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Synthesis and Properties of Metal-Substituted Myoglobins

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Myoglobins containing a variety of metallomesoporphyrins (MgP, CdP, SnP, PtP, PdP, H_2P) have been synthesized and characterized. Cadmium was found to be a convenient metal ion for direct insertion or removal from a protein-bound porphyrin. The photochemical instability of SnMb is likely related to the presence of both the proximal and distal histidines in the coordination environment of the Sn. Radical-cation (MP^+)/excited-state ($^3MP^*$) reduction potentials [$E(MP^+/^3MP^*)$] have been estimated for ZnMb (-0.80), MgMb (-0.79), CdMb (-0.77), PtMb (-0.65), PdMb (-0.62), and H_2Mb (-0.45 V vs NHE). The observation of delayed fluorescence in MgMb most likely results from a reduction in the nonradiative decay rate attributable to the rigidity of the protein pocket in which the porphyrin is held.

Distance and driving-force effects on the rate of long-range electron transfer (ET) in proteins are under active investigation in several laboratories.[1-4] Sperm whale myoglobin (Mb), which possesses four modifiable histidine residues at different distances from a labile heme unit,[3,4] is a particularly attractive molecule for systematic studies. In this paper, we describe some of the chemical properties of metal-substituted myoglobins and also explore the photophysical consequences of positioning a metallomesoporphyrin IX in a protein environment. We also will define criteria for estimating the excited-state redox potentials for porphyrin-substituted protein systems.

Experimental Section

Materials and Apparatus. Unless otherwise described, all reagents were used as received. Aqueous solutions were prepared from distilled water that had been filtered through a Barnstead Nanopure water purification system (No. 2794) to a specific resistance >18 MΩ cm. 2-Butanone (MCB) was stored over neutral aluminum oxide. Dichloromethane was distilled from P_2O_5 before use in electrochemical experiments. Carboxymethyl cellulose cation-exchange resin, CM-52 (Whatman, preswollen, microgranular), and Sephadex ion-exchange gel, G-25-80 (Sigma, bead size 20-80 μm), were equilibrated in the appropriate buffer solution before use. Sperm whale myoglobin (Sigma) was purified by standard procedures.[3]

Solutions for emission spectroscopy were degassed and purged with purified argon (passed over a manganese oxide column) on a dual-manifold vacuum-argon line. Protein samples were concentrated, and small molecules were removed, by Amicon ultrafiltration (YM-5 filter, 5000-MW cutoff).

Preparation of Pentaammineruthenium-Modified Myoglobins. The synthesis, isolation, and characterization of these derivatives have been described previously.[3,5]

Preparation of Metalloporphyrin Diacids. Platinum, zinc, tin, and palladium complexes of mesoporphyrin IX dihydrochloride were prepared by the method of Adler.[6] The appropriate metal dichloride (100 mg), or K_2PtCl_4 in the particular case of platinum, was added to a solution of mesoporphyrin IX diacid (100 mg in 10 mL of DMF) and the solution refluxed in the dark: 8 h (Pt); 4 h (Zn); 30 min (Sn); 20 min (Pd). The reaction was monitored by absorption spectroscopy, and additional metal chloride was added if required. Following the addition of H_2O (50 mL), the metalloporphyrin was collected by filtration, washed with water, and dried under vacuum.

Magnesium mesoporphyrin IX dimethyl ester was prepared[7] by refluxing anhydrous $Mg(ClO_4)_2$ (50 mg) with mesoporphyrin IX dimethyl ester (100 mg) in pyridine (20 mL) for 1 h. Water (50 mL) was added and the metalloporphyrin extracted with CH_2Cl_2. After it was washed with copper sulfate solution, to remove excess pyridine, and with water (2 × 50 mL), the organic layer was dried (anhydrous Na_2SO_4). After the solvent was removed under reduced pressure, and finally under high vacuum to remove the remaining trace amounts of pyridine, the metalloporphyrin was recrystallized from CH_2Cl_2/hexane. Cadmium mesoporphyrin IX dimethyl ester was readily obtained by stirring mesoporphyrin IX dimethyl ester (50 mg) and cadmium diacetate (10 mg) in warm DMSO (5 mL) for 10 min. The metalloporphyrin was precipitated by the addition of water and collected by filtration.

Cadmium and magnesium mesoporphyrin dihydrochlorides were prepared by stirring a 1:1 THF (50 mL)/NaOH(aq) (2 M, 50 mL) solution of the appropriate diester (100 mg) for 2 days under N_2(g) in the dark. Following removal of THF under reduced pressure, and addition of NH_4Cl(aq), the desired porphyrin product was obtained by filtration.

Apomyoglobin. Apomyoglobin (ca. 50 mg) was prepared by the standard acid/2-butanone extraction method.[3,8] Following removal of the heme prosthetic group, the apoprotein was dialyzed (Spectrapor dialysis bag, Spectrum Medical Industries, 20.4-mm diameter, 6000-8000-MW cutoff, 3.2 mL/cm) against μ = 100 mM Tris buffer (3 × 2 L). Each dialysis was continued for 4 h at 4 °C, and the apoprotein was immediately used for reconstitution with the desired metalloporphyrin.

Reconstituted Myoglobin.[9-11] Approximately 2 mg of metalloporphyrin was used per 10 mg of apoprotein (assuming efficient conversion of native protein to the apoproduct). The metalloporphyrin diacid was dissolved in 2 mL of μ = 100 mM phosphate buffer containing ca. 10 drops of 0.1 N NaOH. This solution was chilled to 4 °C before adding dropwise to the apoprotein with gentle stirring. For magnesium,

(1) Cowan, J. A.; Upmacis, R. K.; Beratan, D. N.; Onuchic, J. N.; Gray, H. B. *Ann. N.Y. Acad. Sci.* **1988**, *550*, 68.

