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Hydroxide Ion Binding to Methemerythrin. An Investigation by Resonance Raman and Difference Spectroscopy†

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ABSTRACT: The pH dependence for the interconversion of the acid and base forms of methemerythrin from *Themiste dyscritum* was investigated by difference spectroscopy. A new technique was designed to be able to study mixtures without knowledge of extinction coefficients or exact protein concentrations. The resultant pK_a value of 8.4 proved that *T. dyscritum* hemerythrin crystals used for previous X-ray crystallographic studies at pH ≤6.5 were in the acid form. Since this material contains a 5-coordinate iron atom with no evidence of a ligated water molecule, it is more appropriately referred to as methemerythrin than aquomethemerythrin. The presence of an iron-bound hydroxide in the base form of methemerythrin was verified by resonance Raman spectroscopy for both *T. dyscritum* and *Phascolopsis gouldii*. At pH >9, the protein from either species exhibited a new feature at 490 cm^{-1} that shifted to 518 cm^{-1} in D_2O and was assigned to a coupled Fe–OH stretching and O–H bending vibration. Thus, hydroxomethemerythrin is the correct designation for the base form of the protein. The other resonance-enhanced vibration, the Fe–O–Fe symmetric stretch, was observed at 506 cm^{-1} in hydroxomethemerythrin and at 511 cm^{-1} in methemerythrin and was unaffected by deuteration. Addition of perchlorate to methemerythrin had no effect on the Raman spectrum, despite its known role in stabilizing the met form relative to the hydroxomet form.

Hemerythrin is an oxygen transport protein found in several phyla of marine invertebrates. It contains a non-heme binuclear iron center that reversibly binds one molecule of oxygen for every two iron atoms (Boeri & Ghiretti-Magaldi, 1957). Upon oxygenation both iron atoms are oxidized from the ferrous to the ferric state while the oxygen is reduced to peroxide (Kurtz et al., 1977; Loehr & Loehr, 1979). The protein can also be oxidized by chemical reagents such as ferricyanide or hydrogen peroxide (Wilkins & Harrington, 1983) to produce methemerythrin, a form that no longer binds dioxygen but has the binuclear iron center fixed in the ferric state. Methemerythrin has been shown to bind small anions such as chloride, azide, and thiocyanate (Keresztes-Nagy & Klotz, 1965; Garbett et al., 1969). All anionic adducts of

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methemerythrin have similar electronic, circular dichroism, resonance Raman, and Mössbauer spectra as well as magnetic susceptibilities (Garbett et al., 1969; Freier et al., 1980; Moss et al., 1971), and these characteristics are shared with inorganic μ-oxo bridged iron dimers (Garbett et al., 1969; Murray, 1974; Loehr et al., 1980).

Methemerythrin that has been prepared without small anions present has been referred to as aquomethemerythrin, the implication being that a water molecule occupies the site where the anions bind in the other methemerythrins. Aquomethemerythrin, however, appears to be slightly different in structure than the other methemerythrin adducts. The electronic spectrum of aquomethemerythrin shows only one absorption band between 300 and 400 nm whereas the other forms all have two absorption bands in that region (Keresztes-Nagy & Klotz, 1965); the Mössbauer spectrum shows an isomer shift and a quadrupole splitting that are both significantly below the range of values for the other methemerythrins though still interpretable as high-spin Fe(III) (Garbett et al., 1971c); the resonance Raman spectrum shows no iron–ligand vibration in the 200–600-cm^{-1} region (Freier et al., 1980); the circular dichroism spectrum is markedly different from all other forms of methemerythrin (Garbett et al., 1969). Crystallographic studies have recently revealed a possible structural basis for these observations.

According to X-ray structure determinations the binuclear iron center in methemerythrin can exist in at least two different coordination environments. In both cases the two iron atoms are ligated to five histidines and two bridging carboxylates from the protein and to a bridging oxo group derived from the aqueous solvent. The differences arise in the occupancy of the sixth coordination site, the anion binding site, on one of the two iron atoms. In azidomethemerythrin (Stenkamp et al., 1981), thiocyanatomethemerythrin (Stenkamp et al., 1978), and oxyhemerythrin (R. E. Stenkamp, L. C. Sieker, and L. H. Jensen, personal communication) from *Themiste dyscritum* as well as azidometmyohemerythrin from *Themiste zostericola* (Hendrickson, 1981), the anion binding site is occupied and both iron atoms are 6-coordinate. Model compounds that closely reproduce the coordination geometry in this form of the protein have recently been synthesized (Armstrong & Lippard, 1983; Wieghardt et al., 1983). In the other crystalline form of the protein previously referred to as either aquomethemerythrin (Stenkamp et al., 1976, 1978) or hydroxomethemerythrin (Stenkamp et al., 1981, 1982), the anion site is vacant and the resulting pentacoordinate iron has a geometry slightly distorted from octahedral (Stenkamp et al., 1983, 1984). Thus, it is clear that neither term was a correct description of these crystals. The results reported in the present paper establish that the protein samples used for the crystallographic studies were in the *acid form* (hereafter referred to as methemerythrin) and that conversion to the *base form* involves the formation of a ferric hydroxide complex (hereafter referred to as hydroxomethemerythrin).

