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Metal Ion Facilitated Dissociation of Heme from *b*-Type Heme Proteins

Marcia R. Mauk, Federico I. Rosell, and A. Grant Mauk*

Department of Biochemistry and Molecular Biology and the Centre for Blood Research, Life Sciences Centre, 2350 Health Sciences Mall, University of British Columbia, Vancouver, BC V6T 1Z3 Canada

Received September 3, 2009; E-mail: mauk@interchange.ubc.ca

Abstract: Addition of Ni^{2+}, Cu^{2+}, or Zn^{2+} (10−40 equiv) to metMb in sodium bicarbonate buffer (25 °C) at alkaline pH (7.8−9.5) results in a time-dependent (2−6 h) change in the electronic absorption spectrum of the protein that is consistent with dissociation of the heme from the active site and that can be largely reversed by addition of EDTA. Similar treatment of cytochrome b_5, indoleamine 2,3-dioxygenase, and cytochrome $P450_{cam}$ (in the presence or absence of camphor) produces a similar spectroscopic response. Elution of metMb treated with Ni^{2+} in this manner over an anion exchange column in buffer containing Ni^{2+} affords apo-myoglobin without exposure to acidic pH or organic solvents as usually required. Bovine liver catalase, in which the heme groups are remote from the surface of the protein, and horseradish peroxidase, which has four disulfide bonds and just three histidyl residues, exhibit a much smaller spectroscopic response. We propose that formation of carbamino groups by reaction of bicarbonate with protein amino groups promotes both protein solubility and the interaction of the protein with metal ions, thereby avoiding precipitation while destabilizing the interaction of heme with the protein. From these observations, bicarbonate buffers may be of value in the study of nonmembrane proteins of limited solubility.

Introduction

Stabilization of ferriprotoporphyrin IX binding to the active sites of *b*-type heme proteins results primarily from coordination of the heme iron with ligands provided by the protein, from hydrogen-bonding interactions of the heme propionate groups with the apoprotein and from hydrophobic interactions of the porphyrin prosthetic group with the protein environment in which the heme group resides. As a result, heme protein variants or solution conditions that compromise these interactions diminish the stability of heme binding.

The two classic methods for removal of heme from *b*-type heme proteins destabilize heme binding by protonation of the axial ligands to the heme iron and protonation of the propionate groups to disrupt any hydrogen bonding interactions in which they participate through use of acidic conditions (pH 2−4). At acidic pH, the heme bound to such proteins can usually be extracted readily into the nonmiscible apolar solvent 2-butanone[1] or into acetone (in this case, the resulting apoprotein is isolated as a precipitant, and the dissociated heme remains in the acidified acetone[2,3]). These procedures usually permit preparation of apoprotein that can be reconstituted with a variety of porphyrins, metalloporphyrins, or other macrocycles and that when reconstituted with ferriprotoporphyrin IX affords native proteins with functional properties characteristic of protein from which the heme had never been removed. Alternatively, heme release from myoglobin can be achieved with surfactants[4,5] but these reagents and other denaturants (e.g., urea, guanidinium chloride) induce major structural transitions that may not be tolerated (i.e., reversible) in the case of proteins with less robust structures.

During our studies of a variety of *b*-type heme proteins, we have observed that the stability of heme binding to some well-characterized members of this group is compromised in the presence of transition metal ions and that this behavior can be studied without precipitation of the protein in the presence of bicarbonate buffer. These observations have led us to a more systematic evaluation of the linkage of specific metal ions and the stability of heme binding to *b*-type heme proteins. The results provide evidence for what appears to be a general property of *b*-type heme proteins and a new means by which heme may be dissociated from such proteins in a preparative manner at neutral or alkaline pH without the use of organic solvents or denaturing agents.

Experimental Section

Horse heart Mb (Sigma) was converted to metmyoglobin (metMb) with potassium ferricyanide and then passed over a column of Dowex 1X-8 as described by Linder et al.[6] Bovine liver tryptic cytochrome b_5 was prepared as described previously.[7,8] Human

(1) Teale, F. W. *Biochim. Biophys. Acta* **1959**, *35*, 543.
(2) Theorell, H.; Åkeson, A. *Ann. Acad. Sci. Fennicae, Ser. A.* **1955**, *II*, 303–312.
(3) Harrison, S. C.; Blout, E. R. *J. Biol. Chem.* **1965**, *240*, 299–303.
(4) Tofani, L.; Feis, A.; Snoke, R. E.; Berti, D.; Baglioni, P.; Smulevich, G. *Biophys. J.* **2004**, *87*, 1186–1195.
(5) Feis, A.; Tofani, L.; De Sanctis, G.; Coletta, M.; Smulevich, G. *Biophys. J.* **2007**, *92*, 4078–4087.
(6) Linder, R. E.; Records, R.; Barth, G.; Bunnenberg, E.; Djerassi, C.; Hedlund, B. E.; Rosenberg, A.; Benson, E. S.; Seamans, L.; Moscowitz, A. *Anal. Biochem.* **1978**, *90*, 474–480.
(7) Reid, L. S.; Mauk, A. G. *J. Am. Chem. Soc.* **1982**, *104*, 841–845.
(8) Mauk, M. R.; Mauk, A. G. *Eur. J. Biochem.* **1989**, *186*, 473–486.

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indoleamine 2,3-dioxygenase (IDO) was expressed in *Escherichia coli* and purified as previously described.[9] Cytochrome $P450_{cam}$ was expressed in *E. coli* DH5α cells from a pTZ19u plasmid encoding this protein that was the kind gift of Professor Lindsay Eltis, and the enzyme was purified as previously described.[10] Crystalline bovine liver catalase (Sigma) and horseradish peroxidase (HRP) (Fluka) were used without further purification. Protein concentrations were determined from the molar absorptivities of $\varepsilon_{408} = 188\ 000\ M^{-1}\ cm^{-1}$ (pH 6.4) for Mb,[11] $\varepsilon_{413} = 117\ 000\ M^{-1}\ cm^{-1}$ for cytochrome b_5,[12] $\varepsilon_{404} = 172\ 000$ for IDO,[13] $\varepsilon_{391} = 102\ 000\ M^{-1}\ cm^{-1}$[14] for cytochrome $P450_{cam}$ in the presence of camphor, $\varepsilon_{417} = 115\ 000\ M^{-1}\ cm^{-1}$[14] for cytochrome $P450_{cam}$ in the absence of camphor, $\varepsilon_{406} = 120\ 000\ M^{-1}\ cm^{-1}$ per monomer for catalase,[15] and $\varepsilon_{403} = 100\ 000\ M^{-1}\ cm^{-1}$ for HRP.[16]

Glass-distilled water passed through a Barnstead Nanopure Diamond Life Sciences purification system was used throughout. Solutions of metal ions were prepared by volumetric dilution from Titrosol (E. Merck) standards in water ($CoCl_2$, $CuCl_2$, $MnCl_2$) or gravimetrically for $ZnSO_4$ (Fisher) and $NiCl_2$ (Sigma). Hemin (Frontier Scientific) solutions were prepared immediately before use by dissolving in 0.1 M NaOH and then diluting rapidly into buffer.[17] Air saturated bicarbonate (Sigma), HEPES (Acros), and borate (Sigma) buffers were maintained at 25 °C.

