Resonance Raman Studies and Structure of a Sulfide Complex of Methemerythrin†

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ABSTRACT: The complex of sulfide and methemerythrin has been characterized by resonance Raman spectroscopy. At pH 8.0 the complex contains two irons and one S^{2-} at the active site. The resonance Raman spectrum of the sulfidomethemerythrin complex contains only one vibration, at 444 cm^{-1}. This vibration is assigned to an iron–sulfide stretch. The possibility that sulfidomethemerythrin contains a μ-sulfido bridge, Fe^{III}–S^{2-}–Fe^{III}, analogous to the proposed μ-oxo bridge in azidomethemerythrin is discussed.

Hemerythrin, the oxygen binding protein of sipunculid worms, is an octamer of molecular weight 108 000. Each identical subunit contains two iron atoms and binds one molecule of oxygen. The structure of the oxygen binding site has been the subject of much investigation. [For a review see Kurtz et al. (1977) and Loehr & Loehr (1979).]

In addition to its biologically active forms, oxyhemerythrin and deoxyhemerythrin, hemerythrin exists in an inactive oxidized state, methemerythrin, which reversibly binds any of a number of anionic ligands. Because these ligands bind in or near the oxygen binding site, studies of methemerythrin have been used to elucidate the active-site structure.

On the basis of X-ray crystallographic evidence (Hendrickson et al., 1975; Stenkamp et al., 1976; Stenkamp & Jensen, 1979), magnetic susceptibility measurements (Okamura et al., 1969; Moss et al., 1971; Dawson et al., 1972), Mössbauer spectroscopy (Okamura et al., 1969; York & Bearden, 1970; Garbett et al., 1971a), and electronic absorption spectroscopy (Garbett et al., 1969), several models for the active site of hemerythrin have been proposed (Kurtz et al., 1977; Hendrickson, 1978).

The two iron atoms, located at the oxygen binding site, are 3.0–3.5 Å apart. In oxyhemerythrin and methemerythrin, both irons are in the ferric oxidation state. At least one of the two irons is coordinatively bound to peroxide or to the reversibly bound anionic ligand in oxyhemerythrin or methemerythrin, respectively. Other ligands that have been suggested to coordinate to the iron include some provided by the protein framework, e.g., the imidazole nitrogen of histidine, the phenolic oxygen of tyrosine, and the carboxylate groups of aspartate or glutamate, and others provided by the solvent, water, hydroxide, and O^{2-} in an Fe^{III}–O^{2-}–Fe^{III} μ-oxo bridge. It is the object of this research to examine bound ligands by resonance Raman spectroscopy.

Although deoxyhemerythrin is colorless, oxyhemerythrin and its methemerythrin derivatives have charge-transfer absorption bands in the visible or near-ultraviolet region (Keresztes-Nagy & Klotz, 1965). With laser excitation in or near these transitions, resonance Raman spectroscopy has been used to characterize in detail the binding of O_2, N_3^-, SCN^-, and $SeCN^-$ to hemerythrin (Dunn et al., 1973, 1975; Kurtz et al., 1977). Spectra of hemerythrin complexes with unsymmetrically isotopically labeled ligands (Kurtz et al., 1976, 1977) have demonstrated the end-on binding of O_2, N_3^-, and SCN^-.

All derivatives of hemerythrin that have been studied except deoxyhemerythrin exhibit a Raman vibration near 510 cm^{-1}, the region assigned to an iron–oxygen stretching frequency (Kurtz, 1977). In oxyhemerythrin, this band is at 504 cm^{-1} and has been assigned to an iron–peroxide stretch (Dunn et al., 1973). In methemerythrin, however, there is no bound molecular oxygen so this vibration must be due to a bond (or bonds) between iron and one of the oxygen-containing ligands mentioned above.