In the course of these investigations a new difference spectroscopic method was developed that enables the determination of equilibrium constants without prior knowledge of molar absorptivities or accurate protein concentrations.

Theory

The electronic spectra of methemerythrin and hydroxomethemerythrin are shown in Figure 1. The marked spectral differences in the protein from *Phascolopsis gouldii* have been used previously to determine an equilibrium constant for hydroxide ion binding (Garbett et al., 1971a; Gorman & Darnall, 1981). Although the spectral changes in *T. dyscritum* hemerythrin are smaller, they can also be used to obtain thermodynamic parameters and equilibrium constants.

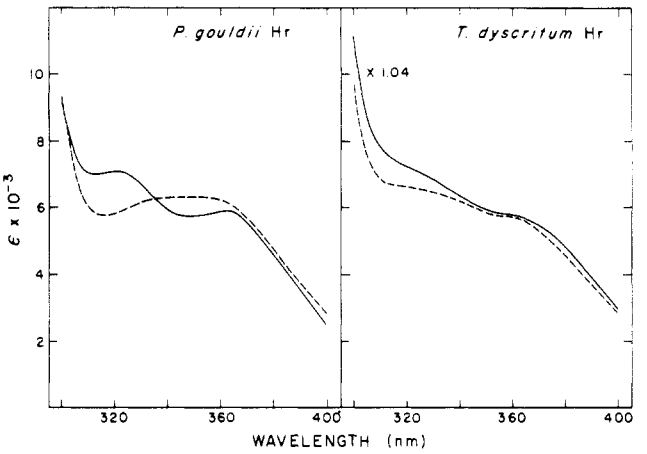

FIGURE 1: Absorption spectra of methemerythrins as a function of pH. (Left) *P. gouldii* methemerythrin, pH 7.25, in 5 mM $NaClO_4$ and 0.04 M Tris–sulfate (---) and hydroxomethemerythrin, pH 8.95, in 0.04 M Tris–sulfate (—). (Right) *T. dyscritum* methemerythrin, pH 7.28 (---), and hydroxomethemerythrin, pH 8.98 (—). The pH 8.98 spectrum has been multiplied by 1.04 for spectral clarity. ϵ in M^{-1} cm^{-1} per hemerythrin monomer.

The transition from methemerythrin to hydroxomethemerythrin can be written as a hydroxide binding reaction per hemerythrin (Hr) subunit

$$Hr + nOH^- \rightleftharpoons Hr(OH)_n \quad (1)$$

with the formation constant given by

$$K_f = [Hr(OH)_n]/([Hr][OH]^n) \quad (2)$$

where [OH] represents the activity of hydroxide ion. The absorbance of a mixture of Hr and Hr(OH) is

$$A = \epsilon_a[Hr] + \epsilon_b[Hr(OH)_n] \quad (3)$$

where ϵ_a and ϵ_b are the molar absorptivities of the acid and base forms, respectively, for a path length of 1 cm. If different mixtures of Hr and Hr(OH) are in the reference and sample beams, the measured difference absorption is given by

$$\Delta A = \epsilon_a[Hr]_s + \epsilon_b[Hr(OH)_n]_s - \epsilon_a[Hr]_r - \epsilon_b[Hr(OH)_n]_r \quad (4)$$

where the subscripts s and r refer to sample and reference, respectively.

If the total protein concentration, $[Hr_T]$, is the same in the sample and reference cells, this equation can be written as

$$\Delta A = \epsilon_a[Hr]_s + \epsilon_b([Hr_T] - [Hr]_s) - \epsilon_a[Hr]_r - \epsilon_b([Hr_T] - [Hr]_r) \quad (5)$$

Equation 5 simplifies to

$$\Delta A = \epsilon_a[Hr]_s - \epsilon_b[Hr]_s - \epsilon_a[Hr]_r + \epsilon_b[Hr]_r \quad (6)$$

and further simplifies to

$$\Delta A = \Delta\epsilon([Hr]_s - [Hr]_r) \quad (7)$$

where $\Delta\epsilon$ represents $\epsilon_a - \epsilon_b$. Equation 2 can be rewritten as

$$[Hr]_s = [Hr_T]/(K_f[OH]^n_s + 1) \quad (8)$$

$$[Hr]_r = [Hr_T]/(K_f[OH]^n_r + 1) \quad (9)$$

Combining eq 7–9

$$\Delta A = \Delta\epsilon[Hr_T]/(K_f[OH]^n_s + 1) - \Delta\epsilon[Hr_T]/(K_f[OH]^n_r + 1) \quad (10)$$

$$\Delta A/[\Delta\epsilon[Hr_T]] = (K_f[OH]^n_r - K_f[OH]^n_s)/[(K_f[OH]^n_s + 1)(K_f[OH]^n_r + 1)] \quad (11)$$

$$\Delta A/[\Delta\epsilon[Hr_T]K_f([OH]^n_r - [OH]^n_s)] = 1/[(K_f[OH]^n_s + 1)([Hr_T]/[Hr]_r)] \quad (12)$$

$$\Delta A/([OH]^n_r - [OH]^n_s) = \Delta\epsilon K_f[Hr]_r/(K_f[OH]^n_s + 1) \quad (13)$$

