 5′ and 3′ tobacco mosaic virus UTR sequences, which confer similar stability across the different transcripts. Thus, the ribosomes in the cell-free system are probably “saturated” with stable mRNAs at the levels present in our assays.

In addition, previous studies using electron microscopy have also shown efficient recycling of multiple ribosomes translating mRNA simultaneously through the formation of circular polysomes linking the mRNA’s 3′ and 5′ ends in the cell-free wheat germ extract (57). Poly(A)-binding protein I, present in all eukaryotic cells, is believed to place the disengaged ribosome at the 3′ back to the 5′ front of the mRNA, and Madin et al. (57) have shown in vitro that this phenomenon is not dependent on the presence of the 5′ cap and 3′ poly(A) tail for the mRNA (16, 58–60). Therefore, continuous ribosomal recycling on a cell-free pool of mRNA most likely decouples the translation yield dependence on the mRNA transcript concentration, above a certain threshold level.

**Equilibrium Unfolding of ApoMb Variants**—To look for correlations between holoMb expression yield and globin stability, we measured the folding parameters for the Mb variants listed in Table 1. Intrinsic protein stability was measured by examining GdnHCl-induced unfolding of the apoMb forms of the variants, as shown in Fig. 5.

The changes in the CD and fluorescence signals are concerted and suggest two major unfolding processes. Intrinsic fluorescence emission at 341 nm for all Mb species (except gray seal) increases initially with increasing GdnHCl concentration and then decreases as the protein completely unfolds. The result is a well-defined bell-shaped curve (Fig. 5A). The peak in the fluorescence curve helps to define the emission of the molten globule intermediate (1) (2, 4, 11), complementing the CD profiles, in which the I state is defined by an inflection point in the decrease in negative CD$_{222}$ nm signal amplitude. The intrinsic fluorescence of Mb originates from Trp-7 and Trp-14, and previous studies have proposed that the increase in fluorescence for the I state is due to either the movement of a quenching amino acid side chain away from Trp-7 or an increase in the flexibility of the indole side chain, allowing it to move into a more apolar environment. The fluorescence of the exposed Trp

This lack of correlation between the total mRNA measured and expression of holoMb (Fig. 4) might be attributed to several factors that are most likely unique to the eukaryotic wheat germ-based cell-free expression system. In comparison with the prokaryotic mRNAs, eukaryotic mRNAs generally are much more stable, with globin mRNAs in reticulocytes having half-lives of 50 h (52–56). The stability, translation efficiency, and regulation of degradation of eukaryotic mRNAs are often attributed to their 5′- and 3′-untranslated regions (UTRs). The in vitro mRNA transcribed was engineered to include UTRs at the 5′ (Ω sequence) and 3′ ends derived from tobacco mosaic virus-positive sense RNA, but without the 5′ end cap and poly(A) tail for in vitro translation efficiency and to bypass the issues of decapping and shortening of the poly(A) tail of the mRNAs during cell-free expression (Fig. 2) (16, 24, 26).

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FIGURE 5. GdnHCl-induced equilibrium unfolding of selected apoMbs was followed by fluorescence (A) and CD changes (B). Full circles are the observed data, and the solid lines are the fitted curves using Equation 2 and the parameters listed in Table 1. Unfolding measurements were done with 10 μM protein in 10 mM potassium phosphate, pH 7, at 20 °C to be comparable with the work of Culbertson and Olson (2).

residues is quenched by surrounding solvent in the completely unfolded U state (11, 61–64).

A bell-shaped fluorescence curve was not observed for the unfolding of gray seal apoMb. Instead, higher fluorescence was observed for the N state and decreased monotonically with increasing [GdnHCl], suggesting the absence of Trp-7 quenching in the N apoMb state.

The overall apoMb unfolding constant, \( K_{\text{NU}} \), is defined as \( K_{\text{NU}} K_{\text{IL}} \) and overall stability is empirically represented as \( -\log(K_{\text{NU}}) \) or \( \log(K_{\text{UN}}) \), which is proportional to the free energy released during folding to the native conformation, where \( K_{\text{UN}} \) is the equilibrium constant for the U → N reaction.

Our results confirm that wild-type Mbs originating from deep diving mammals have significantly higher apoglobin stabilities, with dwarf sperm whale Mb being one of the most stable native Mbs investigated. Similar results were observed under high salt unfolding conditions (200 mM potassium phosphate, pH 7) by Scott et al. (12). ApoMb variants constructed with large apolar substitutions at the E7 and E11 helical positions showed significantly higher apoglobin stabilities than the original wild-type proteins (Fig. 5 and Table 1). For example, the H64F/V68F mutation increased the overall \( K_{\text{UN}} \) over 50-fold in dwarf sperm whale Mb, even though the hemin affinity of the double mutant was much less than that of the wild-type globin (Table 1). The H64F/V68F double mutation in both dwarf sperm whale Mb and sperm whale Mb and the H64L single mutation in dwarf sperm whale Mb stabilized the N state significantly more than the I state as shown by the large decreases in the \( K_{\text{NI}} \) values for the unfolding of the native to molten globule intermediate, with little change in the \( K_{\text{IU}} \) values (Table 1).