In azidomethemerythrin, the band at 507 cm^{-1} was assigned by Kurtz et al. (1977) to an oxygen exchangeable with water, probably a μ-oxo bridge. By adding N_3^- to a solution of oxyhemerythrin in oxygen-18 water, they found approximately half of the intensity of the 507-cm^{-1} vibration shifted to 491 cm^{-1}. The 50:50 ratio of peak intensities was independent of the number of times the oxyhemerythrin was converted to deoxyhemerythrin and reoxygenated before addition of azide. They speculated that the residual intensity at 507 cm^{-1} in the $H_2{}^{18}O$ sample is due to an iron–tyrosine or an iron–carboxylate vibration or possibly a bridging oxygen which does not exchange with water during the deoxy–reoxy cycle.

In order to further characterize these oxygen-containing ligands to the irons in hemerythrin, we have studied complexation of aquomethemerythrin with hydrogen sulfide. It was assumed that S^{2-}, HS^-, or H_2S might be substituted for O^{2-}, OH^-, or H_2O, respectively, in aquomethemerythrin and that characterization of the sulfide–hemerythrin complex would shed light on the nature of some of the oxygen-containing ligands in hemerythrin.

Materials and Methods

Preparation of Hemerythrin Samples. Oxyhemerythrin was isolated from live *Phascolopsis* (syn. *Golfingia*) *gouldii* (Marine Biological Laboratory, Woods Hole, MA) according to the procedure of Klotz et al. (1957). Methemerythrin was prepared by oxidation of oxyhemerythrin with $Fe(CN)_6^{3-}$, followed by extensive dialysis. Oxygen-free methemerythrin was obtained by dialysis against oxygen-free, argon-saturated buffer.

The sulfide complex of methemerythrin was formed either by addition of a hydrogen sulfide solution to a well-buffered solution of oxygen-free aquomethemerythrin or by dialysis of oxygen-free aquomethemerythrin against a buffered solution of hydrogen sulfide.

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0006-2960/79/0418-5372$01.00/0

Concentrations of hemerythrin are reported as monomer concentrations. For the determination of the concentration of aquomethemerythrin solutions, solid azide was added to the sample, and the absorbance at 447 nm of the resulting azidomethemerythrin solution was measured ($\epsilon = 3.7 \times 10^3$ M^{-1} cm^{-1}).

The buffer used for all hemerythrin solutions was tris(hydroxymethyl)aminomethane (Tris)–acetate at pH 8.0. To ensure good buffering when H_2S was added, we usually made the buffer concentration 0.5 M in Tris.

The sulfide–methemerythrin complex in D_2O was prepared by dialysis of aquomethemerythrin against D_2O-exchanged Tris–acetate buffer (pD 8.0), followed by introduction of a small amount of D_2S gas above the sample. The HOD concentration in the sample was determined by proton magnetic resonance to be about 12%.

Standardization of H_2S Solutions. Solutions of H_2S were made by bubbling H_2S through oxygen-free water for at least 1 h. The concentration of H_2S was determined by iodometric titration. An aliquot of the H_2S solution was withdrawn in a gas-tight syringe, added to a solution of excess triiodide, and back-titrated with thiosulfate. All titrations were performed in triplicate, immediately before and after the H_2S was added to the hemerythrin. The results were most reproducible when the H_2S was continuously bubbled through a saturated (about 0.1 M) stock solution and small aliquots of this solution were added directly to the hemerythrin with a gas-tight syringe.

Synthesis of $H_2{}^{34}S$. Isotopically labeled $H_2{}^{34}S$ was made from elemental sulfur according to the procedure of Seel (1956). Fourteen milligrams of 90% enriched ^{34}S (Mound Laboratories) was combined with 25 mg of melted paraffin and 7 mg of Celite. The mixture was heated by a Bunsen burner and the evolved gas was trapped in liquid nitrogen. The yield of $H_2{}^{34}S$ was 80%. Raman spectra of gaseous H_2S showed a shift from 2613 cm^{-1} for $H_2{}^{32}S$ to 2609 cm^{-1} for $H_2{}^{34}S$.

Resonance Raman and Absorption Spectra. Resonance Raman spectra were obtained by excitation with a Coherent Radiation CR-3 or a Spectra Physics 164 Ar^+ laser. The spectra were collected with either a Spex 1400-II or a Spex 1401 double monochromator equipped with a cooled RCA C31034A photomultiplier tube. Backscattering geometry (Shriver & Dunn, 1974) was used throughout. Incident laser intensity was usually 50 mW and the samples were spun during illumination.