Spectroscopic and kinetics experiments were performed with Cary Models 6000 and 3 spectrophotometers. All protein samples were thermostatted in Teflon stoppered cuvettes (Hellma) at 25 °C unless otherwise indicated. Kinetics of heme release were monitored at 411 nm for Mb, 404 nm for IDO, 412.5 nm for cytochrome b_5, 405 nm for catalase and 403 nm for HRP.

Results

Effects of Metal Ions on the Stability of Heme Binding to Apo-Myoglobin. Addition of Ni^{2+} (100 μM) to a solution of horse heart metMb (5 μM, pH 9.5, $NaHCO_3$ (20 mM), NaCl (0.1 M)) results in a slow but marked decay in the electronic absorption spectrum of metMb that is essentially complete in 24 h (Figure 1). Specifically, the Soret maximum shifts from 411 to 385 nm such that the absorbance intensity at 411 nm decreases by 63%, and well-defined isosbestic points are observed at 388.5, 511.5, and 619 nm. Minimal change (<1%) is observed in the spectrum of metMb under identical solution conditions over the same period of time in the absence of Ni^{2+}. These observations are consistent with dissociation of the heme prosthetic group from its well-known binding site by addition of Ni^{2+} to metMb under these conditions. No further change in spectrum occurred in the subsequent four days at 25 °C, and there was no evidence of precipitation of either protein or heme. Increasing the bicarbonate or NaCl concentration resulted in no significant acceleration of this spectroscopic transition. The final Soret and visible spectrum observed after 24 h is nearly the same as that of heme alone in the metal ion-buffer mixture

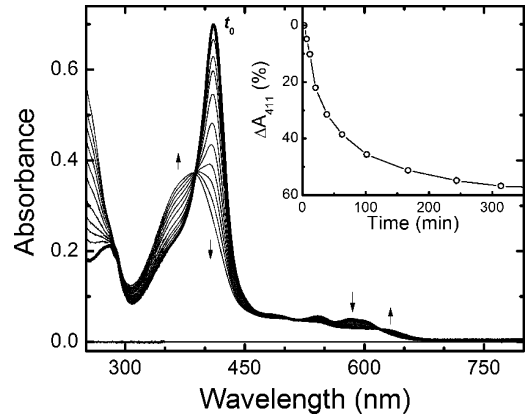

Figure 1. Time dependent response of the Mb spectrum to Ni^{2+}. Mb (5.50 μM) in $NaHCO_3$ (20 mM) with NaCl (0.1 M) at pH 9.50, $NiCl_2$ (100 μM), 25 °C. The percentage decrease in the absorbance at 411 nm is shown in the inset. The arrows indicate the direction of change in spectral intensity with time.

although the absorption maxima are more intense in the presence of the protein. The Soret spectrum of heme alone in the metal containing buffer continued to decrease significantly in intensity with time which is indicative of aggregation,[18,19] but this spectroscopic change is not observed in the presence of protein. The stability of the absorption spectrum to further change 24 h after adding Ni^{2+} to metMb indicates that the heme released from the active site probably remains associated with the protein through ill-defined, adventitious binding to the surface of the protein.

The significant absorbance increase below 280 nm (Figure 1) results from binding of Ni^{2+} by protein residues as indicated by the fact that a solution of Ni^{2+} at the same concentration (100 μM) in this buffer has an absorbance at 250 nm of ~0.02. The same experiment (5 μM Mb with 100 μM Ni^{2+}) conducted in 20 mM borate buffer (0.1 M NaCl pH 9.5) rather than bicarbonate resulted in precipitation of ~65% of the protein. The Soret maximum of the protein that remained in solution shifted <1 nm, and the electronic transitions observed below 280 nm were again indicative of Ni^{2+} binding to Mb. In the absence of protein, Ni^{2+} (100 μM) in the borate buffer exhibits a gradual increase in light scattering ($A_{411} \approx 0.03$ after 24 h) presumably owing to Ni^{2+}-hydroxide polymer formation.

The time dependent effect of varying concentrations of Ni^{2+}, Zn^{2+}, and Cu^{2+} on the Soret absorbance of metMb in bicarbonate buffer (pH 9.5) is shown in Figure 2. The concentration dependencies shown in this figure indicate that under these conditions, slightly more than 15 equiv of Ni^{2+} are required to achieve complete dissociation of the heme from its normal binding site and that 3.3 equiv of Cu^{2+} produced 50% of the maximum reduction in Soret absorbance. No evidence of protein instability or precipitation was observed during these experiments. Alternatively, addition of Co^{2+} (100 μM) to Mb (5 μM) in bicarbonate buffer (pH 9.5) resulted in precipitation of some protein (<10%) but no change in spectrum. Addition of Mn^{2+} under identical conditions led to precipitation of >24% of the protein within 24 h at 25 °C, again with no change in spectrum of the protein that remained in solution.

The very rapid release of heme from metMb in the presence of higher molar ratios (≥17 equiv) of Cu^{2+} (Figure 2B) led to

(9) Vottero, E. R. Ph.D. Dissertation, University of British Columbia, Vancouver, 2007.
(10) Westlake, A. C.; Harford-Cross, C. F.; Donovan, J.; Wong, L. L. *Eur. J. Biochem.* **1999**, *265*, 929–935.
(11) Antonini, E.; Brunori, M. In *Hemoglobin and Myoglobin in Their Reactions with Ligands*; North Holland: New York, 1971; p 22.
(12) Ozols, J.; Strittmatter, P. *J. Biol. Chem.* **1964**, *239*, 1018–1023.
(13) Papadopoulou, N. D.; Mewies, M.; McLean, K. J.; Seward, H. E.; Svistunenko, D. A.; Munro, A. W.; Raven, E. L. *Biochemistry* **2005**, *44*, 14318–14328.
(14) Gunsalus, I. C.; Wagner, G. C. *Methods Enzymol.* **1978**, *52*, 166–188.
(15) Vlasits, J.; Jakopitsch, C.; Schwanninger, M.; Holubar, P.; Obinger, C. *FEBS Lett.* **2007**, *581*, 320–324.
(16) Delincee, H.; Radola, B. J. *Eur. J. Biochem.* **1975**, *52*, 321–330.
(17) de Villiers, K. A.; Kaschula, C. H.; Egan, T. J.; Marques, H. M. *J. Biol. Inorg. Chem.* **2007**, *12*, 101–117.
(18) Brown, S. B.; Lantzke, I. R. *Biochem. J.* **1969**, *115*, 279–285.
(19) Kuzelova, K.; Mrhalova, M.; Hrkal, Z. *Biochim. Biophys. Acta* **1997**, *1336*, 497–501.