Rates of Hemin Dissociation—To investigate the relationship between cell-free expression yield and hemin affinity, kinetic experiments were performed to measure rates of hemin dissociation (\( K_{\text{H}} \)) at pH 5 and pH 7. At pH 7, the rates of dissociation are very slow, and precipitation of the resultant apoprotein can interfere with analyses of the time courses, whereas at pH 5.0 the rate of hemin loss is much faster and easier to measure (46).

Hemin affinity is determined primarily by the rate of dissociation. The rate of association (\( K_{\text{H}} \)) is driven primarily by non-specific hydrophobic effects that prescribe a bimolecular association rate constant that is roughly \( -1 \times 10^8 \text{ M}^{-1} \text{ s}^{-1} \) for all apoMb variants that have been examined, regardless of their overall affinities and exact structures (23).

The distal heme pocket mutations, H64L and H64F/V68F, increased the hemin dissociation rates significantly at both pH 5 and pH 7 (Table 1 and Fig. 6). As shown previously (15, 20), filling up the heme pocket with large apolar residues increases apoglobin stability but compromises the physiological function of Mb by decreasing O₂ affinity, heme affinity, and resistance to autoxidation (1, 22, 65).

Interestingly, the naturally occurring variants with the highest apoMb stabilities did not have the lowest hemin loss rates. For example, the rates of hemin loss from dwarf sperm whale metMb were higher at both pH 5 and pH 7 than those of the other naturally occurring Mbs listed in Table 1 (see Fig. 6).

Equilibrium Unfolding of HoloMb Variants—The H64L and H64F/V68F mutations in dwarf sperm whale Mb increase both overall globin stability (\( K_{\text{UN}} \)) and the rate constant for hemin dissociation (Table 1 and Fig. 6). Thus, although apoprotein folding is enhanced, hemin affinity is decreased. The \( k_{\text{H}} \) and \( K_{\text{H}} \) values for the Mbs from deep diving mammals were more variable than expected. Estimates of the equilibrium hemin dissociation constants at pH 7 (\( K_{\text{H}} \)) were computed as the ratio of

FIGURE 6. Kinetic traces for the dissociation of hemin from selected Mbs at pH 7 and 37 °C. The lines represent the observed time courses that were fit to a simple exponential decay with a first order rate constant equal to \( k_{\text{H}} \). The upward trends in the mutant traces represent light scattering due to precipitation of the apoglobin product. Experimental conditions are as follows: 6 μM metMb and 60 μM H64F/V68F Sw apoMb in 0.45 M sucrose in 0.15 M sodium phosphate.
$k_{-\text{H}}$ measured at pH 7 to $k'_{\text{H}}$, the association rate constant for bimolecular hemin binding. The value of $k'_{\text{H}}$ for the binding of heme monomers is roughly the same for all apoMb variants, independent of pH, and equal to $\sim 1 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ (23). The estimated values for $K_{-\text{H}}$ are listed in Table 2, column 1.

An alternative method for estimating hemin affinity is to examine holoMb unfolding using the analyses developed by Culbertson and Olson (2) and the scheme in Fig. 1. ApoMb unfolding parameters obtained from independent experiments and analyses (Fig. 5 and Table 1) are used to analyze holoMb unfolding curves (Fig. 7) and estimate equilibrium hemin dissociation constants for the native holo- and intermediate holo-Mb states (i.e. $K_{\text{NH}}$ and $K_{\text{IH}}$ in Fig. 1 and Table 2). Titration curves for the unfolding of six key holo-Mb variants are shown in Fig. 7 where the lines represent fits to Equation 3 using the apoMb unfolding parameters in Table 1 and the $K_{\text{NH}}$ and $K_{\text{IH}}$ values listed in Table 2.

As observed by Culbertson and Olson (2), there are discrepancies between the absolute values of $K_{\text{NH}}$ obtained from the analyses of holoMb unfolding and the $K_{-\text{H}}$ dissociation constant computed from the ratio of rate constants. However, both estimates of the overall equilibrium hemin dissociation constants for the N apoMb states are in the $10^{-12}$ to $10^{-14}$ M range. In addition, the overall trends with respect to the mutants and variants are the same, with the distal histidine mutants showing 5–10-fold higher $K_{\text{NH}}$ and $K_{\text{IH}}$ values. The differences in the absolute values could be attributed to differences in pH and temperature between the holoMb unfolding titrations and the kinetics measurements (2). Another cause is the difficulty of defining the individual $K_{\text{NH}}$ and $K_{\text{IH}}$ values from the observed curves for the variants in Fig. 7 where all the spectral transitions appear highly concerted with little evidence for intermediates. In contrast, Culbertson and Olson (2) used mutations that preferentially stabilized the intermediate I and the apoMb N states, which allowed better definition of the individual parameters.