Taking the reciprocal of both sides of eq 13 gives

$$\frac{[OH]^n_r - [OH]^n_s}{\Delta A} = \frac{1}{\Delta\epsilon[Hr]_r}[OH]^n_s + \frac{1}{\Delta\epsilon[Hr]_r K_f} \quad (14)$$

The values of $[OH]_r$ and $[OH]_s$ can be readily determined from pH measurements. If the left side of eq 14 is plotted vs. $[OH]^n_s$, K_f can be determined from the ratio of the slope to the intercept. The value of n, the number of hydroxide ions bound to the active site, can be empirically determined by finding the integer value that gives the most linear fit to eq 14.

The work of Gorman & Darnall (1981) first introduced the use of difference spectroscopy without knowledge of molar extinction coefficients. This technique is here expanded to being a method in which protein concentrations, which are often difficult to obtain without accurate extinction coefficients, are also not needed. The major advantage of this method is that it can be used to determine equilibrium constants without ever producing a pure solution of either reactant or product.

Experimental Procedures

Hemerythrin for these studies was purified from the hemolymph of two species of sipunculid, *P. gouldii*, obtained live from Marine Biological Laboratories in Woods Hole, MA, and *T. dyscritum*, obtained live from the Oregon Institute of Marine Biology in Charleston, OR. The hemerythrin was extracted and crystallized as previously described (Klotz et al., 1957; Dunn et al., 1977). The crystals were dissolved in 0.2 M Tris[1]–sulfate (pH 8.0) and then converted to methemerythrin by addition of solid potassium ferricyanide in a 4-fold molar excess per binuclear iron unit, followed by dialysis to remove the spent reagents. Protein concentrations were determined from the ϵ_{280} values of 35 400 and 33 300 M^{-1} cm^{-1} per binuclear iron for hemerythrin from *P. gouldii* and *T. dyscritum*, respectively (Dunn et al., 1977).

Difference Spectra. Methemerythrin from *T. dyscritum* (0.2 mM in binuclear iron) was dialyzed against 0.02 M potassium hydrogen sulfate that had been adjusted to pH 7.2–7.8 with solid Tris base. The pH of the hemerythrin solution was varied by mixing 3.0 mL (delivered by a Micrometric syringe buret) with 0.3 mL of concentrated Tris–sulfate buffer prepared by adding varying amounts of solid Tris base to 0.3 M $KHSO_4$. The ionic strength of the resulting solution (excluding the contribution of the protein) was 0.14 for all samples. The solution with the lowest pH was used in the reference cell, and solutions at four higher pH values were used in the sample cell. Difference spectra were recorded after $^1/_2$–1-h equilibration at the desired temperature.

Measurements were made on a Cary-14 spectrophotometer using the 0–0.1 slide wire and jacketed cell holders attached to a circulating water bath as described previously (McCallum, 1982). The slit width was maintained below 0.15 mm. At low temperatures the cell compartments were continually flushed with dry nitrogen to prevent moisture condensation. The temperature and pH of each sample were measured in the spectrophotometer cell. The pH values were corrected for the temperature dependence of the calibration buffers (pHydrion, Microessential Laboratories). The hydroxide ion activity was determined from the pH and the pK_w of water, corrected for temperature and ionic strength (Harned & Copson, 1933).

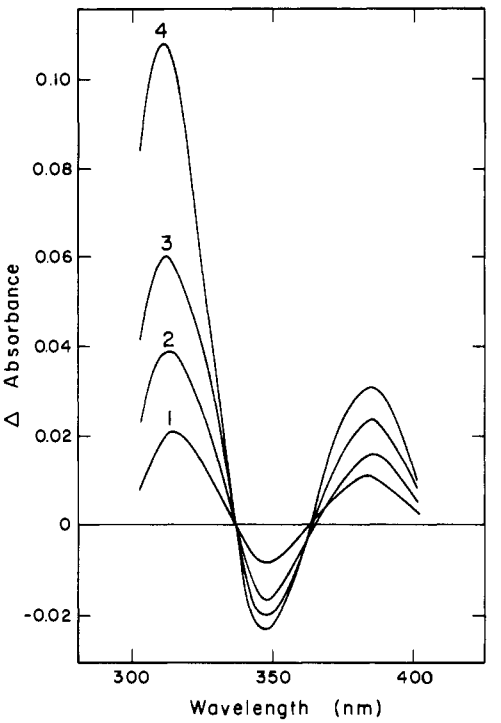

FIGURE 2: Difference absorption spectra of methemerythrin from *T. dyscritum* at 9.2 °C and varying pHs. Reference cell: pH 7.47. Sample cell: (1) pH 7.87; (2) 8.16; (3) 8.38; (4) 8.82. Protein concentration is 0.25 mM in hemerythrin monomer. Reproducibility of absorbance values is within ±15%.