(2) Hoffman, B. M.; Ratner, M. A. *J. Am. Chem. Soc.* **1987**, *109*, 6237. Liang, N.; Mauk, A. G.; Pielak, G. J.; Johnson, J. A.; Smith, M.; Hoffman, B. M. *Science* **1988**, *240*, 311. McLendon, G.; Pardue, K.; Bak, P. *J. Am. Chem. Soc.* **1987**, *109*, 754. McLendon, G. *Acc. Chem. Res.* **1988**, *21*, 160. Gray, H. B. *Chem. Soc. Rev.* **1986**, *15*, 17. Mayo, S. L.; Ellis, W. R., Jr.; Crutchley, R. J.; Gray, H. B. *Science* **1986**, *233*, 948. Nocera, D. G.; Winkler, J. R.; Yocom, K. M.; Bordignon, E.; Gray, H. B. *J. Am. Chem. Soc.* **1984**, *106*, 5145. Elias, H.; Chou, M. H.; Winkler, J. R. *J. Am. Chem. Soc.* **1988**, *110*, 429. Scott, R. A.; Mauk, A. G.; Gray, H. B. *J. Chem. Educ.* **1985**, *62*, 932. Karas, J. L.; Lieber, C. M.; Gray, H. B. *J. Am. Chem. Soc.* **1988**, *110*, 599. Bechtold, R.; Gardineer, M. B.; Kazmi, A.; van Hemelryck, B.; Isied, S. S. *J. Phys. Chem.* **1986**, *90*, 3800. Bechtold, R.; Kuehn, C.; Lepre, C.; Isied, S. S. *Nature* **1986**, *322*, 286. Isied, S. S.; Vassilian, A.; Wishart, J. F.; Creutz, C.; Schwarz, H. A.; Sutin, N. *J. Am. Chem. Soc.* **1988**, *110*, 635. Ho, P. S.; Sutoris, C.; Liang, N.; Margoliash, E.; Hoffman, B. M. *J. Am. Chem. Soc.* **1985**, *107*, 1070. Liang, N.; Pielak, G. J.; Mauk, A. G.; Smith, M.; Hoffman, B. M. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 1249. McLendon, G.; Miller, J. R. *J. Am. Chem. Soc.* **1985**, *107*, 7811. McLendon, G.; Guarr, T.; McGuire, M.; Simolo, K.; Strauch, S.; Taylor, K. *Coord. Chem. Rev.* **1985**, *64*, 113. Guarr, T.; McLendon, G. *Coord. Chem. Rev.* **1985**, *68*, 1-52. Cheung, E.; Taylor, K.; Kornblatt, J. A.; English, A. M.; McLendon, G. L.; Miller, J. R. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 1330. Conklin, K. T.; McLendon, G. *Inorg. Chem.* **1986**, *25*, 4804 and references therein.

(3) Axup, A. W.; Albin, M. A.; Mayo, S. L.; Crutchley, R. J.; Gray, H. B. *J. Am. Chem. Soc.* **1988**, *110*, 435.

(4) Cowan, J. A.; Gray, H. B. *Chem. Scr.* **1988**, *28A*, 21.

(5) Crutchley, R. J.; Ellis, W. R., Jr.; Gray, H. B. In *Frontiers in Bioinorganic Chemistry*; Xavier, A. V., Ed.; VCH: Weinheim, FRG, 1986; p 679.

(6) Adler, A. D.; Long, F. R.; Kampas, F.; Kim, J. *J. Inorg. Nucl. Chem.* **1970**, *32*, 2443.

(7) Cowan, J. A.; Sanders, J. K. M. *J. Chem. Soc., Perkin Trans. 1* **1987**, 2395.

(8) Teale, F. W. J. *Biochim. Biophys. Acta* **1959**, *35*, 543. Yonetani, T. *J. Biol. Chem.* **1967**, *242*, 5008.

(9) The preparation of ZnMb has been described previously.[3] Here, we not only summarize this procedure, with minor changes in conditions, but also describe the preparation of other novel metallomesoporphyrin-modified Mb's. MgMb has been studied previously by Hoffman and co-workers;[10] however, the reported synthetic procedure is different from that described here.

(10) Zemel, H.; Hoffman, B. M. *J. Am. Chem. Soc.* **1981**, *103*, 1192. Scholler, D. M.; Wang, M.-Y. R.; Hoffman, B. M. *Methods Enzymol.* **1978**, *52*, 487.

(11) Other metal-substituted Mb's (including Cu, Ru, Co, Mn, Cr, and Rh derivatives) have been reported.[12]

(12) Andres, S. F.; Atassi, M. Z. *Biochemistry* **1970**, *9*, 2268. Paulson, D. R.; Addison, A. W.; Dolphin, D.; James, B. R. *Inorg. Chem.* **1979**, *25*, 7002. Hoffman, B. M. In *The Porphyrins*; Dolphin, D., Ed.; Academic Press: New York, 1978; Vol. VII, p 403. McLendon, G.; Murphy, P. *J. Biol. Chem.* **1980**, *255*, 4035.

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zinc, tin, and cadmium, a reaction time of 8 h was satisfactory, whereas 12 h was preferable for palladium, platinum, and free-base porphyrins. The insertion of diesters was aided by the addition of DMSO (0.5 mL) and, in the particular case of Zn mesoporphyrin dimethylester, by the addition of imidazole (~30 mg), in order to improve the solubility of the porphyrin.