**TABLE 2**

Estimates of equilibrium constants for hemin dissociation from holo-Mb variants

| Mb variant                  | Relative expression yield | $K_{+\text{H}}$ | $K_{\text{NH}}$ | $K_{-\text{IH}}$ |
|-----------------------------|---------------------------|----------------|----------------|-----------------|
| Dw H64F/V68F               | 2.27 ± 0.48               | 3.3            | 0.83           | 80              |
| Sw H64F/V68F               | 2.17 ± 0.29               | 1.7            | 0.19           | 10              |
| Dw H64L                    | 1.77 ± 0.30               | 2.2            | 0.54           | 26              |
| Dw                          | 1.62 ± 0.24               | 0.47           | 0.048          | 5.3             |
| Gray seal                  | 1.38 ± 0.23               | 0.23           | 0.023          | 4.4             |
| Goosebeak whale            | 1.34 ± 0.18               | 0.12           | 0.045          | 4.8             |
| Sw                         | 1.00 ± 0.18               | 0.14           | 0.022          | 1.6             |
| Human                      | 0.28 ± 0.05               | 0.028          |                |                 |
| Pig                        | 0.16 ± 0.03               | 0.028          |                |                 |

* Relative expression was computed as described in Equation 4 in the text.

$K_{-\text{H}}$ was estimated at pH 7 from $k_{-\text{H}}$ calculated at pH 7, and the $k_{-\text{H}}$ value of $1 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ as measured by Hargrove et al. (23). $k_{-\text{H}}$ was measured at 37°C in 0.45 M sucrose in 0.15 M sodium phosphate, pH 7. The $k_{-\text{H}}$ values for human and pig Mbs were taken from Hargrove et al. (22).

* The holoMb unfolding experiments were performed with 10 μM Mb in 10 mM potassium phosphate, pH 7, at 20°C.

**FIGURE 7.** GdnHCl (GuHCl)-induced equilibrium unfolding of selected holoMbs. The solid circles are the observed data, and the solid lines are fitted curves using Equation 2, and the $K_{\text{NH}}$ and $K_{\text{IH}}$ parameters are listed in Table 2. The final $m_{\text{NH}}$ and $m_{\text{IH}}$ values for the variants other than Sw are as follows: Dw Mb (4.0 and 3.4 kcal mol$^{-1}$ M$^{-1}$); goosebeak whale Mb (4.3 and 3.3 kcal mol$^{-1}$ M$^{-1}$); Gray seal Mb (5.7 and 4.7 kcal mol$^{-1}$ M$^{-1}$); Dw H64F/V68F Mb (4 and 2.3 kcal mol$^{-1}$ M$^{-1}$); and Dw H64L (4.2 and 3.2 kcal mol$^{-1}$ M$^{-1}$). Unfolding measurements were done with 10 μM protein in 10 mM potassium phosphate, pH 7, at 20°C as in Ref. 2.
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![Graph: Correlation between holoMb cell-free expression yield and overall apoMb stability](image)

**FIGURE 8. Correlation between holoMb cell-free expression yield and overall apoMb stability (\(-\log(K_{\text{NH}})\)) (A); stability of the apoMb N state relative to the I state (\(-\log(K_{\text{NI}})\)) (B); and stability of the apoMb I state relative to the U state (\(-\log(K_{\text{IU}})\)) (C).** The apoMb equilibrium unfolding constants for all species except pig (refer to Table 1) were measured with 10 \(\mu\)M protein in 10 mM potassium phosphate, pH 7, at 20 °C. The pig Mb data points were taken from Table 1, are represented by black circles, and were not included in the simple linear regression analysis of the data in all three panels.

and spectral signatures. Furthermore, it is difficult to determine the \(m_{\text{NH}}\) and \(m_{\text{IH}}\) values for the different Mb variants due to structural and charge variations.

From the holoMb unfolding experiments, the \(K_{\text{NH}}\) values for the naturally occurring holoMb variants were determined to be approximately \(10^{-14} \text{ M}\), whereas \(K_{\text{IH}}\) is \(100\)-fold larger (\(10^{-12} \text{ M}\)). The H64F/V68F and H64L mutants of the dwarf sperm whale Mb had larger \(K_{\text{NH}}\) values on the order of \(10^{-13} \text{ M}\) and an \(100\)-fold higher \(K_{\text{IH}}\) value on the order of \(10^{-11} \text{ M}\).