Raman Spectra. Methemerythrin solutions 1.0–2.0 mM in hemerythrin monomer were dialyzed vs. the appropriate buffer. Raman spectra were obtained by pumping the sample through a capillary at ~10 °C in a recirculating system. Sample integrity was checked by the maintenance of spectral intensity during multiple scans and by the UV/visible absorption spectrum at the end of the experiment. Raman spectra were recorded on a computerized Jarrell-Ash spectrophotometer (Loehr et al., 1979) using 90° scattering geometry, an RCA C31034 photomultiplier tube (photocathode at ~245 K), and an Ortec Model 9302 amplifier–discriminator. Samples were illuminated with 457.9- or 363.8-nm excitation from a Spectra-Physics Model 164 Ar laser.

Results and Discussion

Interconversion of Methemerythrin and Hydroxomethemerythrin. The spectrum of methemerythrin from *P. gouldii* or *T. dyscritum* is characterized by a broad absorbance maximum at ~355 nm (Figure 1). Conversion to the hydroxomet form results in the appearance of two separate peaks at ~320 and ~370 nm, a pattern that is typical of methemerythrins with coordinated anions (Garbett et al., 1969). In difference absorption spectra of samples at high pH relative to a reference solution at lower pH, hydroxomethemerythrin from *T. dyscritum* displays distinctly increased absorbance at 310 and 380 nm as well as a decrease in absorbance at 347 nm (Figure 2). The protein appears to exhibit isosbestic behavior. For the application of eq 14, a maximal ΔA was measured from the peak at 310 nm to the trough at 347 nm. (Measurement of ΔA_{max} permitted this value to be obtained from a single difference spectrum, whereas measurement of ΔA at a single λ would have introduced an additional error in the estimation of the base line.) A plot of the resulting data as a function of the hydroxide ion concentration in the sample

[1] Abbreviations: MES, 2-(*N*-morpholino)ethanesulfonic acid; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

Table I: Effect of Temperature on the Equilibrium Constant for Hydroxide Binding to Methemerythrin from *T. dyscritum*

temp (°C)	slope[a]	intercept[a] (×10⁶)	log K_f[b]
4.2	−4.35	−3.81	6.06 ± 0.16
9.2	−5.40	−2.99	6.25 ± 0.14
12.0	−7.85	−11.12	5.85 ± 0.19
16.5	−7.91	−12.27	5.81 ± 0.21
21.0	−5.20	−8.30	5.80 ± 0.20
30.0	−6.92	−13.28	5.72 ± 0.34
34.5	−5.37	−23.57	5.34 ± 0.35

[a] Values were obtained from a plot of $([OH]_r - [OH]_s)/\Delta A$ vs. $[OH]_s$ as depicted in Figure 3. [b] log K_f was calculated from substituting the slope and intercept into eq 14. The error range represents the 95% confidence interval.

Table II: Thermodynamic Parameters for Reaction of Methemerythrin with Hydroxide Ion To Form Hydroxomethemerythrin

source of hemerythrin	ΔH (kcal/mol)	ΔS (cal mol^{-1} K^{-1})	ΔG (kcal/mol)	log K_f	pK_a
T. dyscritum[a]	−9.32	−5.17	−7.78	5.71	8.28
P. gouldii[b]	−11.10	−6.60	−9.13	6.70	7.29

[a] ΔH and ΔS values were from a weighted linear least-squares fit of a van't Hoff plot of the data in Table I. ΔG at 25 °C was from ΔH and ΔS. log K_f for hydroxide binding at 25 °C was from ΔG. pK_a for proton dissociation during formation of hydroxomethemerythrin was from −log ($K_f K_w$). Experiments were performed at $I = 0.14$. [b] Based on 25 °C, $I = 0.10$, data of Gorman & Darnall (1981) for a reaction involving proton dissociation. Values for ΔH and ΔS were recalculated by subtracting ΔH and ΔS for the ionization of water. ΔG and log K_f were calculated as in footnote *a*.

is shown in Figure 3 for three different temperatures with $n = 1$ in eq 14. Larger values of n gave nonlinear behavior. log K_f values obtained from the slopes and intercepts of these linear plots are listed in Table I. Similar log K_f values were obtained for a ΔA based on the difference peak at 380 nm instead of the peak at 310 nm.