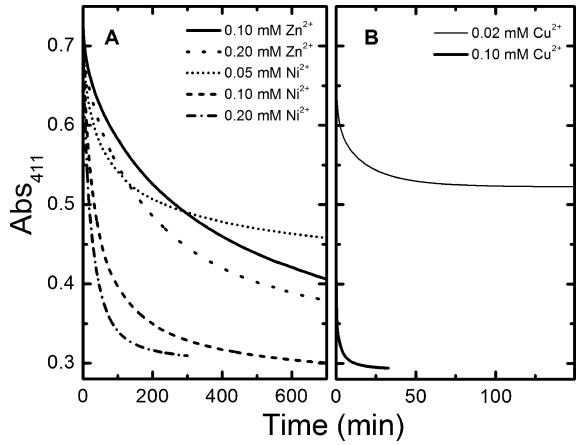

Figure 2. The change in absorbance at 411 nm as a function of time following the addition of metal ions to Mb (5.75 μM) in $NaHCO_3$ buffer (20 mM) with NaCl (0.1 M) at pH 9.5 and 25 °C. The absorbance of each solution prior to addition of metal ion was 0.73. Panel A: Reactions with Zn^{2+} and Ni^{2+}. Panel B: Reactions with Cu^{2+}. The concentration of each metal ion is shown adjacent to its trace.

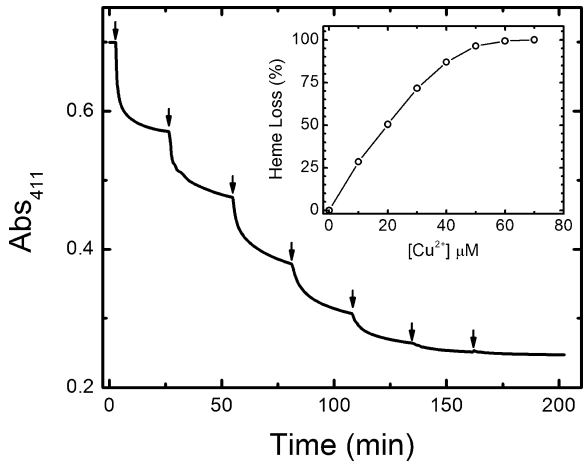

Figure 3. Effect of incremental addition of Cu^{2+} (10 μM each) on the Soret absorbance of metMb (5.3 μM) in $NaHCO_3$ buffer (20 mM, pH 9.5) with NaCl (0.1 M) (25 °C). The arrows indicate each addition of $CuCl_2$ solution. The inset shows the fraction of heme lost from metMb under these conditions as a function of total Cu^{2+} added.

consideration of the kinetics of heme release following incremental addition of smaller concentrations of copper ion (Figure 3). Each addition is accompanied by a release of heme that can be fit as multiple exponential processes. These incremental additions of Cu^{2+} result in the same changes in the Soret and visible spectrum of metMb that result from addition of Ni^{2+} (Figure 1), and the extent of heme release is a function of the total amount of Cu^{2+} added (Figure 3, inset). Overall, the efficiency of heme release from metMb by metal ions differed in the order $Cu^{2+} > Ni^{2+} > Zn^{2+}$. All three metal ions facilitate release of heme from metMb (5 μM) in $NaHCO_3$ buffer (20 mM) containing NaCl (0.1M)(25 °C) at a significant rate under moderately alkaline conditions. NaCl is not required in these experiments, but it enhances the stability of the apo-Mb and promotes the recovery of the apoprotein during ion exchange chromatography (vide infra).[20]

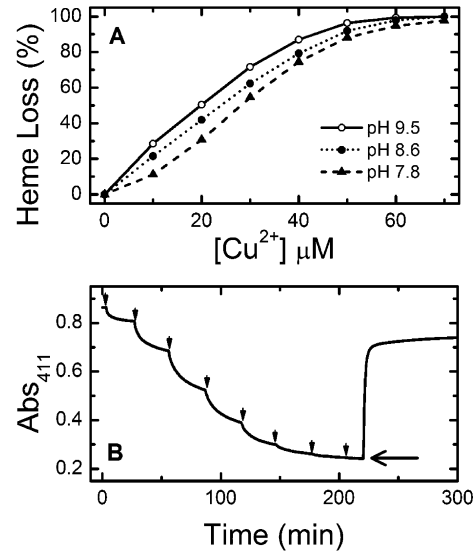

Figure 4. Heme release from metMb as a function of pH and $[Cu^{2+}]$. (A) Release of heme from metMb (5 μM) as monitored by the decrease in Soret absorbance following addition of Cu^{2+} at pH 9.5, 8.6, and 7.8. (B) Heme release following incremental addition of Cu^{2+} (each increasing final $[Cu^{2+}]$ by 10 μM) to metMb (4.9 μM) and reconstitution following addition of EDTA solution (final [EDTA] = 200 μM) at pH 7.8 as reflected by the intensity of the Soret absorbance ($NaHCO_3$, buffer (20 mM) with NaCl (0.1 M)) at pH 7.8 and 25 °C). The vertical arrows indicate addition of $CuCl_2$ solution, and the horizontal arrow indicates addition of EDTA solution.