**Correlations of HoloMb Expression with ApoMb Stability**—HoloMb expression yields in bacteria and animal cells depend on the copy number of the Mb gene, transcriptional regulation, mRNA stability, codon bias, amount of hemin present, and cellular homeostasis. In principle, these variables can be controlled explicitly in the cell-free expression assay and should be the same for all the Mb variants (16, 30, 66). Additionally, there should be no variability due to nutritional source and growth phase. As shown with in vivo Mb expression in E. coli (16, 30).

Thus, we hoped that the cell-free expression assay would allow more straightforward determination of what biochemical properties of Mb govern the production of the holoprotein.

As shown in Fig. 8A, there is a strong linear correlation between the expression yields of soluble holoMb measured using Equation 1 and the logarithm of overall globin stability \((-\log(K_{\text{NH}})) or \(\log(K_{\text{NU}}))\), with \(R^2 = 0.93\). This correlation verifies quantitatively the previous suggestion by Scott et al. (12) that deep diving whale Mbs are much easier to express than Mbs from terrestrial mammals. These results are also consistent with the work by Smith and by others (11, 12, 14, 15) on expression of holoMb variants in E. coli, where both qualitative and semi-quantitative measurements suggested that apoglobin stability was a key factor in governing expression yields. In this earlier work, however, the correlations were much poorer, and the scatter in the observed data were much larger (see under “Discussion” and Fig. 10A). The results in Fig. 8 indicate that the cell-free expression assay has enabled us to decrease the variability in expression yields enough to observe strong correlations with key biochemical parameters.

The expression yields of the more stable, naturally occurring holoMbs from deep diving mammals are significantly higher than those of the less stable holoMbs from the terrestrial mammals. The expression yields of pig and human holoMbs were only barely detectable from their Soret absorbance signals and SDS gel bands (Figs. 2, 3, and 8). Again, these quantitative observations verify previous qualitative observations of the difficulty of expressing pig and human Mbs in E. coli in fully folded hologlobin forms. When expressed in E. coli, these less stable proteins either undergo proteolytic degradation or aggregate very rapidly to form insoluble inclusion bodies (12, 67).

The stability of the apo-N state relative to the intermediate molten globule, as measured by \(\log(K_{\text{NI}})\), has a more significant
The figure shows a correlation between holoMb cell-free expression yield and $-\log(K_{\text{MW}})$ (A) and $-\log(k_{\text{on}})(B)$. $K_{\text{MW}}$ was determined from the analyses of the holoMb unfolding experiments done with 10 μM protein in 10 mM potassium phosphate, pH 7, at 20 °C, and $k_{\text{on}}$ was obtained from analyses of hemin dissociation time courses measured at pH 5 as described in Table 1. The $k_{\text{on}}$ values at pH 5 for Sw, human, and pig Mbs were taken from Hargrove et al. (22).

Expression yields were computed from Equation 1, and blank values were $-0.06$ as shown in Fig. 8.

effect on expression yield than the relative stability of the apo-I state, as measured by $-\log(K_{\text{UN}})$ (Fig. 8, B and C). This observation is consistent with structural interpretations of the apoMb I state, in which most of the heme pocket is highly disordered and hemin is more weakly bound, forming a nonspecific hemichrome complex (2, 4, 5, 68–72). Thus, the stability of the N apoMb state is more critical for high expression of the holo-protein than the stability of the molten globule state.

Culbertson and Olson (2) and Hargrove and Olson (49) have shown that the enhanced resistance of holo-metMb to denaturation is primarily due to the high affinity of the apoprotein for hemin. Thus, it seemed reasonable to think that higher hemin affinity might also enhance the expression of soluble holoMb. However, as shown in Fig. 9, there was no positive correlation between hemin affinity and cell-free expression yields. The distal pocket H64F/V68F and H64L mutants of dwarf sperm whale and sperm whale Mbs have the highest expression yields but also the largest hemin dissociation rate and equilibrium constants (Tables 1 and 2 and Fig. 9). If anything, there is a negative correlation between holoMb expression and hemin affinity as measured by $-\log(k_{\text{on}})$ or $-\log(K_{\text{MW}})$ obtained from independent kinetic and GdnHCl titration experiments, respectively (Fig. 9).