After reaction, the solutions were loaded onto a Sephadex G-25-80 column that had previously been equilibrated with μ = 100 mM phosphate buffer (Biogel P2 200–400 mesh is also satisfactory) and eluted with the same buffer at 4 °C. The protein band was collected and concentrated by ultrafiltration before further purification by isoelectric focusing (LKB, Multiphor). For this step an ampholine (LKB) pH range of 7–9 was found to be suitable for reconstituted native protein, whereas pH 8–10 was used for ruthenium-labeled (ruthenated) derivatives. The isoelectric point for native protein reconstituted with metalloporphyrins containing divalent metal ions was similar to that for ferromyoglobin (pI 7.4 ± 0.2). For free-base reconstituted Mb, a slightly higher pI 7.5 ± 0.1 was found, while SnMb had pI 8.1 ± 0.1. The ruthenated derivatives had pI values in the range pI 8.7 ± 0.1, or pI 9.4 ± 0.2 in the case of SnMb.

Final purification by column chromatography (CM-52, 2 × 10 cm) yielded the pure protein. Native protein that had been substituted with various metalloporphyrin diacids was generally eluted with μ = 30–40 mM Tris buffer, while the diester derivatives were eluted with μ = 50 mM Tris. The ruthenated proteins were eluted with μ = 100 and 130 mM Tris for the diacid and diester, respectively. It was found that prior purification by preparative IEF eliminated most of the sample impurities, and only occasional trace impurities were detected during column chromatography.

General yields were in the range of 70–80% for magnesium, tin, and cadmium derivatives and ~60–70% for platinum, palladium, and free base. These calculations were based both on the extinction coefficients for the Soret bands of the metalloporphyrins in organic solvents with added imidazole or pyridine (which were found to be similar to those for the porphyrin in the protein interior) and on the intensity of the aromatic peptide absorption at 280 nm, although the latter method was less accurate because of frequent overlap with porphyrin absorption bands. Binding of the porphyrin in the myoglobin pocket, rather than the protein surface, was verified in the case of magnesium and platinum myoglobins (representing coordinatively unsaturated and saturated metalloporphyrins, respectively) by emission spectroscopy. Addition of the fluorescent probe 8-anilino-1-naphthalenesulfonic acid (ANSA) to a solution of apomyoglobin led to an increase in the intensity, and 35-nm blue shift, of the ANSA emission band relative to that found in aqueous phosphate buffer. No change was observed following addition to a solution of reconstituted protein, indicating that the myoglobin pocket was already occupied by porphyrin. In the particular case of magnesium, cadmium, tin, and zinc Mb's, splitting and/or broadening of the Q-bands were observed (Table I). The rationalization of this effect is related to heme orientational disorder.[13] No such perturbation was found in the case of platinum, palladium, or free base Mb.

Steady-state emission spectra recorded in this laboratory were obtained on a home-built instrument that has been described elsewhere (see Nocera et al. in ref 2), and samples were prepared ($A_{Soret} \approx 0.6$) and degassed as described above, in 1 × 1 cm cuvettes.

Electrochemical Measurements. Electrochemical measurements of metalloporphyrin reduction potentials were made by differential pulse polarography (DPP) and/or cyclic voltammetry (CV). DPP measurements used a Princeton Applied Research (PAR) Model 174A polarographic analyzer, whereas CV was carried out with a PAR Model 175 universal programmer in conjunction with a PAR Model 173 potentiostat/galvanostat. Results were recorded on a Houston Instruments Omnigraphics 2000 recorder. Electrode potentials were measured with a Keithley 177 microvoltmeter, relative to a SSCE reference. A gold working electrode [Bioanalytical Systems (BAS), surface area 0.25 cm^2] and platinum-wire counter electrode were routinely used. Samples were dissolved in dichloromethane, with 0.1 M tetrabutylammonium hexafluorophosphate as supporting electrolyte, and degassed by flushing the surface of the stirred solution with argon (previously bubbled through dry CH_2Cl_2) for 15 min. Titrations with pyridine (or imidazole) employed spectrograde solvent that had been dried over 4-Å molecular sieves (or analytical grade reagent).

Results and Discussion

Synthesis and Chemical Properties of Porphyrin-Reconstituted Myoglobins. The porphyrin-modified proteins are listed in Table I with electronic absorption data. The stability of reconstituted myoglobins was found to depend on whether the metallomesoporphyrin was used as the diester or diacid derivative and on whether or not the metal center was capable of binding axial ligands. From studies of a series of metallomesoporphyrin diacids and diesters,[14] containing coordinatively saturated and unsaturated metal ions, it has become clear that two factors dominate the stability of the porphyrin–myoglobin complex. With porphyrin diacids, the electrostatic interaction between the carboxylate functionality and the positively charged His-97 and Arg-45 surface residues at the edge of the hydrophobic pocket plays an important role in complex formation, while the ability of the metal ion to coordinate to the proximal histidine will also serve to lock the porphyrin into the myoglobin cavity (Figure 1). We have found that a metalloporphyrin having one or the other of these two characteristics will be successfully incorporated into apomyoglobin. If a porphyrin has neither feature, however, it will not, and so hydrophobic interactions alone will not bind the porphyrin to the protein. The overall stability of those proteins containing metalloporphyrins that can participate in both types of binding is therefore greater than those that feature only one binding interaction. Over a period of ca. 1 week, some porphyrin loss was evident from the latter. In related studies, we have found greater stability for Co(III) and Mn(III) myoglobins,[15] which is pre-

Table I. Absorption Spectral Data[a]