The bicarbonate buffer has a profound effect on promoting the solubility of the excess metal ions and the stability of the metal ion-protein complexes. Replacement of Na^+ with NH_4^+ in the bicarbonate buffer system (i.e., 20 mM NH_4HCO_3, 0.1 M NH_4Cl, pH 9.5) greatly reduced the effects of metal ions on the spectrum of metMb. For example, the spectrum of metMb (6.5 μM) in the presence of Cu^{2+} (100 μM) or Ni^{2+} (200 μM) in NH_4HCO_3 buffer exhibited a decrease in A_{411} after 1000 min of just 1% or 1.5%, respectively. This reduction in rate of heme release could result from diminished binding of metal ions or from increased stability of heme binding to metMb as the result of coordination of ammonia to the heme iron at alkaline pH.[21,22]

pH Dependence of Metal Ion Facilitated Release of Heme from metMb. The pH-linkage of metal ion induced release of heme from metMb was considered (e.g., Figure 3). Reducing pH from 9.5 to 8.6 decreases the extent of heme release observed for initial increments, but at sufficiently high concentrations of metal ions (e.g., 70 μM), the extents of heme release are essentially identical (Figure 4A), and incremental addition of Cu^{2+} at the lower pH again demonstrates saturable behavior. Lowering the pH to 7.8 introduces another small reduction in the rate of initial heme release and results in a slight lag phase in the release of heme upon incremental Cu^{2+} addition (Figure 4B). Following complete Cu^{2+}-induced release of heme from metMb at this pH in bicarbonate buffer, addition of EDTA results in rapid restoration of the original metMb spectrum with typical recovery of >88% in the Soret intensity within 1 h (Figure 4B). Failure to achieve 100% recovery presumably results from irreversible aggregation of some of the heme following its release. The sodium bicarbonate buffer is a key factor in the reversibility of this process. In HEPES buffer (20

(20) The term holoprotein as used here refers to a protein containing Fe^{3+}-protoporphyrin IX, while the term apoprotein refers to a protein devoid of heme. Neither term is related to the binding of metal ions.

(21) Chance, B.; Rumen, N. M. *Eur. J. Biochem.* **1976**, *61*, 409–415.
(22) Marques, H. M.; Byfield, M. P.; Pratt, J. M. *J. Chem. Soc., Dalton Trans.* **1993**, 1633–1639.

Table 1. Analysis of Heme Release from metMb as a Function of pH and Metal Ion Concentration[a]

									% heme loss	
ion	conc. (μM)	pH	k_1[b] (min^{-1})	ΔAbs_1[c] (%)	k_2 (min^{-1})	ΔAbs_2(%)	k_3 (min^{-1})	ΔAbs_3 (%)	1h	12 h
Ni^{2+}	50	9.5	0.0918	23	0.0153	38	0.00229	38	31	55
	100	9.5	0.178	20	0.0227	50	0.00429	31	64	92
	200	9.5	0.187	23	0.0391	54	0.0117	23	77	92
	200	8.6	0.231	21	0.0238	25	0.00134	54	11	21
	400	8.6	0.372	11	0.0376	19	0.00166	69	18	48
	200	7.8	0.411	17	0.0245	33	0.00191	50	6	11
Zn^{2+}	100	9.5	0.119	9	0.0135	18	0.00226	72	25	70
	200	9.5	0.292	14	0.0157	20	0.00334	66	32	77
	100	8.6	0.461	27	0.0473	22	0.00933	51	25	34
	100	7.8	2.93	29	0.384	14	0.0266	58	28	31
Cu^{2+}	20	9.5	1.35	64	0.222	12	0.0438	24	51	52
	100	9.5	4.88	88	0.456	8	0.111	5	97	97
	100	8.6	4.54	79	0.688	16	0.0967	5	99	99
	100	7.8	2.33	69	0.390	21	0.0613	10	98	98

[a] Addition of metal ions to Mb (5.75 μM) in $NaHCO_3$ buffer (20 mM) with 0.1 M NaCl at 25 °C. [b] The errors in rate constants are ≤1% of the numerical values. [c] % of the change in Abs_{411nm} contributed by each rate process.

mM with 0.1 M NaCl, pH 7.8, 25 °C), incremental addition of Cu^{2+} to Mb (5 μM) resulted in slight turbidity at [Cu^{2+}] < 30 μM. On increasing Cu^{2+} concentration to 70 μM Cu^{2+} under these conditions, the heme appears to be released from Mb, but about a third of the protein is precipitated, and the protein remaining in solution recovers only ~50% of the original Soret intensity following treatment with EDTA.

Rate of Metal Ion-Induced Heme Release from metMb. The rate of heme release induced by addition of metal ions varies with the identity of the metal ion (Figure 2), with the concentration of the metal ion (Figure 2, Table 1), and with pH (Table 1). In all cases, the kinetics of heme dissociation from metMb can be fitted as the sum of three exponentials, and the rate constants obtained from such an analysis are set out in Table 1. For Ni^{2+} and Cu^{2+}, the absorbance change associated with the fastest rate process decreases with decreasing pH, while for Zn^{2+}, the effect of pH is the opposite. The complexity of the trends observed for each rate process is consistent with the expectation that these three metal ions bind at different sites on metMb and with different affinities. Interestingly, the rate of heme dissociation is not necessarily correlated with the final extent of heme release as clearly seen for Zn^{2+} (Table 1). To simplify identification of conditions that best promote heme dissociation, we summarize these results in terms of the % of heme released 1 and 12 h after addition of metal ions (Table 1).

Metal Ion Facilitated Release of Heme from Other *b*-Type Heme Proteins. Observation of metal ion facilitated release of heme from metMb raises the question of whether this phenomenon is unique to myoglobin or is a common feature of *b*-type heme proteins. In the case of ferricytochrome b_5, metal ion-induced heme release is relatively rapid at pH 7.8 ($NaHCO_3$ buffer (20 mM) with NaCl (0.1 M)) and 25 °C (Figure 5 inset) but slower than observed for Mb (cf. Figure 4). Close inspection reveals that the isosbestic points that are observed initially for metal ion induced release of heme from cytochrome b_5 are not perfectly maintained after extended time (e.g., 22 h) (Figure 5) as the result of a slight amount of light scattering that we attribute to aggregation of released heme. Nevertheless, the kinetics of heme release from ferricytochrome b_5 (5 μM) following addition of Cu^{2+} (100 μM)(Figure 5 inset) is described best as a biexponential process with k_1 (0.0292 min^{-1}) contributing 18% and k_2 (0.00405 min^{-1}) contributing 81% of the $\Delta A_{412.5}$ so that 34% of the heme was released after 1 h and 97% was released after 12 h. Heme release from ferricytochrome b_5 (5 μM) was complete in 24 h following addition of Cu^{2+} (50 μM) but just 30% complete following similar treatment with Zn^{2+} (50 μM) at pH 7.8 ($NaHCO_3$ buffer (20 mM) with NaCl (0.1 M)). In NH_4HCO_3 buffer, just 10% of the heme was released from ferricytochrome b_5 following exposure to Cu^{2+} (50 μM) for 24 h.