At 25 °C, the log K_f for hydroxide binding to methemerythrin is 5.71 (Table II). If the reaction is written as Hr-(H_2O) ⇌ Hr(OH) + H^+, then the corresponding log K_a = log ($K_f K_w$) can be calculated by using a value of 13.99 for log K_w at 25 °C and $I = 0.14$ (Harned & Copson, 1933). The resulting pK_a value of 8.28 gives an indication of the pH required for the formation of hydroxomethemerythrin from *T. dyscritum*. The corresponding pK_a value for a similar temperature and ionic strength for hydroxomethemerythrin from *P. gouldii* is 7.4–7.8 (Gorman & Darnall, 1981; Bradič & Wilkins, 1983). However, these studies differ in the nature of the major anion in solution: the results for *T. dyscritum* hemerythrin were obtained in Tris–sulfate buffer, whereas the results for *P. gouldii* hemerythrin were obtained in sodium phosphate buffer (Gorman & Darnall, 1981; Bradič & Wilkins, 1983). Previous work has indicated that phosphate has a minimal effect on the hydroxide binding equilibrium in *P. gouldii* methemerythrin (Garbett et al., 1971a,b). We found that 0.3 M sulfate caused a slight shift in the optical spectrum of hydroxomethemerythrin from *P. gouldii* at pH 8.8. Thus, it is likely that the pK_a for methemerythrin from *T. dyscritum* would be slightly lower in the absence of sulfate but still significantly higher than the pK_a for the *P. gouldii* protein.

The observed pH dependence for hydroxide binding to methemerythrin indicates that in both species the hydroxide-free form predominantes below pH 7.4. The supernatant from the *T. dyscritum* methemerythrin crystals used for X-ray

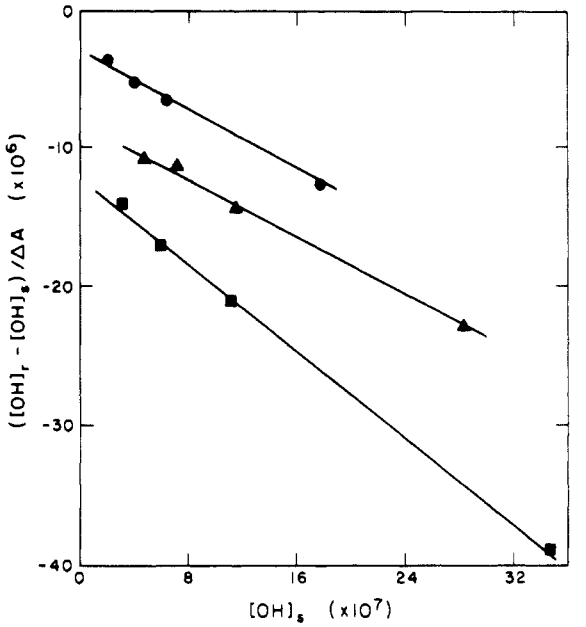

FIGURE 3: Dependence of the function $([OH]_r - [OH]_s)/\Delta A$ on the hydroxide ion concentration in the sample. Sample and reference cells contain identical concentrations of *T. dyscritum* hemerythrin but different amounts of hydroxide, $[OH]_s$ and $[OH]_r$, respectively. ΔA is $A_{310} - A_{347}$ for the sample cell minus the reference cell at 9.2 (●), 16.5 (■), and 21.0 °C (▲). The data have been subjected to a least-squares fitting routine with the points being weighted by $1/\sigma^2$. The good fits to a linear relationship are indicative of $n = 1$ in eq 14. The individual slopes and intercepts do not show a correlation with temperature because the reference cells were at different [OH]'s in the three data sets.

diffraction studies (Stenkamp et al., 1976, 1978, 1981, 1982, 1983, 1984) ranged from pH 4.5 to pH 6.5 in the presence of 20–30% 2-methyl-2,4-pentanediol based on short-range pH indicator paper. Thus, these crystals must be anion-free methemerythrin. All attempts to produce crystals of hydroxomethemerythrin from crystals of methemerythrin have failed due to the enhanced solubility of the protein above pH 8.5 (L. C. Sieker and M. W. Makinen, personal communications).

The determination of a single ionization being involved in the formation of the hydroxide derivative of methemerythrin in *T. dyscritum* (Figure 3) is in agreement with the recent results for acid/base interconversion in *P. gouldii* methemerythrin (Gorman & Darnell, 1981; Bradič & Wilkins, 1983). Further similarities between the proteins from the two species are seen in the thermodynamic parameters for hydroxide ion binding (Table II). Although there is a small difference in the pH at which the reaction occurs, it is likely that the same reaction is taking place in both species.