	MP-DME		MP-DME + pyridine[b]		MP-diacid/protein	
M	λ, nm	rel OD	λ, nm	rel OD	λ, nm	rel OD
Mg					280	0.049
	397	1.0	411	1.0	409	1.0
	524	0.041	529	0.046	542	0.037
	561	0.070	562	0.032	584 (573 sh)	0.022
Zn					280	0.060
	400	1.0	412	1.0	414	1.0
	531	0.046	541	0.052	541	0.047
	568	0.066	577	0.040	583 (573 sh)	0.024
Cd					280	0.127
	406	1.0	419	1.0	421	1.0
	541	0.085	550	0.072	551	0.076
	576	0.066	585	0.031	591 (582 sh)	0.027
Pt					280	0.020
	380	1.0	380	1.0	380	1.0
	498	0.078	499	0.079	499	0.052
	535	0.161	535	0.161	534	0.172
Pd					280	0.0175
	392	1.0	392	1.0	391	1.0
	511	0.073	511	0.074	510	0.083
	546	0.229	546	0.231	544	0.235
H_2					282	0.136
	398	1.0	398	1.0	395	1.0
	497	0.084	497	0.084	496	0.080
	531	0.058	531	0.063	532	0.058
	567	0.039	567	0.042	562	0.042
	620	0.028	620	0.030	614	0.030
Sn					283	0.094
	404	1.0	407	1.0	404	1.0
	537	0.081	538	0.084	537	0.050
	574	0.071	575	0.077	577 (569 sh)	0.031

[a] P = mesoporphyrin IX. MP-DME spectra were measured in CH_2Cl_2 solution; protein spectra were measured in μ = 0.1 M sodium phosphate buffer (22–24 °C). For each metal derivative, the peak intensities for the absorption bands are given as a ratio relative to the Soret band (OD = 1.0). This is to facilitate comparison of data for each metal derivative. The ratios are not intended to be used between metal derivatives; that is, the Soret bands for the metal Mb complexes do not have exactly the same extinction coefficients. [b] Mg mesoporphyrin IX was titrated with imidazole in order to give a five-coordinate species.

(13) Cowan, J. A.; Gray, H. B. Submitted for publication in *Inorg. Chem.*

(14) The stabilities of coordinatively saturated [Pt, Ag (used in other studies currently in progress)] and unsaturated (Mg, Zn) mesoporphyrin diacids and diesters were examined.

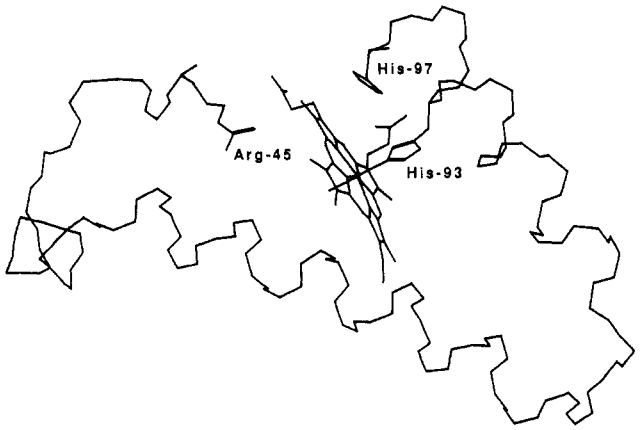

Figure 1. Porphyrin binding to myoglobin, stabilized by coordination to the proximal histidine (His-93) and by salt bridges from the porphyrin carboxylate side chains to His-97 and Arg-45. The protein backbone from residue 45 to 97 and the individual residues noted above are shown.

sumably related to stronger binding to the proximal histidine.

The chemistry of cadmium myoglobin is of particular interest. Cadmium mesoporphyrin is very acid labile. At moderately low pH (pH <5) the metal ion is removed, offering an alternative procedure for the synthesis of the free-base porphyrin derivative of myoglobin. In pH 5.2 phosphate buffer, no demetalation was observed. At pH 4.7, however, relatively slow loss of metal ion was detected ($t_{1/2} \approx 20$ min), whereas complete demetalation occurred over a period of only a few minutes at pH 4.[16] Cadmium ion also was found to be readily inserted into protein-bound free-base porphyrin by addition of cadmium acetate to a Tris-buffered solution of the protein. Phosphate buffer could not be used, owing to the low solubility of cadmium phosphate. Cadmium might, therefore, be readily inserted into proteins such as cytochrome *c*, or other heme-bound cytochrome systems, where the porphyrin unit is nonlabile but the porphyrin core is sterically accessible to incoming metal ions. In comparison with cadmium, magnesium porphyrin displays greater stability in the presence of acid and did not suffer demetalation until pH ~3.7, when loss of the metal ion occurred over a period of around 40 min. In comparison with cadmium, this resulted after loss of the porphyrin unit from the protein pocket, as determined from the shift of the Soret band.[17] There was essentially no loss of metal ion for pH ≥4.4, although at pH 4.3 the splitting of the Q(0,0) band was less pronounced, probably as a result of some porphyrin loss from the protein cavity, but the minor component was still clearly evident as a shoulder on the major Q(0,0) absorption.