Resonance enhancement was determined by comparison of the area of the Raman band to that of 0.2 M sodium sulfate which was added as an internal standard. Corrections were made for self-absorption (Shriver & Dunn, 1974).

All absorption spectra were measured in a Cary 14 spectrophotometer.

Results

When H_2S or Na_2S is added to an oxygen-free solution of aquomethemerythrin at pH 8.0,[1] a red complex with an absorption maximum at 510 nm is formed (Keresztes-Nagy & Klotz, 1965). The spectrum of this complex is shown in Figure 1. We will call it sulfidomethemerythrin. This complex is not formed when H_2S is added to deoxyhemerythrin or when Na_2S is added to either deoxyhemerythrin or oxygen-free aquomethemerythrin.

[1] The pK_a of aquomethemerythrin is 7.8 (Garbett et al., 1971b). Therefore, at pH 8.0 methemerythrin is a 60:40 mixture of hydroxymet- and aquomethemerythrin.

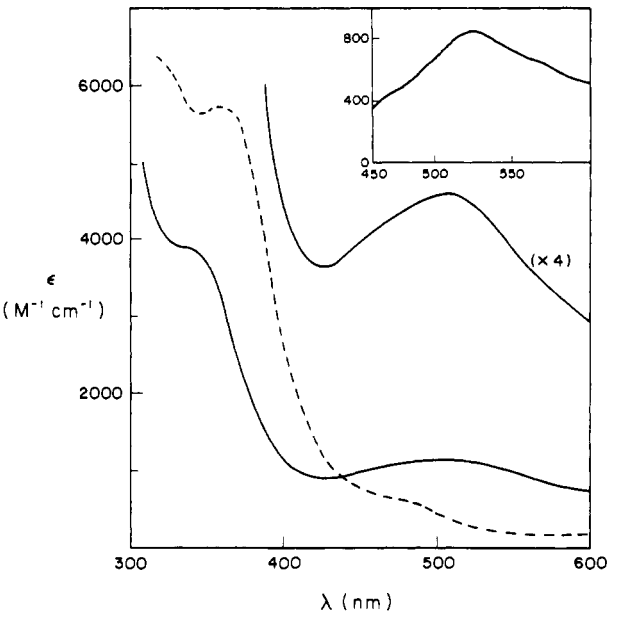


FIGURE 1: Absorption spectrum of the complex of H_2S with methemerythrin. Solid line, sulfidomethemerythrin; dashed line, aquomethemerythrin. Both samples are in 0.5 M Tris–acetate at pH 8.0. The insert shows the difference of the absorptivities between sulfido- and aquomethemerythrin.

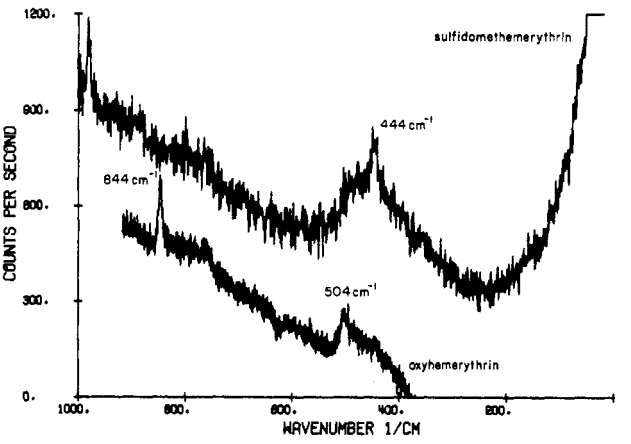


FIGURE 2: Resonance Raman spectra of 4×10^{-3} M sulfidomethemerythrin and the product generated after addition of oxygen to sulfidomethemerythrin. Laser excitation, 514.5 nm, 50 mW; 4-cm^{-1} spectral slit. The 981-cm^{-1} band is due to sulfate added as an internal standard. In both spectra, the very broad band centered at 500 cm^{-1} is background Raman scattering of the Pyrex sample tube.