Resonance Raman Spectra. In an earlier study of *P. gouldii* hemerythrin, Freier et al. (1980) observed a single resonance-enhanced vibration at 509 cm^{-1} in methemerythrin and a pair of resonance-enhanced vibrations at 507 and 492 cm^{-1} in hydroxomethemerythrin. Table III shows our values for these vibrations to be at 511 cm^{-1} in methemerythrin and at 506 and 490 cm^{-1} in hydroxomethemerythrin, in good agreement with the previous results. The peak at 506–511 cm^{-1} is assigned to the symmetric stretch of the Fe–O–Fe bridge on the basis of its being present in all methemerythrins, its being exchangeable with $H_2{}^{18}O$ from solvent under certain conditions (Freier et al., 1980), and the structural evidence from X-ray crystallography and X-ray absorption spectroscopy for a μ-oxo bridge in methemerythrins (Stenkamp et al., 1984; Elam et al., 1982; Hendrickson et al., 1982). The additional

Table III: Resonance Raman Spectral Frequencies in Hemerythrins from *P. gouldii*[a]

hemerythrin sample	H_2O (cm^{-1})	assignment	D_2O (cm^{-1})	assignment
met	511	ν_s(Fe-O-Fe)	511	ν_s(Fe-O-Fe)
perchloromet	510	ν_s(Fe-O-Fe)	510	ν_s(Fe-O-Fe)
hydroxomet	506	ν_s(Fe-O-Fe)	506	ν_s(Fe-O-Fe)
	490	ν(Fe-OH) + δ(Fe-O-H)	518	ν(Fe-OD)
			466	δ(Fe-O-D)

[a] Raman frequencies relative to the 981-cm^{-1} peak of sulfate are accurate to ±2 cm^{-1}; ν_s is the symmetric stretching mode.

peak at 490 cm^{-1} in hydroxomethemerythrin can be assigned to the Fe–O vibration of the bound hydroxide ion on the basis of its deuterium isotope dependence (see below). The resonance Raman spectra of the met and hydroxomet forms of *T. dyscritum* hemerythrin show essentially identical values for ν_s(Fe–O–Fe) and ν(Fe–OH) to those from the *P. gouldii* protein. Similar observations with regard to species similarity have been made previously from the resonance Raman spectra of the azidomet and oxy forms of hemerythrin (Dunn et al., 1977).

A resonance-enhanced mode ascribed to the ν(Fe–L) of the exogenous ligand has been observed in the following methemerythrins: azido (376 cm^{-1}), thiocyanato (298 cm^{-1}), selenocyanato (275 cm^{-1}), and cyanamido (388 cm^{-1}) as well as peroxo in oxyhemerythrin (504 cm^{-1}) (Freier et al., 1980). The peak at 490 cm^{-1} in hydroxomethemerythrin (Table III; Figure 4) is consistent with this pattern and its frequency is in the range expected for an Fe–OH vibration (Sjöberg et al., 1980). Further evidence for this assignment was obtained from the behavior of the protein after exposure to D_2O. In deuterated hydroxomethemerythrin from *P. douldii* there is a marked decrease in intensity at 490 cm^{-1} and a corresponding increase in intensity at 518 cm^{-1} (Figure 4). A similar shift to higher frequency was observed upon deuteration of hydroxomethemerythrin from *T. dyscritum*. In both cases the width of the peak in D_2O is consistent with the continued presence of the Fe–O–Fe vibration at 506 cm^{-1} (at ~50% the intensity of the major peak) as well as a small contribution at 490 cm^{-1} due to residual OH^- in the sample. Studies of oxyhemerythrin and azidomethemerythrin (Kurtz et al., 1977; Shiemke et al., 1984) as well as methemerythrin ± perchlorate (Table III) all show that the ν(Fe–O–Fe) is generally unaffected by protein deuteration.

The drop in intensity at 490 cm^{-1} in deuterated hydroxomethemerythrin is clearly an indication of a vibration involving a protonated moiety. However, the 28-cm^{-1} shift to higher frequency is unexpected since mass considerations alone predict an 11-cm^{-1} decrease for an Fe–OD vibration and M–OH vibrations often decrease 20–25 cm^{-1} in D_2O (Hewkin & Griffith, 1966; Griffith & Wickins, 1966). Hydrogen bonding of the bound hydroxide appears to be ruled out by the lack of suitable acceptors within a 6-Å radius of the exogenous ligand site in azidomethemerythrin (R. E. Stenkamp, personal communication). A plausible explanation for the observed behavior is that the 490-cm^{-1} mode in hydroxomethemerythrin is actually a coupled Fe–OH stretching and Fe–O–H bending vibration. Deuterium substitution would have a much greater effect on the latter and result in a separation of the two modes, with the pure ν(Fe–OD) occurring at 518 cm^{-1} and the pure δ(Fe–O–D) at 466 cm^{-1} (Table III; Figure 4). Although such behavior is not common for metal hydroxides, it is well documented in organic alcohols and acids. In formic acid (Millikan & Pitzer, 1957; Nakamoto & Kishida, 1964; Redington, 1977) the strong feature at 1105 cm^{-1} [ν(C–O) + δ(C–O–H)] shifts to 1177 cm^{-1} [ν(C–O)] in D_2O while a weak feature at 1223 cm^{-1} [δ(C–O–H)] shifts to 990 cm^{-1} [δ(C–O–D)] in D_2O. In methanol (Falk & Whalley, 1961) the corresponding intense peak is observed at 1034 cm^{-1} in H_2O and 1040 cm^{-1} in D_2O, whereas the weaker peak is at 1346 cm^{-1} in H_2O and 942 cm^{-1} in D_2O. In metal hydroxides δ(M–O–H) generally occurs between 600 and 1200 cm^{-1} (Nakamoto, 1970) and shifts by a factor of $2^{-1/2}$ to lower frequency in D_2O. Thus, the 466-cm^{-1} peak assigned to δ(Fe–O–D) in deuterated hydroxomethemerythrin would have had its corresponding δ(Fe–O–H) at ~660 cm^{-1} in hydroxomethemerythrin, which is within the expected range for this vibration but is apparently too weak to be observed.