In the case of SnMb, it is possible that the Sn center might be six-coordinate through binding by both the proximal and distal histidines. Sn(IV) porphyrins are known to favor coordination of two nitrogenous aromatic ligands,[19] while the possibility of coordination by the distal histidine has been discussed by Hoffman in relation to studies of CoMb.[20] Unfortunately, ruthenium derivatives of Sn^{IV}Mb were not found to be of value in ET studies, owing to the chemical instability of the excited-state Sn(IV) porphyrin. Distinct bands were found to appear in the absorption spectrum after irradiation with only a few hundred laser pulses (40 mJ/pulse). It is known that Sn(IV) porphyrins with axially bound nitrogenous bases are subject to photodecomposition to chlorin and tetrahydroporphyrin derivatives[19] in the presence of electron-donor functionality. This is a likely reaction pathway in a protein environment containing residues capable of acting as electron donors, and the occurrence of a six-coordinate Sn(IV) center would more readily explain the photochemistry displayed following laser excitation. It is worth noting that in the case of Sn(IV) cytochrome *c*, where only one histidine coordinates to the metal center, no such photochemical instability has been reported.[21]

Table II. Reduction Potentials and Excited-State Energies

M	$E(MP^+/MP)$, V[a]	$E(MP^+/MP)_{cor}$, V[b]	$E(^3MP^*)$, eV	$E(MP^+/^3MP^*)$, V[b]
Zn	0.53 (1)	0.98 (1)	1.78[c]	−0.80 (1)
Mg	0.48 (1)	0.93 (1)	1.72[c]	−0.79 (1)
Cd	0.49 (1)	0.94 (1)	1.71[c]	−0.77 (1)
Pt	0.81 (1)	1.26 (1)	1.91[d]	−0.65 (1)
Pd	0.79 (1)	1.24 (1)	1.86[d]	−0.62 (1)
H_2	0.75 (1)	1.20 (1)	1.65[c]	−0.45 (1)

[a] These potentials for P-DME derivatives in CH_2Cl_2 solution were determined after the addition of a trace of pyridine (imidazole was added in the case of Mg). [b] The results have been systematically corrected to give values for the reduction potentials for MP-diacid in myoglobin (vs NHE). [c] Energy of T(0,0) emission band (obtained at 12 K). [d] Energy of T(0,0) emission band (obtained at 77 K).

Metalloporphyrin as Excited-State Electron Donors. A principal reason for synthesizing a series of porphyrin-reconstituted myoglobins was the possibility of systematically tuning the driving force for ET in ruthenated protein derivatives by using a series of metalloporphyrins of varying radical-cation (MP^+)/excited-state ($^3MP^*$) reduction potentials. Employing excited-state porphyrins, we hoped to generate driving forces large enough to allow the study of ET in the longer range (19–22 Å^{-1}) ruthenium-labeled myoglobins[1,3,5] and also provide a wider range of available $\Delta G°$'s (Table II). The driving force in ruthenated native heme protein[22] is too small for longer range ET experiments.

There has previously been a measure of uncertainty in the evaluation of driving force for reactions involving photoexcited porphyrins in protein systems due to a lack of reliable data for the ground-state MP^+/MP reduction potentials of the porphyrin in the protein environment.[23] In part, this is attributable to experimental difficulties in the direct measurement of protein-bound MP^+ reduction potentials. The problems undoubtedly arise from the low stability of the radical cation in the partially aquated protein environment on the time scale of the electrochemical measurement.[24] Although it might appear difficult to obtain a set of absolute potentials for protein-bound porphyrins, it ought to be possible to derive a set of potentials that are correct in a relative sense by consideration of the porphyrin redox potentials determined under standard solution conditions. Unfortunately, literature values for porphyrin redox potentials have normally been determined with different supporting electrolytes and solvents.[25] It is also important to consider the need to axially ligate the metalloporphyrin with, for instance, pyridine or imidazole, in order to mimic ligation by the proximal histidine in the Mb pocket, depending on the porphyrin under study.

The $MP^+/^3MP^*$ reduction potential is defined as

$$E(MP^+/^3MP^*) = E(MP^+/MP) - E(^3MP^*) \qquad (1)$$

(15) The Co(III) and Mn(III) myoglobins were prepared by procedures similar to those described in the Experimental Section.
(16) At this low pH, some porphyrin loss from the protein would be expected[17] but was not significant, as evidenced by the red-shifted Soret band of the cadmium porphyrin due to coordination by the proximal histidine.
(17) Myoglobin is increasingly unstable to heme loss below pH 4.5.[18]
(18) Antonini, E.; Brunori, M. *Hemoglobin and Myoglobin in Their Reactions with Ligands*; Frontiers of Biology 21; North-Holland: Amsterdam, 1971; p 104.
(19) Whitten, D. G.; Yau, J. C.; Carroll, F. A. *J. Am. Chem. Soc.* **1971**, *93*, 2291.
(20) Hoffman, B. M. In *The Porphyrins*; Dolphin, D., Ed.; Academic Press: New York, 1978; Vol. VII, p 413.
(21) Barboy, N.; Feitelson, J. *Biochemistry* **1987**, *26*, 7240. Koloczek, H.; Horie, T.; Yonetani, T.; Anni, H.; Maniara, G.; Vanderkooi, J. M. *Biochemistry* **1987**, *26*, 3142.
(22) Crutchley, R. J.; Ellis, W. R., Jr.; Gray, H. B. *J. Am. Chem. Soc.* **1985**, *107*, 5002.
(23) The measurements of Hoffman and McLendon are for Hb and cyt *c*, respectively.[24]
(24) McGourty, J. L.; Blough, N. V.; Hoffman, B. M. *J. Am. Chem. Soc.* **1983**, *105*, 4470. Peterson-Kennedy, S. E.; McGourty, J. L.; Kalweit, J. A.; Hoffman, B. M. *J. Am. Chem. Soc.* **1986**, *108*, 1739. McLendon, G. M.; Rogalsky, J. S.; Magner, E.; Conklin, K. T. In *4th International Symposium on Oxidases and Related Redox Systems*; Mason, H., Ed.; A. Liss: New York, in press.
(25) Davis, D. G. In *The Porphyrins*; Dolphin, D., Ed.; Academic Press: New York, 1978; Vol. V, p 127. Kadish, K. M. *Prog. Inorg. Chem.* **1986**, *34*, 435.