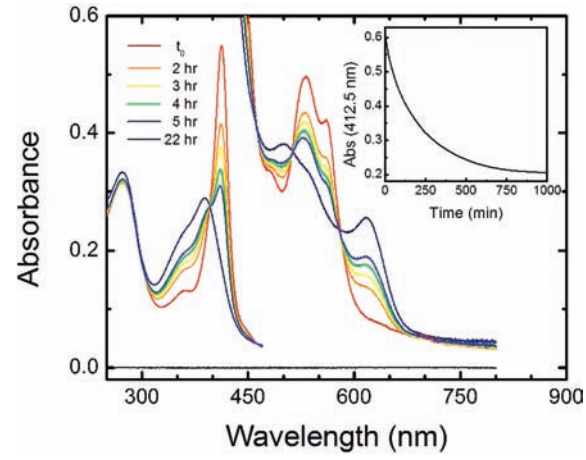

Figure 5. The change in electronic absorption spectrum of ferricytochrome b_5 (4.8 μM) as a function of time following addition of Cu^{2+} (50 μM) in $NaHCO_3$ buffer (20 mM, pH 7.8) containing NaCl (0.1 M) at 25 °C. The inset shows the change in intensity of the Soret maximum (412.5 nm) with time after addition of Cu^{2+} (100 μM) to ferricytochrome b_5 (4.8 μM) under otherwise identical solution conditions.

In the case of IDO, decreasing the reaction temperature to 15 °C and increasing the NaCl concentration dramatically improves the stability of the apoprotein, so the reaction conditions for reaction with metal ions were adjusted accordingly for this protein. Addition of Cu^{2+} (100 μM) to IDO (5 μM) (pH 7.8, $NaHCO_3$ buffer (20 mM) with NaCl (0.3 M)) led to release of 86% of heme in 1 h at 15 °C, which compares with 98% release from metMb and 34% release from ferricytochrome b_5 at 25 °C under conditions that were otherwise identical. At this lower temperature, there was no evidence of precipitation or apo-IDO instability for at least 48 h after addition of Cu^{2+}. After 24 h at 15 °C, the same concentration of Ni^{2+} induced loss of 28% of the heme from IDO, and the reaction was not yet complete.[23]

The cytochrome $P450_{cam}$−camphor complex exhibited a change from its characteristic high-spin electronic absorption spectrum to the spectrum characteristic of low-spin protein immediately following addition of Zn^{2+} (28.5 equiv) at pH 7.8

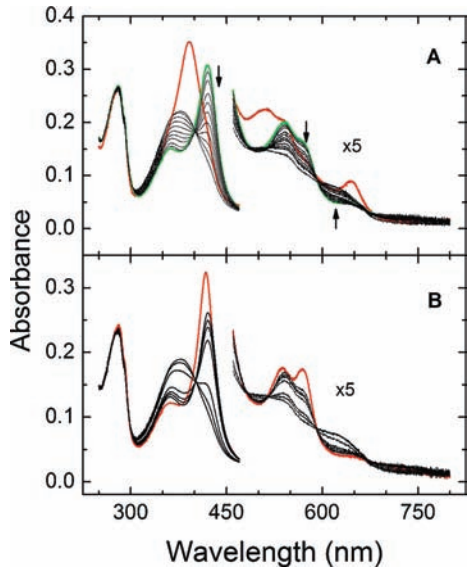

Figure 6. The release of heme from cytochrome $P450_{cam}$ following addition of Zn^{2+} in $NaHCO_3$ buffer (20 mM, pH 7.8) containing NaCl (0.1 M) at 25 °C. (A) The electronic absorption spectrum of the cytochrome $P450_{cam}$−camphor complex (3.5 μM protein with 500 μM camphor) before (red spectrum) and immediately after (green spectrum) addition of Zn^{2+} (100 μM). The black spectra demonstrate the subsequent change in spectrum as a function of time. (B) The electronic absorption spectrum of cytochrome $P450_{cam}$ (2.8 μM) in the absence of camphor before (red spectrum) and as a function of time (black spectra) after addition of Zn^{2+} (100 μM).

($NaHCO_3$ buffer (20 mM) with NaCl (0.1 M) 25 °C) (Figure 6A). This change in spectrum occurs within the time required for manual addition of Zn^{2+} and recording the first spectrum, and it is followed by a change in spectrum that continues for the next 4−5 h that is similar to the changes observed following similar treatment of Mb, cytochrome b_5, and IDO. The rapid conversion to the low-spin spectrum may signify release of bound camphor, but more evidence is required to evaluate this possibility. In the absence of camphor, a prompt but subtle change is induced in the low-spin spectrum of cytochrome $P450_{cam}$ that involves a decrease in intensity of the Soret absorbance (~30%), a slight shift in Soret maximum to the red, and a decrease in the intensity of the absorption maximum at 570 nm immediately following addition of Zn^{2+} (35.7 eqiv). Over the next 6 h, the spectrum continues to change (Figure 6B) in a manner consistent with dissociation of heme from the active site. These spectroscopic changes could be reversed ~90% overnight following addition of EDTA.

Addition of metal ions to catalase produced a change in spectrum at a much lower rate than that observed for metMb or ferricytochrome b_5. For example, after three hours at pH 7.8 (25 °C), Cu^{2+} (200 μM) produced a decrease in Soret band intensity of just 15 and 28% for 5 μM and 2 μM catalase (concentrations based on heme content), respectively. However, this rate of change was increased by raising the temperature to 37 °C. For catalase (2 μM) in sodium bicarbonate buffer (20 mM, pH 8.2, 37 °C), the change in spectrum was complete in 2 h following addition of Cu^{2+} (200 μM) and >22% complete in 6 h with Zn^{2+} (200 μM). The concentration of metal ions required for this spectroscopic response by catalase is significantly greater than that required for the other proteins studied here. Increasing the concentration of metal ions to promote more extensive and rapid spectroscopic responses was limited by the precipitation of the protein at metal ion concentrations >0.5 mM.

(23) After 48 and 68 h, 45% and 56% of the heme is dissociated under these conditions, respectively.