These results confirm previous observations by Olson et al. (20) and Smith (15) who showed that the highest expressing mutants observed through in vivo E. coli expression in both random and rationally designed libraries of sperm whale Mb include variants containing H64L, H64F, and H64F/V68F mutations. Smith’s measurements (15) were based on the appearance of the Soret band in E. coli suspensions where the holoMb was kept reduced in the presence of cell media or dithionite and equilibrated with CO gas. Then, the amplitude of the derivative signal in the 420 nm region was recorded as an indirect measure of the amount of MbCO present in the cells (15, 18). The level of expression was recorded as the “peak to trough” derivative signal of the variant to that for wild-type sperm whale Mb, which was measured as a control in each set of experiments. Thus, in Smith’s measurements (15), the enhanced affinity of the H64L and H64F mutants for CO could have been compensating for their decreased hemin affinity and resulting in higher expression yields.

In the cell-free expressions, no reductants were added, and the H64L and H64F/V68F mutants were expressed in the ferric state without having a stabilizing H2O bound to the pentacoor-


dinate hemin. This result can be seen directly by the broader 395-nm Soret absorbance peak of the H64F/V68F metMb mutants shown in Fig. 3 (1). Thus, the high levels of expression of the H64L and H64F/V68F mutants are due exclusively to their greater apoglobin stability.

ELISA measurements of the total amount of soluble, folded holoMb in the wheat germ extract lysate correlated with and thereby confirmed the holoMb cell-free expression yields defined by Equations 1 and 4 for the wild-type and heme pocket mutants of sperm whale and dwarf sperm whale Mb (Table 3). The more stable Mbs such as the dwarf sperm whale H64F/V68F and H64L Mbs had a higher amount of polypeptide in the soluble form. Minimization of proteases in the pretreated wheat germ extract (17) also allowed measurement of unfolded proteins and enabled a better estimate of the total polypeptide (both folded and unfolded/aggregated protein) synthesized using the indirect ELISA measurements. The total amount of polypeptide synthesized for both sperm whale Mb and dwarf sperm whale Mb are within a similar range. However, the H64F/V68F mutation in both sperm whale Mb and dwarf sperm whale Mb and the H64L mutation in dwarf sperm whale Mb appeared to double the total amount of polypeptide synthesized. The cause of this effect is unclear; however, ELISA is difficult to quantitate because of the differences in specificity of the Mb antibody.

Discussion

Heterologous Expression in E. coli—In his survey of rationally designed heme pocket mutants of sperm whale Mb, Smith (15) observed that globin stability was critical for heterologous expression of Mbs in E. coli. A summary of Smith’s results is shown in Fig. 10A, where expression relative to sperm whale Mb is plotted versus $\log(K_{\text{UN}})$ for values measured at high (200 mM) and low (20 mM) potassium phosphate concentrations at pH 7, 25 °C.

The large scatter in the E. coli data could be due to variations in cellular metabolism, the amount of plasmid DNA, codon usage, mRNA transcript levels and stability, state of reduction of the heme, CO production, and differential resistance of the apoglobin to proteolysis (15, 16, 25, 53, 66). For example, Smith (15) argued that the H64G and H64R mutants, which have similar $K_{\text{UN}}$ values to wild-type sperm whale apoMb, show poor expression because they are more prone to proteolysis and degradation (Fig. 10A) (73).
Expression of Hologlobins

TABLE 3
ELISA measurements of total Mb synthesized in the cell-free expression system

Unpurified cell-free translation mixtures were spun down after expression incubation to obtain pellets containing precipitated protein and a lysate containing soluble protein. FL-154 rabbit polyclonal IgG Mb antibody (Santa Cruz Biotechnology) was incubated with the polypeptides contained in the lysates and pellets at 1:50 dilution in 1% bovine serum albumin (BSA), 0.02% azide solution in PBS. For the detection of Mb polypeptides, incubation was next done against goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology) at 1:2000 dilution in 1% BSA in PBS. Quantification of Mb polypeptides was next done through absorbance measurements following incubation with Thermo Scientific Pierce 1-Step TMA Ultra TMB-ELISA substrate.

| Species Mb | Total moles in lysate | Total moles in pellet | Total protein synthesized | Ratio of Mb in lysate to pellet |
|------------|----------------------|----------------------|--------------------------|-----------------------------|
| Sw         | 0.057 ± 0.015        | 0.15 ± 0.02          | 0.11 ± 0.03              | 1.04 ± 0.35                 |
| Sw H64F/V68F | 0.15 ± 0.02          | 0.09 ± 0.009         | 0.24 ± 0.024             | 1.67 ± 0.28                 |
| Dw         | 0.096 ± 0.027        | 0.041 ± 0.0098       | 0.14 ± 0.03              | 2.34 ± 0.86                 |
| Dw H64F/V68F | 0.18 ± 0.07          | 0.091 ± 0.052        | 0.27 ± 0.12              | 1.98 ± 1.37                 |
| Dw H64L    | 0.20 ± 0.065         | 0.094 ± 0.012        | 0.29 ± 0.08              | 2.13 ± 0.74                 |