Table III. Ambient-Temperature Emission Data for MP(diacid)-Modified Myoglobins[a]

M	λ_f, nm		λ_p, nm
Mg	585	638	
Zn	586	635	
Cd	569	631	
H_2	625	690	
Pd			668
Pt			650

[a] [protein] ≈ 5–10 μM (OD ≈ 0.6, Soret) in μ = 0.1 M sodium phosphate buffer (22–24 °C).

where $E(^3MP^*)$ is the excited-state triplet energy and $E(MP^+/MP)$ is the ground-state reduction potential. The triplet energy of the protein-bound porphyrin can be determined from the T(0,0) band in the phosphorescence spectrum of the molecule (Table II).[26] The reduction potentials of the metallomesoporphyrins listed in Table II were measured by using similar solvent conditions and supporting electrolyte. The table quotes the appropriate value, either with or without bound nitrogenous ligand, depending on whether the porphyrin is capable of coordinating the proximal histidine in the myoglobin pocket. In those cases where the metalloporphyrin would not bind an axial ligand, the reduction potential was virtually unchanged following titration with pyridine or imidazole, while in the particular case of magnesium, which is capable of assuming a six-coordinate geometry in the presence of a large excess of ligand,[27] the potential for the five-coordinate species was obtained by addition of only a trace of imidazole as nitrogenous base. Since the triplet energies for each porphyrin in a protein environment have been determined by phosphorescence measurements,[26] the reduction potential for each $MP^+/^3MP^*$ system can be obtained from eq 1. Unfortunately, it was not possible to fix the ground-state potential for the heme unit, a metal-centered redox change that can be readily monitored by spectroelectrochemistry,[22] on the same scale by direct experiment; in myoglobin, the Fe(III) center is coordinated by both the proximal His-93 residue and a bound water molecule on the distal side of the porphyrin.[28] Although we were unable to model this Fe(III)-bound H_2O species in the organic solvent system used for the other metalloporphyrin studies, we believe that we have been able to derive an absolute set of potentials for all protein-bound porphyrins, by consideration of driving-force data (vide infra).

Since it is important that a consistent set of driving forces be used if valid comparisons of data and conclusions are to be made between these and other systems, we have scaled our ground-state porphyrin reduction potentials (column 1, Table II) to take account of the distinct environment within the protein cavity (column 2, Table II). This correction involved consideration of the relative values of $k_{ET}^{forward}$ and k_{ET}^{back} in ruthenium-labeled ZnMb and CdMb.[1,3,29] We assume that the distance dependences of ET for $k_{ET}^{forward}$ and k_{ET}^{back} are identical (i.e., $\beta_{forward} = \beta_{back}$). In the particular limiting case of $k_{ET}^{forward} = k_{ET}^{back}$, $\Delta G°_{forward} = \Delta G°_{back}$, and so $E(^3MP^*) - E(MP^+/MP) = E(MP^+/MP) - 2[E(Ru^{3+}/Ru^{2+})]$. Consideration of $k_{ET}^{forward}$ and k_{ET}^{back}, therefore, allows the absolute value of $E(MP^+/MP)$ to be estimated for cases where $k_{ET}^{forward} \approx k_{ET}^{back}$. Since $E(Ru^{3+}/Ru^{2+})$[22] and the relative values of $E(MP^+/MP)$ have been defined, the absolute values of $E(MP^+/MP)$ for the other metalloporphyrins employed can be determined (Table II). We have previously indicated that these values are in line with that measured for the heme center in the native protein.[1] Recent results further suggest that our assumption of $\beta_{forward} \approx \beta_{back}$ is likely to be valid in many other cases of ET through proteins, and in particular in those modified systems employing excited-state porphyrin electron donors or radical-cation acceptors.[29]

It should be noted that since both $E(MP^+/MP)$ and $E(MP^+/^3MP^*)$ for zinc protoporphyrin and zinc mesoporphyrin are different,[30,31] the resulting ET driving forces (ET from MP* and ET to MP^+) involving these two porphyrins will differ (e.g., excited zinc mesoporphyrin is ~160 mV more reducing than excited zinc protoporphyrin). It is important to keep these driving-force differences in mind when making comparisons of the rates of ET reactions in which these two porphyrins are excited-state electron donors and radical-cation electron acceptors.

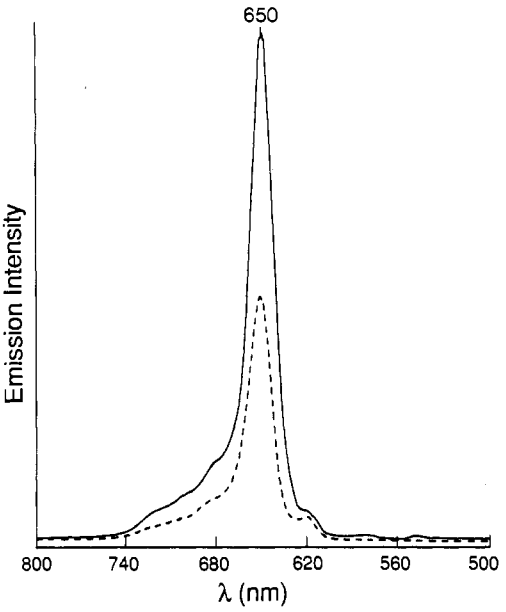

Figure 2. Emission spectra of PtMb (—) and its a_5Ru(His-48) derivative (---). [protein] ≈ 10 μM in μ = 0.1 M sodium phosphate buffer (22–24 °C).