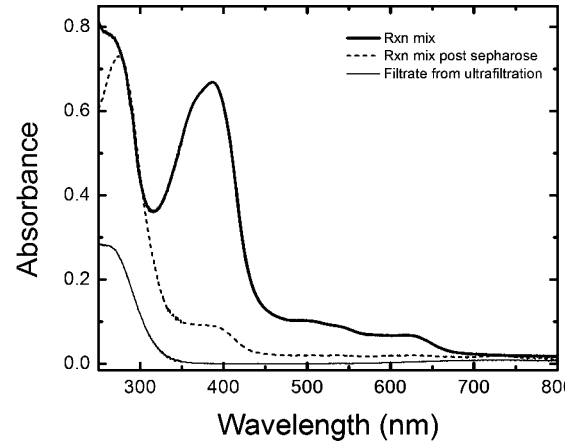

Figure 7. Removal of heme by chromatography on Q-Sepharose. MetMb (10 μM) was incubated with $CuCl_2$ (100 μM) at pH 7.8 in $NaHCO_3$ buffer (20 mM) with NaCl (0.1 M) for 50 min, 25 °C (reaction mix spectrum, heavy line) and then passed over Q-Sepharose (eluent spectrum, dashed line, corrected for dilution). EDTA was then added (to 200 μM, pH 7.8) to the eluted apoprotein, and the Cu^{2+} was removed by centrifugal ultrafiltration (spectrum of filtrate from centrifugal ultrafiltration (Microcon), thin line).

HRP proved to be relatively resistant to Ni^{2+} or Zn^{2+} induced spectroscopic change from pH 7.8 to 9 but responded rapidly to Cu^{2+} addition. For example, addition of Cu^{2+} (20 μM) to HRP (4 μM) in $NaHCO_3$ buffer (20 mM, pH 7.8) containing NaCl (0.1 M) induced a 30% decrease in the Soret intensity within 10 min with no shift in the position of the Soret maximum. The spectroscopic changes induced by Cu^{2+} differ from those seen for the other proteins studied here in that the resulting spectrum does not resemble that of unbound heme. Instead, it appears that addition of Cu^{2+} in some way disrupts heme binding at the active site in a spectroscopically detectable but structurally ill-defined manner.

Purification of Apoprotein and Reconstitution with Heme. The dissociation of heme from *b*-type heme proteins by metal ions may have practical implications for preparation of apoproteins that do not tolerate exposure to acid pH or organic solvents as required by the traditional procedures. However, use of this method for preparation of apoproteins also requires identification of a method for separating the unbound heme from the resulting apoprotein because heme released from such proteins undergoes a variety of aggregation and/or nonspecific interactions that prevent separation of unbound heme from apoprotein by simple buffer exchange methods.[24,25] Nevertheless, apoprotein can be separated rapidly from unbound heme by elution of a protein-metal ion-buffer mixture that exhibits the spectrum of unbound heme over a column of Q-Sepharose Fast Flow resin (GE Healthcare) that has been equilibrated with the same bicarbonate buffer/metal ion composition prior to application of the protein-containing reaction mixture. Under these conditions, the apoprotein elutes with the equilibrating metal-ion buffer solution, and the heme remains bound to the resin (Figure 7). If the p*I* of the apoprotein is substantially lower than the buffer pH, as is the case for cytochrome b_5, addition of higher concentrations of NaCl to the metal ion-buffer may be required to elute the apoprotein completely from the resin.

(24) Cann, J. R. *Biochemistry* **1964**, *3*, 714–722.
(25) Blum, O.; Haiek, A.; Cwikel, D.; Dori, Z.; Meade, T. J.; Gray, H. B. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 6659–6662.

Heme is satisfactorily retained by the Q-Sepharose even with buffers containing as much as 0.3 M NaCl. Other anion exchange resins can be used for this separation but generally do not bind heme as tenaciously as Q-Sepharose. This procedure for removing heme is scalable, and the amount of ion-exchange resin used for this separation should be appropriate for the amount of protein processed. For example, a Q-Sepharose column containing 0.5 mL of resin is sufficient to retain the heme released from 3 mL of 10 μM protein.

Reconstitution of the resulting purified apoprotein can be achieved following removal or simple chelation of metal ions with EDTA. Removal of the EDTA-metal ion complex is not required for reconstitution but can be accomplished by buffer exchange through centrifugal ultrafiltration, gel filtration (on PD-10 columns (GE Healthcare), or protein desalting spin columns (Pierce)) if required. ApoMb prepared by this method can be fully reconstituted by addition of hemin (in a variety of buffers including phosphate, borate, and bicarbonate) to produce metMb that is spectroscopically indistinguishable from the initial metMb sample (data not shown). In the case of metMb, for example, metal ion induced release of heme from metMb, purification of the apoprotein, and reconstitution with fresh heme as described here can be completed in less than an hour.

Discussion

The current results provide evidence that metal ion induced destabilization of heme binding to *b*-type heme proteins may be a general property of this class of protein. It is also clear, however, that each protein responds differently on exposure to various metal ions and that the optimal conditions required for metal ion induced dissociation of heme from each protein must be defined individually. For the proteins included in the current study, the most fundamental common feature of the conditions employed is that heme dissociation could be induced at alkaline pH in bicarbonate buffer in contrast to standard methods for preparation of apoproteins at acidic pH.[1−3] Myoglobin has been the focus of this work, and the insights gained from this protein have been applied to the other members of this protein family to evaluate the generality of this phenomenon. From this initial survey, it is clear that each *b*-type heme protein exhibits a distinct selectivity regarding which metal ion(s) can destabilize heme binding and which have no effect.

The binding of Cu^{2+},[26,27] Zn^{2+}[24,28] and Mn^{2+}[29] to metMb and some resulting spectroscopic consequences were discovered in the 1960s, and binding sites for Cu^{2+} and Zn^{2+} were identified by X-ray crystallographic studies of sperm whale metMb around the same time.[30] The results we have obtained in bicarbonate buffer are in distinct contrast to those in these earlier studies in that the earlier work was performed in unbuffered KCl solution or acetate buffer or *N*-ethylmorpholine buffer.[26,31,32] Under such conditions (pH < 7), $\geq$2 eqiv of Cu^{2+} were sufficient to precipitate sperm whale Mb. This behavior led these authors to conclude that binding of Cu^{2+} to metMb resembles acid-induced denaturation and to the discussion of Cu^{2+} as a denaturing agent for this protein.[26,32,33] Binding of Cu^{2+} or selected metal ion complexes to metMb has been reported to induce a loss of tertiary structure with retention of secondary structure[25,27,31] such that heme is bound nonspecifically to the protein in a manner that prevents its removal by dialysis, ultrafiltration, or desalting columns.[31,34,35] Presumably, acidic pH was used in these previous studies to avoid problems arising from insolubility of metal ions at alkaline pH. The solution conditions used in our work avoid insolubility of the protein and/or metal ions at alkaline pH and permitted recognition that a major consequence of metal ion binding to metMb and other *b*-type heme proteins is the destabilization of heme binding.