During the biosynthesis of heme proteins, there is competition between heme binding and the irreversible aggregation and degradation of the corresponding apoproteins. Thus, expression yield is thought to be dependent on the availability of heme and the affinity of the apoglobin for it (74, 75). However, Smith (15) observed no correlation between relative expression in E. coli and hemin dissociation rate and equilibrium constants measured in vitro, and in our work there appears to be a weak inverse relationship (Fig. 9). In both sets of experiments, H64L and H64F mutants with low heme affinity but high globin stability show the highest level of relative expression. These observations also suggest that heme binding occurs post-translationally to fully folded N-state apomyoglobin (Fig. 1). This model is consistent with the strong linear dependence on the log(K_UN) for both the bacterial and cell-free eukaryotic expression systems.

Mechanistic Interpretation of Cell-free Expression—Our proposed models for the cell-free expression of holoMb are shown in Fig. 11A. The rate constant for translation, k_translation, should be zero order until all the translation cofactors and nutrients are consumed. The newly formed apombo will either bind free heme to form the stable holometMb product or aggregate and precipitate irreversibly into a separate insoluble phase (Fig. 11A). In our models, we have assumed that heme binding is effectively irreversible. At pH 7–8, the rate of hemin dissociation is extremely slow (t_H ≈ 1–20 h), and the K_H value for heme binding is on the order of 10^{-12} to 10^{-14} M (Table 1) (22, 23).

HoloMb has been shown to be highly resistant to precipitation and proteolysis (76, 77), whereas the apo U state, which has only residual or transient local helical structure (2, 5, 78), is prone to rapid self-aggregation and precipitation through its various extended conformations (5, 79, 80).

In the simplest model, the rate constants for folding to the native N state (k_S) and unfolding to the U state (k_U) are assumed to be much lower than the rate constants for hemin binding (k_H[H]) and precipitation (k_ppt). Under these conditions, the rate of holoMb formation is given by Equation 5:

\[
\frac{d[\text{holoMb}]}{dt} = k_S[H][N] = k_H[H] \left( \frac{K_{UN}}{K_{UN} + 1} \right) [\text{apoMb}]
\]

A steady state approximation is then assumed for the total concentration of translated apoMb ([U] + [N]) during the expression process.

If the precipitation process is first order, i.e. \( \frac{d[\text{ppt}]}{dt} = k_{ppt}[U] \) or \( k_{ppt}(1/(K_{UN} + 1))[\text{apoMb}] \), then the steady state expression rate for the rate of holoMb formation is as shown in Equation 6,

\[
\frac{d[\text{holoMb}]}{dt} = \frac{k_{translation}K_{UN}}{K_{UN} + k_{ppt}} \left( \frac{[\text{apoMb}]}{K_{UN} + [H]} \right)
\]

Thus, the rate of holoMb formation should depend hyperbolically on K_UN with the exact dependence being determined by the ratio of the rate constants for precipitation and heme binding (k_ppt/k_S[H] in the denominator of Equation 6). Assuming that this rate applies throughout the assay, the relative expression yield can be computed from \( \frac{d[\text{holoMb}]}{dt} \) for a variant divided by the computed \( \frac{d[\text{holoMb}]}{dt} \) for sperm whale Mb. The dotted line in Fig. 11B shows a fit of the observed relative expression data to Equation 6 and indicates that, in principle, the rate of holoMb formation and the relative expres-
sion yield should not show a strictly linear dependence on log-
(K_{UN}) but reach a limiting value equal to \( \bar{k}_{\text{translation}} \).

This nonlinear dependence on log(K_{UN}) will also occur if the
aggregation process is modeled more realistically as a bimolec-
ular process with a second order rate constant \( k'_{\text{aggregation}} \) (Fig.
11B), which is what most workers in the field assume (81). In
this case, the steady state equation for [apoMb] is a quadratic as
shown in Equation 7.

\[
\frac{d[\text{holoMb}]}{dt} = \frac{(\sqrt{K_{UN}K_{UN}}^2 + 4k_{\text{aggregation}}k_{\text{translation}} - k_{U}K_{UN})}{2k_{\text{aggregation}}} \quad \text{(Eq. 7)}
\]

This more complex expression also asymptotically approaches \( k_{\text{translation}} \) at very high \( K_{UN} \), fits the observed data,
and is shown in Fig. 11B as the dashed line. This square root
function shows a steeper dependence on \( K_{UN} \) and an increased
differential expression between the more stable variants and
the less stable ones.

For both the unimolecular precipitation and bimolecular
aggregation models, \( k_{\text{ppt}} \) and \( k'_{\text{aggregation}} \) have to be greater than the
effective rate of hemin binding \( (k'_{\text{I[H]}}) \) to obtain the almost
linear dependence on log(K_{UN}) observed experimentally.