Table IV. Rate Constants for PdMb and PtMb

Mb deriv	k_{obs}, s^{-1} [a]	Mb deriv	k_{obs}, s^{-1} [a]
PdMb	9.0×10^2	PtMb	9.7×10^3
a_5Ru(His-48)PdMb	1.0×10^4	a_5Ru(His-48)PtMb	2.2×10^4

[a] $k_{obs} = k_d + k_{ET}$. [protein] ≈ 10 μM (OD ≈ 0.6, Soret) in μ = 0.1 M sodium phosphate buffer (22–24 °C). Error in measurements is ±10%.

Emission Spectroscopy. The emission wavelengths for protein-bound porphyrins set out in Table III are similar to those found for the unbound metalloporphyrin in an organic solvent with appropriate axial coordination.[32] The decreases in the phosphorescence emission intensity in the emission spectra of the Ru(His-48) derivatives of PtMb (Figure 2) and PdMb are therefore attributable to ET from the porphyrin triplet state to the surface ruthenium. [In previous work on ruthenated protein derivatives, we have shown by control studies on related systems (reduced Ru(His-48)MgMb) and calculations of energy-transfer efficiency that the porphyrin excited state is quenched by an ET mechanism.[1,3,4]] The decreases in intensity (~2-fold for Pt; ~10-fold for Pd) are in line with the observed decay rates (k_{obs}) in both the "native" and ruthenium-labeled proteins (Table IV).

(26) The triplet energies were determined from the porphyrin phosphorescence spectrum. Spectra of Pt and Pd Mb's at 77 K were obtained on a home-built instrument (Nocera et al., ref 2). Samples requiring deconvolution of the phosphorescence from the strong fluorescence (H_2, Mg, Zn, and Cd Mb's) were run by L.-H. Zang (University of California, Davis, CA) at 12 K.

(27) Miller, J. R.; Dorough, G. D. *J. Am. Chem. Soc.* **1952**, *74*, 3977. Storm, C. B.; Corwin, A. H.; Arellano, R. R.; Martz, M.; Weintraub, R. *J. Am. Chem. Soc.* **1966**, *88*, 2525.

(28) Takano, T. *J. Mol. Biol.* **1977**, *110*, 537, 569.

(29) Cowan, J. A.; Upmacis, R. K.; Winkler, J. R.; Gray, H. B. Unpublished results. Meade, T. J.; Winkler, J. R.; Gray, H. B. *J. Am. Chem. Soc.*, in press.

(30) Gingrich, D. J.; Nocek, J. M.; Natan, M. J.; Hoffman, B. M. *J. Am. Chem. Soc.* **1987**, *109*, 7533.

(31) Stanienda, A.; Biebl, G. *Z. Phys. Chem.* (*Frankfurt am Main*) **1967**, *52*, 254.

(32) Gouterman, M. In *The Porphyrins*; Dolphin, D., Ed.; Academic Press: New York, 1978; Vol. III, pp 24–36. Allison, J. B.; Becker, R. S. *J. Chem. Phys.* **1960**, *32*, 1410. Becker, R. S.; Allison, J. B. *J. Phys. Chem.* **1963**, *67*, 2669.

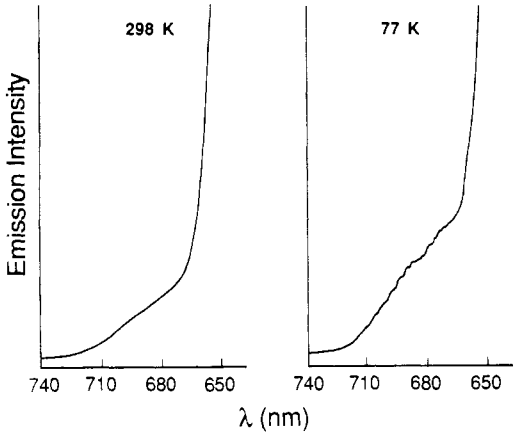

Figure 3. Low-temperature (77 K) phosphorescence displayed by MgMb. [protein] ≈ 3 μM in μ = 0.1 M sodium phosphate buffer.

Table V. Protein-Bound-Porphyrin Decay Rates

M	MP-diacid/protein k_d, s^{-1}	M	MP-diacid/protein k_d, s^{-1}
Mg	18	Pd	9.0×10^2
Zn	44	Pt	9.7×10^3
Cd	3.5×10^2	H_2	76

[a] Lifetimes were measured in μ = 0.1 M sodium phosphate buffer (MP-diacid/protein); [sample] ≈ 2–8 μM (OD ≈ 0.6, Soret); 22–24 °C. Error in measured rates is ±10%.