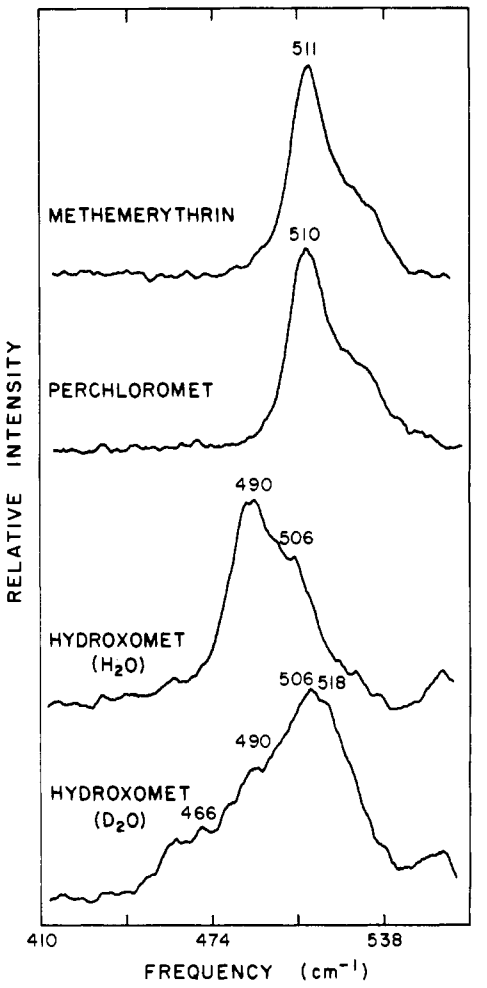

FIGURE 4: Resonance Raman spectra of methemerythrins from *P. gouldii*. Spectra were obtained with ~20 mW of 363.8-nm excitation and are accumulations of ~10 scans: scan rate 0.1–0.2 cm^{-1} s^{-1}; slit width 6 cm^{-1}. Spectra have been subjected to background subtraction and a 25-point smooth. Methemerythrin: 1.2 mM (in monomer) in 0.05 M MES and 0.1 M Na_2SO_4 (pH 6.2). Methemerythrin (ClO_4): 1.1 mM in 0.05 M MES, 0.1 M Na_2SO_4, and 0.02 M $NaClO_4$ (pH 6.2). Hydroxomethemerythrin: 1.7 mM in 0.01 M Tris and 0.1 M sulfate (pH 9.0). Deuterated hydroxomethemerythrin: 1.4 mM prepared by passage of hydroxomethemerythrin through a 2.5-mL Sephadex G-25 column equilibrated with 0.01 Tris and 0.1 M sulfate in D_2O (pD = 9.4).

Preparation of methemerythrin from either species in the presence of perchlorate ion tends to stabilize the met form relative to the hydroxomet form between pH 7 and pH 9 and results in a derivative whose electronic spectrum is indistinguishable from that of methemerythrin as has been previously shown for *P. gouldii* (Garbett et al., 1971a). The resonance Raman spectrum of perchloromethemerythrin is also indis-

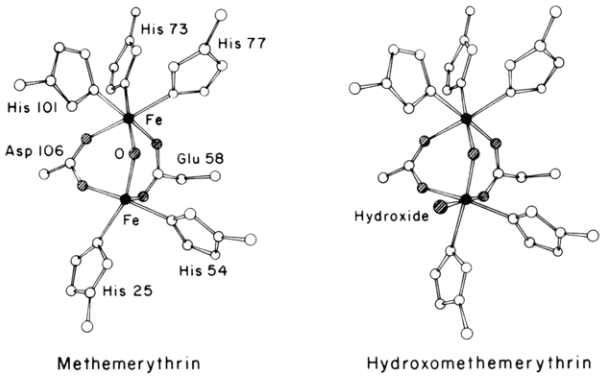

FIGURE 5: (Left) Structure of the binuclear iron center in methemerythrin at 2.0-Å resolution (Stenkamp et al., 1983). (Right) Proposed structure of the binuclear iron center in hydroxomethemerythrin based on 2.0-Å resolution data for azidomethemerythrin (Stenkamp et al., 1984).

tinguishable from that of methemerythrin for *P. gouldii* (Table III; Figure 4) and *T. dyscritum*. The major resonance-enhanced feature in the spectrum is the Fe–O–Fe vibrational mode at ~510 cm^{-1}. The spectra show no evidence of an additional Fe–O vibration from a bound water molecule, which would be expected to appear in the 500-cm^{-1} region (Sjöberg et al., 1980). The resonance Raman spectra of methemerythrin and perchloromethemerythrin do exhibit a shoulder at ~530 cm^{-1}, but this feature is unaffected by deuteration and, thus, unlikely to be due to a ligated water molecule. Although we cannot rule out a lack of resonance Raman enhancement of a coordinated H_2O molecule, our failure to observe an Fe–OH_2 vibrational mode is in agreement with the crystallographic evidence for a 5-coordinate iron atom free of exogenous ligands in both methemerythrin (Stenkamp et al., 1983) and perchloromethemerythrin (Stenkamp et al., 1978).