Excitation within the 510-nm absorption band of sulfidomethemerythrin made with $H_2{}^{32}S$ generates one Raman band at 444 cm^{-1} (Figure 2). The resonance Raman spectrum of sulfidomethemerythrin made with $H_2{}^{34}S$ is shown in Figure 3. The 444-cm^{-1} band has shifted to 438 cm^{-1}. Upon replacement of H_2S with D_2S in the formation of sulfidomethemerythrin, no shift in the 444-cm^{-1} band could be detected (Figure 3).

When oxygen is added to sulfidomethemerythrin, the band at 444 cm^{-1} disappears and two bands at 844 and 504 cm^{-1} characteristic of oxyhemerythrin appear (Figure 2).

Because backscattering geometry was used, the background Raman band of the Pyrex sample tube is substantial (Figure 2). Near 440 cm^{-1}, this background is approximately linear and can easily be subtracted. For examination of the 500-cm^{-1} region, however, a borosilicate sample tube should be used. Although the background Raman spectrum is larger than that of Pyrex, it is flat in the 500-cm^{-1} region.

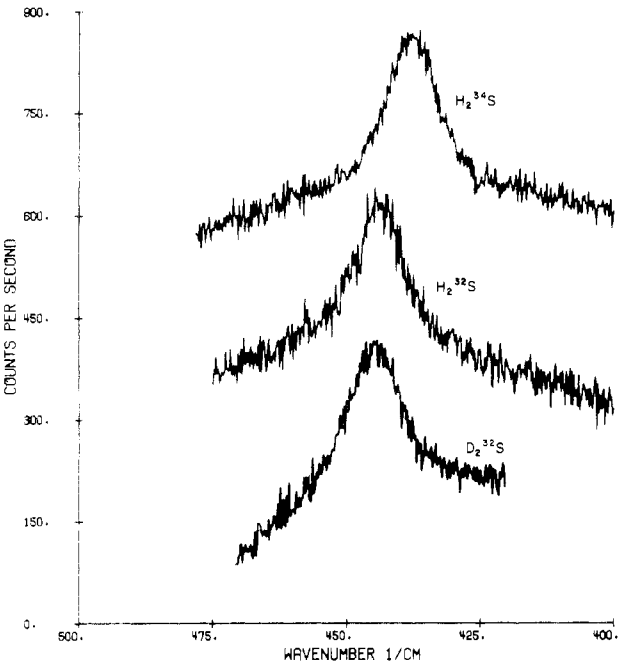


FIGURE 3: Resonance Raman spectra of 4×10^{-3} M sulfidomethemerythrin made from $H_2{}^{32}S$, $H_2{}^{34}S$, and $D_2{}^{32}S$. Laser excitation. 514.5 nm, 60 mW; 3-cm^{-1} spectral slit.

When a borosilicate sample tube was used to examine the 500-cm^{-1} region of the resonance Raman spectrum of sulfidomethemerythrin, no peaks other than the 444-cm^{-1} band were observed.

The proposed structure of azidomethemerythrin contains both azide and O^{2-} as ligands to the iron. To test if sulfide could replace either of these ligands, we added sulfide to azidomethemerythrin and azide was added to sulfidomethemerythrin.

The resonance Raman spectrum of oxygen-free azidomethemerythrin to which H_2S was added is identical with that of azidomethemerythrin. Conversely, the resonance Raman spectrum of sulfidomethemerythrin to which azide was added is identical with the upper spectrum in Figure 2. Azide does not appear to perturb the active-site structure of sulfidomethemerythrin, and sulfide does not appear to perturb the active-site structure of azidomethemerythrin.

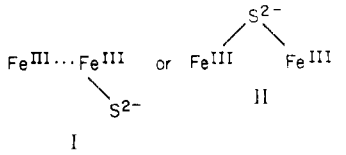
Although an excitation profile of the 444-cm^{-1} band in sulfidomethemerythrin was not measured over the range of all visible frequencies, the resonance enhancement of this vibration is greater at 514.5 nm than at the other, shorter wavelengths available on an Ar^+ laser. It appears this vibration is coupled to the 510-nm charge-transfer transition.