The main emission band in these spectra corresponds to the T(0,0) component. When one goes from 298 to 77 K, the bands sharpen slightly [e.g., for PdMb $\lambda_{1/2}$ = 27 nm (298 K), $\lambda_{1/2}$ = 15 nm (77 K)] and λ_{max} T(0,0) is relatively unchanged. Zinc, cadmium, magnesium, and free-base porphyrins do not phosphoresce at room temperature but do emit at cryogenic temperatures.[26] Figure 3 shows part of the 77 K emission spectrum of MgMb, highlighting the shoulder on the fluorescence band that is attributable to phosphorescence. The individual triplet components are well resolved at 12 K after deconvolution of the singlet emission.[26]

A necessary prerequisite for the utilization of excited-state porphyrins as electron donors is the existence of a suitably long triplet lifetime. From a kinetic analysis developed elsewhere,[33] it can be shown that

$$k_{obs} = k_{ET} + k_d \quad (2)$$

where k_{obs} is the observed decay rate of the porphyrin triplet state, and k_{ET} and k_d are the contributions to k_{obs} from electron-transfer and natural decay processes, respectively. Clearly, if k_d is large relative to k_{ET} (i.e., the triplet lifetime is short), the possibility arises that the ET rate, determined by subtraction of k_d from k_{obs}, may be lost in the substantial error inherent to the experiment (±10%). It has already been established[3,4] that only MgMb and ZnMb have triplet lifetimes of sufficient length to allow the measurement of ET rates to the longer range ruthenium derivatives of Mb. For the porphyrins described here, however, the ET rate to the Ru(His-48) residue was measurable in every case.[1,3,4] This was possible since the triplet states of the metallomesoporphyrins listed in Table V were found to be significantly long-lived when held in the protein pocket. As a direct consequence, a delayed fluorescence[34] may also be evident (shown in Figure 4 for the particular case of MgMb, although a similar effect has previously been documented for proteins containing zinc porphyrins).[21,35] The observations of relatively long triplet lifetimes and delayed fluorescence are, we believe, interrelated and can be explained in terms of a rigid protein pocket in which porphyrin motion is restricted. For instance, the excited-state lifetimes for porphyrins

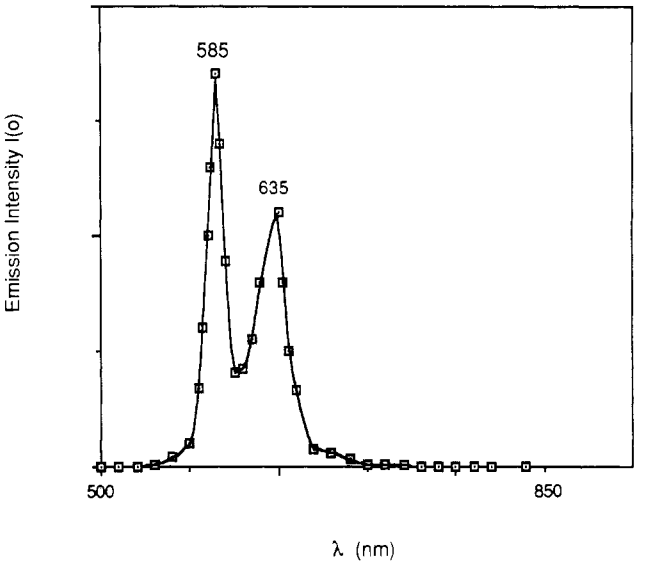

Figure 4. Delayed fluorescence spectrum of MgMb, determined by time-resolved emission spectroscopy. [protein] ≈ 50 μM in μ = 0.1 M sodium phosphate buffer (22–24 °C). The initial intensity of the delayed emission decay profile is plotted against wavelength (500–840 nm, in 5–15-nm increments).

in frozen matrices[36] are comparable to those found for the modified proteins in aqueous buffer at ambient temperature, whereas delayed fluorescence has been observed for zinc, palladium, platinum, and free-base porphyrins embedded in solid films.[37]

The assignment of delayed fluorescence in the case of MgMb was based on several features, including the similarity in the decay rate for the ambient delayed emission at the fluorescence wavelength ($k_d \approx 30 \pm 20$ s^{-1})[38] and the triplet decay rate ($k_d \approx 18 \pm 4$ s^{-1}) and the decrease in signal intensity on lowering the sample temperature, indicative of an activated process.[39] Bimolecular interactions are unlikely, since the porphyrins are held in isolation and the observed k_d is similar to τ_p, while the possibility of charge- or energy-transfer mechanisms can be eliminated by consideration of the energetics involved; there is no energy loss during two successive charge- or energy-transfer processes. At 0 °C, the intensity of the delayed emission was about half of that at 25 °C (Figure 4), whereas at 77 K no delayed emission, and only a weak phosphorescence (~720 nm), were observed. This is consistent with a lowering of the singlet–triplet interconversion rate for a thermally activated process. We therefore attribute the observation of delayed fluorescence in metalloporphyrin derivatives of proteins[21,35] to the rigidity of the pocket in which the porphyrin resides in the protein interior. Delayed fluorescence should also be evident in other porphyrin-substituted myoglobins (e.g., PdMb, PtMb, CdMb, H_2Mb); however, owing to the lower quantum yields for decay from the singlet state in these systems,[40] the emission was not detected.

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(33) Axup, A. W. Ph.D. Thesis, California Institute of Technology, 1986.
(34) Feitelson, J.; Barboy, N. *J. Phys. Chem.* **1986**, *90*, 271.
(35) Upmacis, R. K. Unpublished results.
(36) Gouterman, M. In *The Porphyrins*; Dolphin, D., Ed.; Academic Press: New York, 1978; Vol. III, p 33.
(37) Callis, J. B.; Knowles, J. M.; Gouterman, M. *J. Phys. Chem.* **1973**, *77*, 154. Callis, J. B.; Gouterman, M.; Jones, Y. M.; Henderson, B. H. *J. Mol. Spectrosc.* **1971**, *39*, 410.
(38) The large error in the delayed emission measurement was due to the weak signal observed.
(39) Fluorescence and phosphorescence normally become more favorable at low temperatures.
(40) Gouterman, M. In *The Porphyrins*; Dolphin, D., Ed.; Academic Press: New York, 1978; Vol. III, pp 28–36.