It is of interest to consider the electronic transition responsible for the resonance enhancement of the vibrational spectrum. The Fe–O–Fe peak of methemerythrin and the Fe–O–Fe and Fe–OH peaks of hydroxomethemerythrin increase ~15× and ~4× in intensity, respectively, with ultraviolet excitation (e.g., 363.8 nm) relative to visible excitation (e.g., 457.9 nm). Similar behavior has been observed for the Fe–O–Fe mode in oxyhemerythrin and azidomethemerythrin (Shiemke et al., 1984). Single-crystal spectra of the latter two derivatives show that the electronic transitions below 400 nm are polarized parallel to the iron–iron axis (Gay & Solomon, 1978). Thus, the observed enhancement of ν(Fe–O–Fe) with ultraviolet excitation is consistent with a substantial μ-oxo → Fe(III) charge-transfer contribution in this region of the electronic spectrum. The same is likely to be true for the smaller enhancements observed with visible excitation. In single-crystal spectra of methemerythrin (M. W. Makinen, personal communication) the electronic transitions at ~480 and ~590 nm are polarized parallel to the Fe–Fe axis and, thus, contain significant charge-transfer character associated with the μ-oxo bridge. Although the absorption bands at ~480 and ~600 nm in hydroxomethemerythrin had been assigned as the $^6A_1 \rightarrow [^4A_1,^4E](^4G)$ and $^6A_1 \rightarrow {}^4T_2(^4G)$ transitions of octahedrally coordinated high-spin Fe(III) (Loehr et al., 1980), the present Raman and polarized optical results indicate that the unusually high absorptivity of these bands derives in part from the superexchange through the μ-oxo bridge.

Conclusions

Methemerythrin is the predominant form of ferric hemerythrin at neutral pH in the absence of added anions. X-ray crystallography has shown that this species lacks electron density at the exogenous ligand site and that the remaining five protein ligands (particularly His-25) have rearranged to give a geometry approaching trigonal bipyramidal (Figure 5, left). Binding of an exogenous ligand such as azide converts the 5-coordinate iron in methemerythrin to a 6-coordinate, octahedral structure. The unusual coordination geometry in methemerythrin relative to all of the ligand-bound methemerythrins including oxyhemerythrin is supported by the optical absorption spectrum (it is the only form that lacks a peak at 320 nm) and by the observed kinetics of anion binding. At neutral pH (i.e., <7), anions bind rapidly to methemerythrin in a single second-order reaction (Meloon & Wilkins, 1976; Bradič & Wilkins, 1983).

At pHs above neutrality, methemerythrin is slowly converted to hydroxomethemerythrin at a rate ($t_{1/2} \simeq 1$ min) that is consistent with a requirement for a protein structural change (Bradič & Wilkins, 1983). The appearance of a new deuterium-sensitive vibrational mode at 490 cm^{-1} is indicative of the presence of an Fe–OH bond. The resulting complex is likely to be analogous to the other octahedral, 6-coordinate methemerythrin derivatives on the basis of the similarity of the optical spectra between 300 and 400 nm (Garbett et al., 1969) and on the almost identical EXAFS patterns for hydroxomet, azidomet, and oxy forms of hemerythrin (Elam et al., 1982). Although there are no crystallographic data on hydroxomethemerythrin, difference density maps of chloromethemerythrin minus methemerythrin at 3-Å resolution give a clear indication of chloride binding to iron at the exogenous ligand site (R. E. Stenkamp, L. C. Sieker, and L. H. Jensen, personal communication). The probable structure for the binuclear iron complex in hydroxomethemerythrin is shown in Figure 5, right. This structure is consistent with the slower kinetics for anion binding to hydroxomethemerythrin relative to methemerythrin (Meloon & Wilkins, 1976; Bradič & Wilkins, 1983). In the former case anion binding may require prior dissociation of hydroxide or the formation of a 7-coordinate intermediate.

Perchlorate ion is known to bind to the outside of the methemerythrin molecule about 10 Å away from the active site (Stenkamp et al., 1978) and to thereby stabilize this form of the protein (Garbett et al., 1971a). Although perchlorate binding results in some loss of electron density near the active site, it appears to have no detectable effect on the structure of the binuclear iron complex. Thus, the electronic and circular dichroic spectra (Garbett et al., 1971a) as well as the resonance Raman spectra of methemerythrin and methemerythrin (ClO_4) are virtually indistinguishable.

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