Role of Sodium Bicarbonate. It is clear that sodium bicarbonate buffer is an important participant in these observations because, as indicated above, use of either HEPES or borate buffers prevented any of the metal ion-induced effects observed when using sodium bicarbonate. We were drawn initially to the use of bicarbonate buffer because this is the buffer employed traditionally[36,37] when using the classic procedures of Theorell and Åkeson[2] or Teale[1] for extraction of heme from myoglobin or hemoglobin. The manner in which bicarbonate enables the metal ion induced dissociation of heme from the proteins studied here probably varies from protein to protein but most likely results primarily from reversible modification of lysyl side chains to form carbamino groups similar to those believed to be responsible for transport of CO_2 by deoxyhemoglobin.[38−43] Formation of carbamino groups is favored at alkaline pH and results from reaction of CO_2 with deprotonated α- and lysyl side chain amino groups to introduce a negative charge. For myoglobin, NMR studies have established that in bicarbonate buffer, the CO_2 adduct of the amino terminal residue forms at pH >7.5[44] and that carbamino group formation at lysyl residues increases rapidly as pH is increased above pH 8.[44,45]

We propose that introduction of carbamino groups in the proteins we have studied promotes protein solubility at pH values at which solubility would otherwise be limited in the presence of metal ions and/or the introduction of potential ligands for coordination of exogenous metal ions. The best example of carbamino group participation in coordination of a metal ion is provided by the demonstration by Hausinger, Karplus, and co-workers that Ni^{2+} binding at the active site of urease requires formation of a carbamino adduct of a specific lysyl residue.[46−48] It is also possible that bicarbonate buffer

(26) Breslow, E.; Gurd, F. R. *J. Biol. Chem.* **1963**, *238*, 1332–1342.
(27) Breslow, E.; Beychok, S.; Hardman, K. D.; Gurd, F. R. *J. Biol. Chem.* **1965**, *240*, 304–309.
(28) Cann, J. R. *Biochemistry* **1964**, *3*, 903–908.
(29) Gersonde, K.; Netter, H. *J. Mol. Biol.* **1966**, *22*, 211–221.
(30) Banaszak, L. J.; Watson, H. C.; Kendrew, J. C. *J. Mol. Biol.* **1965**, *12*, 130–137.
(31) Hartzell, C. R.; Hardman, K. D.; Gillespie, J. M.; Gurd, F. R. *J. Biol. Chem.* **1967**, *242*, 47–53.
(32) Breslow, E. *J. Biol. Chem.* **1964**, *239*, 3252–3259.
(33) Lamy, M. T.; Ribeiro, P. C.; Nascimento, O. R.; Bemski, G. *FEBS Lett.* **1976**, *72*, 29–32.
(34) Hargrove, M. S.; Olson, J. S. *Biochemistry* **1996**, *35*, 11310–11318.
(35) Marks, R. H.; Cordes, E. H.; Gurd, F. R. *J. Biol. Chem.* **1971**, *246*, 466–474.
(36) Rossi-Fanelli, A.; Antonini, E.; Caputo, A. *Biochim. Biophys. Acta* **1958**, *28*, 221.
(37) Breslow, E. *J. Biol. Chem.* **1964**, *239*, 486–496.
(38) Ferguson, J. K.; Roughton, F. J. *J. Physiol.* **1934**, *83*, 68–86.
(39) Ferguson, J. K.; Roughton, F. J. *J. Physiol.* **1934**, *83*, 87–102.
(40) Rossi-Bernardi, L.; Roughton, F. J. *J. Physiol.* **1967**, *189*, 1–29.
(41) Morrow, J. S.; Keim, P.; Visscher, R. B.; Marshall, R. C.; Gurd, F. R. *Proc. Natl. Acad. Sci. U. S. A.* **1973**, *70*, 1414–1418.
(42) Kilmartin, J. V.; Fogg, J.; Luzzana, M.; Rossi-Bernardi, L. *J. Biol. Chem.* **1973**, *248*, 7039–7043.
(43) Kilmartin, J. V.; Rossi-Bernardi, L. *Physiol. Rev.* **1973**, *53*, 836–890.
(44) Morrow, J. S.; Keim, P.; Gurd, F. R. *J. Biol. Chem.* **1974**, *249*, 7484–7494.
(45) Wittmann, B.; Gros, G. *J. Biol. Chem.* **1981**, *256*, 8332–8340.
(46) Jabri, E.; Carr, M. B.; Hausinger, R. P.; Karplus, P. A. *Science* **1995**, *268*, 998–1004.
(47) Yamaguchi, K.; Hausinger, R. P. *Biochemistry* **1997**, *36*, 15118–15122.
(48) Pearson, M. A.; Schaller, R. A.; Michel, L. O.; Karplus, P. A.; Hausinger, R. P. *Biochemistry* **1998**, *37*, 6214–6220.

promotes the solubility of some metal ions at alkaline pH that would otherwise be insoluble. Bicarbonate buffer has been reported[49,50] to assist in reducing the extent of heme aggregation, but this effect is insufficient in the absence of protein to prevent heme precipitation when divalent metal ions are present.

Role of Metal Ions. In general, protein binding sites for metal ions involve participation of two or more coordinating groups with His, Cys, Met, and peptide nitrogens most frequently involved,[51−53] so these groups are most likely to participate in the metal ion binding sites related to dissociation of heme in the current work. Some specific residues have been implicated in binding of metal ions[30,54,55] or simple coordination complexes[25] to myoglobin and cytochrome b_5,[56] and it is likely that these sites participate in the metal ion-induced destabilization of heme binding to these proteins. Nevertheless, it is unlikely that these are the only sites responsible for the results reported here. Interestingly, EPR studies of metMb in the presence of 1 equiv of Cu^{2+} indicate that two forms of the metal complex interconvert with a pK of ∼7.9 and could involve separate sites,[57] but related information is not available for the other proteins studied here. The binding of metal ions to cytochrome b_5, IDO, cytochrome P450$_{cam}$, HRP, and catalase has received little or no attention in the literature. For each of these proteins, it is likely that formation of carbamino groups under our conditions participates in additional coordination of metal ions that can introduce further structural perturbation that allows metal ion binding to amino acid side chains that are normally inaccessible.

Use of metal ions to induce heme dissociation as reported here can entail the risk of peptide bond hydrolysis that must be evaluated on a case by case basis. This concern arises because specific amino acid sequences are known to be susceptible to Cu^{2+}-[58−60] and Ni^{2+}-[61−63]catalyzed peptide bond hydrolysis. Although horse heart Mb does possess sequences that have been reported to be subject to Cu^{2+}-catalyzed hydrolysis, no evidence of hydrolysis was detected under the conditions used in these experiments presumably because such hydrolysis requires elevated temperatures (50 °C) and extended reaction time (3 days).[58] For other proteins, we have confirmed the absence of metal ion catalyzed protein hydrolysis by SDS-PAGE (data not shown).