For measurement of the stoichiometry of binding of sulfide[2] to hemerythrin, small aliquots of an H_2S solution were added to a well-buffered solution of oxygen-free aquomethemerythrin. Because the difference in the absorptivities of sulfidomethemerythrin and aquomethemerythrin is greatest at 530 nm, the absorbance at this wavelength was used as a measure of the degree of complexation (Figure 4). Extrapolation of the points at low sulfide concentration to saturating sulfide concentrations indicates a stoichiometry of one sulfide per monomer unit. A fit of the experimental curve suggests the association constant[2] of sulfide to aquomethemerythrin is about 2×10^4 M^{-1} at 25 °C.

[2] Expressed in terms of the stoichiometric sulfide concentration, $[(H_2S) + (HS^-) + (S^{2-})]$.

Discussion

We find a stoichiometry of one S^{2-} per monomer unit and propose the sulfidomethemerythrin complex contains one S^{2-} (or HS^-) and two Fe^{III} atoms at each active site. The sulfide (or bisulfide) may be coordinated to a single iron or may bridge the two irons:

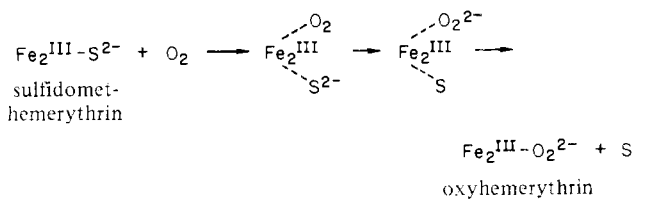


The second structure is analogous to that of S^{2-} in the Fe_2S_2 iron–sulfur proteins except that, in the latter, two such sulfide bridges bond each pair of irons. The optical spectrum of oxidized spinach ferredoxin with absorption maxima at 420 and 463 nm (Lovenberg, 1973) differs substantially from that of sulfidomethemerythrin.

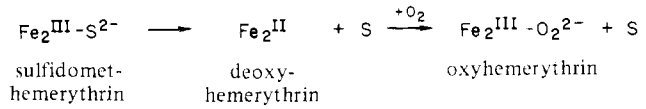
Our formulation, structure I or II, is in disagreement with Bayer et al. (1970), who proposed sulfur is bound as S_2^{2-} in sulfidomethemerythrin, analogous to the binding of O_2^{2-} in oxyhemerythrin. Their suggestion was based on three experimental features: the similarity of the absorption spectrum of sulfidomethemerythrin to that of oxyhemerythrin (maxima at 510 and 500 nm, respectively), indirect chemical evidence that the sulfur is not in the reduced S^{2-} state, and elemental analysis of washed sulfidomethemerythrin crystals showing two excess sulfurs per two irons over that found for aquomethemerythrin.

If a S_2^{2-}–methemerythrin complex were formed when we mixed H_2S and aquomethemerythrin, then two-electron oxidation of H_2S to S_2^{2-} without concurrent reduction of the irons to Fe^{II} would be required. To test this, we added ascorbic acid or dithiothreitol to the aquomethemerythrin before addition of H_2S gas. In the presence of antioxidant, the sulfide–hemerythrin complex was identical with that formed without antioxidant under oxygen-free conditions, suggesting no oxidation of S^{2-} is necessary to form sulfidomethemerythrin.

If the complex is Fe_2^{III}–S^{2-}, one could envision either of the following mechanisms for the conversion of sulfidomethemerythrin to oxyhemerythrin in the presence of O_2:



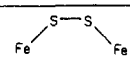
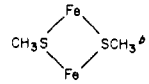
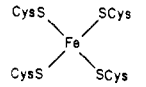
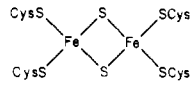
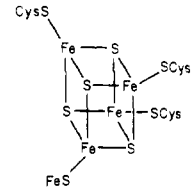
or



Comparison of the 444-cm^{-1} vibration with those of iron–sulfur compounds of known structure does not provide an unambiguous assignment of this frequency. The 444-cm^{-1} band of sulfidomethemerythrin is 50–100 cm^{-1} higher than the iron–sulfur frequencies of iron–sulfur compounds cited in Table I and is at the lower end of the range of observed S–S stretching frequencies [430–600 cm^{-1}; Nakamoto (1978)].