Similar expressions are obtained if the rates of folding and
unfolding are considered in the analysis, keeping the definition
\( K_{UN} = k_{U}/k_{U} \). In these derivations, we assume steady state expres-
sions for [U] and [N] and use either a unimolecular model for
precipitation or a bimolecular process for aggregation. The result-
ing steady state expressions are shown in Equations 8 and 9. (a) For
a first order precipitation process (Equation 8),

\[
\frac{d[\text{holoMb}]}{dt} = \frac{k_{\text{translation}}K_{UN}}{K_{UN} + k_{\text{ppt}}[H]k_{U} / (k_{U} + k_{\text{ppt}}[H])} \quad \text{(Eq. 8)}
\]

and (b) for bimolecular aggregation (Equation 9)

\[
\frac{d[\text{holoMb}]}{dt} = \frac{k_{U}k_{H}[U]}{k_{U} + k_{I[H]}K_{UN}} \left( \frac{k_{\text{aggregation}}k_{\text{translation}}}{k_{U} + k_{I[H]}K_{UN}} \right) \quad \text{versus} \quad \text{log}(K_{UN})
\]

(Eq. 9)

In both cases, the forms of the expression are the same as the
the corresponding equations for rapid interconversion of the
U and N states. What changes is the effective rate of hemin
binding, which is attenuated by \( k_{I}[H] \). This expression describes the competition between the hemin
binding to the N state and its unfolding back to the U state.
When \( k_{I} \) becomes very large, the rapid equilibrium assump-
tion for the N and U states applies, and Equations 8 and 9
reduce to Equations 6 and 7, respectively. When \( k_{U} \) becomes
very small, the rate of folding becomes limiting for holoMb
formation, and Equation 8 reduces to \( \frac{d[\text{holoMb}]}{dt} = k_{\text{translation}}K_{UN}/(k_{U} + k_{\text{ppt}}[U]) \) or \( k_{\text{translation}}K_{N}/(k_{N} + k_{\text{ppt}}) \),
because \( k_{\text{U}}K_{UN} = k_{N} \).

Thus, even when the rates of folding are taken into
account, a hyperbolic-like dependence on \( K_{UN} \) will occur,
and the rate of aggregation must still be very large compared
with the effective hemin-binding rate to fit the observed
data.

The theoretical analyses in Equations 5–9 and Fig. 11 are
clearly first approximations. The I state of apoMb and the IH
hemichrome state were not considered in our models to keep
them simple. Wild-type apo- and holoMb unfolding experi-
ments have shown that the I and IH states are not highly
populated, and their detection can be difficult and requires
multiple spectral measurements (Figs. 5 and 7) (2). The I and
IH states also rapidly appear and decay in kinetic folding
experiments (68, 82). Inclusion of intermediates will alter
the shape of the dependence of relative expression on log-
(K_{UN}) but would not change the asymptotic nature of the
process nor change the conclusion that aggregation of the
unfolded state is very fast.
Our models emphasize the importance of increasing both the rate of translation by adding more amino acids, ATP, and other metabolites and engineering greater apoprotein stability ($K_{\text{UN}}$) to enhance hologlobin expression yields. The availability of free hemin is important, both from a stoichiometry point of view and in terms of the rate of hemin binding versus aggregation, but in most cases, the hemin affinity is so great ($K_d \leq 10^{-12} \text{M}$, see Table 1) that it does not directly influence the expression yields (Fig. 9).

Another key result from these analyses is that the rate of precipitation must be roughly 2500 times faster than the rate of hemin binding to apoMb is given by the rate of dimer dissociation. Hargrove et al. (33, 83). Thus, the effective first order rate of hemin binding to $K_d$ is estimated in previous studies (85).

Free hemin forms dimers with a $K_d \approx 2-3 \times 10^{-7} \text{M}$ at pH 7 (33, 83). Thus, the effective first order rate of hemin binding to apoMb is given by the rate of dimer dissociation. Hargrove et al. (23) have shown that in the micromolar concentration range, the rate of hemin binding to apoMb is a first order process with a rate constant of $\approx 10 \text{s}^{-1}$. Using this first order rate as the effective value of $k'_H[H]$, $k_{\text{ppt}}$ in Equation 6 would be 25,000 $\text{s}^{-1}$ (assuming fast protein conformational transitions), and $k'_{\text{aggregation}}$ in Equation 7 would be $\approx 10^{10} \text{M}^{-1} \text{s}^{-1}$, which is a reasonable estimate for a diffusion controlled bimolecular aggregation process and similar to rates of apoMb aggregation estimated in previous studies (85).