The reversibility of the protein structural changes induced by metal ions is exemplified by prompt restoration of the spectrum of native metMb by addition of EDTA following exhaustive reaction with Cu^{2+} in bicarbonate buffer (Figure 4B). The beneficial role of bicarbonate on this reversibility is at least 2-fold. First, as noted above, bicarbonate reduces the extent of heme aggregation,[49,50] and it is known that heme aggregation impedes heme uptake by apoproteins and precludes detailed kinetic analysis.[19,64,65] In addition, the reversibility of heme binding to Mb likely involves a change in the pI of the apoprotein that accompanies carbamino adduct formation. Formation of carbamino groups in bicarbonate buffer effectively lowers the pI and improves the solubility of the protein, thereby preventing precipitation of the protein that has been reported to accompany addition of metal ions.

While myoglobin, cytochrome b_5, IDO, and cytochrome P450$_{cam}$ responded significantly to the addition of metal ions, the response of HRP and catalase was much smaller. We attribute the poor response of HRP and catalase to structural characteristics of these proteins that distinguish them from the others. Specifically, the three-dimensional structure of bovine liver catalase reveals that the heme prosthetic group of each subunit is buried deeply within the protein and is accessible to bulk solvent only through a channel ∼18 Å in length that is believed to permit access of the substrate, hydrogen peroxide, to the active site.[66] For this reason, the observation of any response of the spectroscopic properties of the heme in this enzyme under the relatively mild conditions we have used is remarkable. However, for its size, HRP possesses a relatively small number of residues that are probable ligands (vide supra) for metal ion binding compared to the other proteins. In addition, HRP is structurally stabilized by the presence of four disulfide bonds.[67]

In combination, these findings lead us to propose that heme binding to many *b*-type heme proteins is sufficiently destabilized by binding of Cu^{2+}, Ni^{2+}, and/or Zn^{2+} to the protein at alkaline pH that the heme is displaced from its normal binding site. Although metal ion binding to most of these proteins has not been considered adequately in the literature, the example provided by myoglobin suggests that these metal ions bind to surface residues of these proteins and that such interactions destabilize the binding of heme. As the concentration of exogenous metal ion is increased, it seems likely, as once proposed by Cann for Zn^{2+} binding to metMb,[24] that these metal ions ultimately bind to the ligand(s) that coordinate the heme iron in the native protein. Normally, metal ions are soluble only at pH < 7 in the absence of chelators, so studies of this type at alkaline pH are not possible. Our use of bicarbonate buffer in the current work overcomes this difficulty and avoids metal ion-induced precipitation of the protein that occurs[24,26] even at acidic pH. Removal of heme under these conditions avoids the use of organic solvents and highly acidic conditions (pH 2−4) required by standard methods for preparation of apoproteins that may

(49) Tripathi, A. K.; Khan, S. I.; Walker, L. A.; Tekwani, B. L. *Anal. Biochem.* **2004**, *325*, 85–91.
(50) Slater, A. F.; Swiggard, W. J.; Orton, B. R.; Flitter, W. D.; Goldberg, D. E.; Cerami, A.; Henderson, G. B. *Proc. Natl. Acad. Sci. U. S. A.* **1991**, *88*, 325–329.
(51) Sigel, H.; Martin, R. B. *Chem. Rev.* **1982**, *82*, 385–426.
(52) Kozlowski, H.; Bal, W.; Dyba, M.; Kowalik-Jankowska, T. *Coord. Chem. Rev.* **1999**, *184*, 319–346.
(53) Holm, R. H.; Kennepohl, P.; Solomon, E. I. *Chem. Rev.* **1996**, *96*, 2239–2314.
(54) Moiseeva, S. A.; Postnikova, G. B. *Biochemistry (Moscow)* **2001**, *66*, 780–787.
(55) Van Dyke, B. R.; Bakan, D. A.; Glover, K. A.; Hegenauer, J. C.; Saltman, P.; Springer, B. A.; Sligar, S. G. *Proc. Natl. Acad. Sci. U. S. A.* **1992**, *89*, 8016–8019.
(56) Reid, L. S.; Gray, H. B.; Dalvit, C.; Wright, P. E.; Saltman, P. *Biochemistry* **1987**, *26*, 7102–7107.
(57) Gurd, F. R.; Falk, K. E.; Malmstrom, B. G.; Vanngard, T. *J. Biol. Chem.* **1967**, *242*, 5731–5735.
(58) Zhang, L.; Mei, Y.; Zhang, Y.; Li, S.; Sun, X.; Zhu, L. *Inorg. Chem.* **2003**, *42*, 492–498.
(59) Humphreys, D. P.; King, L. M.; West, S. M.; Chapman, A. P.; Sehdev, M.; Redden, M. W.; Glover, D. J.; Smith, B. J.; Stephens, P. E. *Protein Eng.* **2000**, *13*, 201–206.
(60) Allen, G. *Met. Ions Biol. Syst.* **2001**, *38*, 197–212.
(61) Karavelas, T.; Malandrinos, G.; Hadjiliadis, N.; Mlynarz, P.; Kozlowski, H.; Barsan, M.; Butler, I. *Dalton Trans.* **2008**, 1215–1223.
(62) Krezel, A.; Mylonas, M.; Kopera, E.; Bal, W. *Acta Biochim. Pol.* **2006**, *53*, 721–727.
(63) Bal, W.; Liang, R.; Lukszo, J.; Lee, S. H.; Dizdaroglu, M.; Kasprzak, K. S. *Chem. Res. Toxicol.* **2000**, *13*, 616–624.
(64) Gibson, Q. H.; Antonini, E. *Biochem. J.* **1960**, *77*, 328–341.
(65) Hargrove, M. S.; Barrick, D.; Olson, J. S. *Biochemistry* **1996**, *35*, 11293–11299.
(66) Reid, T. J.; Murthy, M. R.; Sicignano, A.; Tanaka, N.; Musick, W. D.; Rossmann, M. G. *Proc. Natl. Acad. Sci. U. S. A.* **1981**, *78*, 4767–4771.
(67) Gajhede, M.; Schuller, D. J.; Henriksen, A.; Smith, A. T.; Poulos, T. L. *Nat. Struct. Biol.* **1997**, *4*, 1032–1038.

not be tolerated by some *b*-type heme proteins or by variants of diminished stability. A more general ramification of this work is the proposal that bicarbonate buffers merit evaluation for use in physical studies of nonmembrane proteins of limited solubility as a means of attaining the relatively high concentrations of protein such experiments frequently require.

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