Alternatively, the strong dependence of expression levels on $K_{\text{UN}}$, even when the values are very high, could be due to slow rates of folding compared with heme binding. Under these conditions, the ratios $k_{\text{ppt}}/k_1$ and $k'_{\text{aggregation}}/k_1$ determine the observed dependence of rate of holoMb formation on $K_{\text{UN}}$. However, even if the values of $k_1$ were on the order of only 1 $\text{s}^{-1}$, the values of $k_{\text{ppt}}$ or $k'_{\text{aggregation}}$ would still be very large and on the order of 250,000 $\text{s}^{-1}$ and $10^{10} \text{M}^{-1} \text{s}^{-1}$.

**Physiological Relevance in Mammals—** Mirceta et al. (14) have shown that there is a strong correlation between apparent apoMb stability measured in vitro by Scott et al. (12) and hologlobin expression levels in the muscle tissue of a variety of mammals. Mirceta et al. (14) also showed that enhanced holoMb expression in muscle tissue correlates with increases in net surface charge ($Z_{\text{Mb}}$) at pH 6.5. Previous studies showed that increased net surface charge of proteins also correlates with a decrease in protein aggregation due to electrostatic repulsion (86). This observation led Mirceta et al. (14) to suggest that inhibition of aggregation also plays an important role in enhancing expression levels.

In our analyses, we assumed that the ratios of the rates of precipitation or aggregation and hemin binding were the same for all the variants examined, and only the independently measured value of $K_{\text{UN}}$ was allowed to vary. However, the increased surface charge of the native Mbs from deep diving mammals could be playing a role by decreasing $k'_{\text{aggregation}}$ (or $k_{\text{ppt}}$), and these effects could partially explain the large differences in relative expression of pig and human Mbs ($Z \leq +1$) versus the whale and seal Mbs ($Z \geq +4$) (14). The increased $Z_{\text{Mb}}$ is probably also increasing $K_{\text{UN}}$ through favorable surface electrostatic interactions that stabilize $\alpha$-helices (87–89). The higher surface charge on the whale Mbs probably also prevents crystallization and precipitation of the folded holoMbs when they accumulate to high concentrations in myocytes.

Regardless of the exact interpretation of $Z_{\text{Mb}}$, the marked increases in relative expression of the H64L and H64F/V68F distal pocket mutations for both sperm whale and dwarf sperm whale Mb suggest that overall folding stability, driven in these cases by a more apolar heme pocket, is the dominant parameter in the heterologous cell-free and E. coli expression systems (Fig. 11 and Table 1). These observations support the idea that the hydrophobic effect is a major driving force for folding to the native apoprotein state (84). The results for these distal pocket mutants also show that the presence of His-64 clearly compromises apoglobin stability in favor of functionality as an $O_2$ storage protein. Even though they express well, the H64F and H64L variants bind $O_2$ poorly, autoxidize quickly, and lose heme rapidly (1, 22, 65).

**Conclusions—** The results for cell-free expression of holoMb in Figs. 10 and 11 confirm unambiguously that apoglobin stability is the key factor governing heterologous expression, and similar correlations occur for Mb expression in muscle tissues of mammals (14). In contrast, hemin affinity does not appear to be an important factor as long as $K_d \leq 10^{-11} \text{M}$. These conclusions apply to all globins and provide a quantitative explanation for why Hbs and Mbs with unstable apoproteins express poorly in E. coli.

Perhaps more importantly, our results demonstrate that the cell-free hologlobin expression assay is an ideal platform for high throughput screening of large libraries of any heme protein of interest, from Hb-based oxygen carriers to NO, CO, and $O_2$ gas sensors, for enhanced apoprotein stability as measured by increased holoprotein yields. This assay can also be adapted to examine other properties of heme proteins, including ligand binding, autoxidation, and heme dissociation. For example, a reducing agent could be added to the translation reaction mixture and then removed during purification to allow generation of ferrous samples for $O_2$ binding and/or autoxidation measurements using a microplate spectrophotometer. Similarly, prolonged incubation of partially purified ferric forms could be used to follow heme dissociation, either by the loss of Soret absorbance or by the addition of a heme-scavenging reagent such as H64Y/V68F apoMb.

**Author Contributions—** P. P. S. helped design the study, performed all the experiments except those involving expression in E. coli, analyzed all of the data, and helped to write the initial draft of the paper, all as part of her Ph.D. thesis project. G. N. P. Jr. provided the initial idea that using the cell-free system, helped design key parts of the in vitro expression system and its analysis, and edited the final manuscript. L. P. S. did all the previous work on holoMb expression in E. coli, including the results in Fig. 10A, provided the initial idea that expression yield depends almost exclusively on overall apoMb stability, and edited the paper. J. S. O. helped to design the in vitro unfolding and heme dissociation experiments, analyze the data, derive the theory for rates of expression, write the initial draft of the paper, and edit the final version.
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