On the other hand, isotopic replacement with ^{34}S leads to a frequency shift compatible with assignment of the 444-cm^{-1} band to an Fe–S stretching vibration and inconsistent with a S–S vibration. Calculated and observed isotopic mass depen-

Table I: Observed S-S and Fe-S Frequencies in Iron-Sulfur Proteins and Synthetic Analogues

molecule	structure	ν (cm^{-1})	assignment	ref
$S_2Fe_2(CO)_6$		554 329	S-S[a] Fe-S[a]	Scovell & Spiro (1974)
$(SCH_3)_2Fe_2(CO)_6$		350	Fe-S	Scovell & Spiro (1974)
oxidized rubredoxin (*C. pasteurianum*)		365 311	ν_3 of $Fe-S_4$ tetrahedron ν_1 of $Fe-S_4$ tetrahedron	Long et al. (1971)
oxidized adrenodoxin (beef adrenal glands)		397 297	$Fe-S_{labile}$ $Fe-S_{labile}$	Tang et al. (1973)
oxidized ferredoxin (spinach)		350 395 284 330	$Fe-S_{Cys}$ $Fe-S_{labile}$ $Fe-S_{labile}$ $Fe-S_{Cys}$	Blum et al. (1977)
oxidized ferredoxin (*C. pasteurianum*)		360 345	$Fe-S_{Cys}$ $Fe-S_{Cys}$	Tang et al. (1975)
reduced high-potential iron protein (chromatum)		365 338 250	$Fe-S_{Cys}$ $Fe-S_{Cys}$ $Fe-S_{labile}$	Tang et al. (1975)
$Fe_4S_4(SCH_2Ph)_4^{2-}$		332 275	$Fe-S_{mercaptide}$ $Fe-S_{labile}$	Tang et al. (1975)
Na_2S_2		451	S-S	Janz et al. (1976)
H_2S_2		509	S-S	Nickless (1968)

[a] On the basis of normal coordinate analysis, only about half of this vibration is due to the assigned bond stretch. [b] This structure is based on that of the corresponding ethyl derivative (Dahl & Wei, 1965).

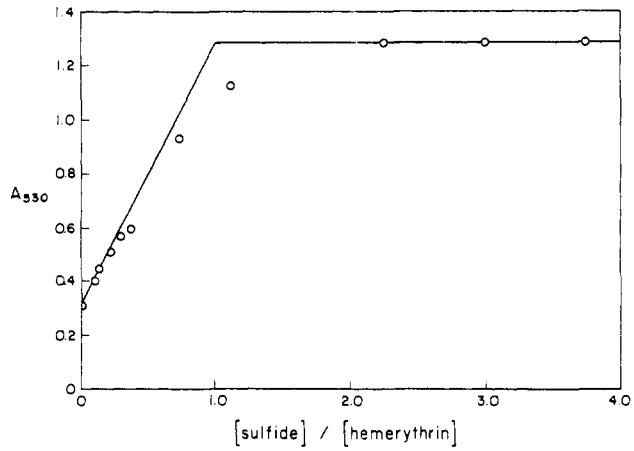


FIGURE 4: Absorbance at 530 nm vs. the ratio of total stoichiometric sulfide concentration, $[(H_2S) + (HS^-) + (S^{2-})]$, to hemerythrin concentration. All samples contained 1.24×10^{-3} M hemerythrin and 0.5 M Tris–acetate at pH 8.0, 25 °C.

dences of vibrational frequencies for several iron–sulfur and iron–oxygen complexes are listed in Table II.

The shifts were computed with the simple valence force field calculation (Wilson et al., 1955). Only the bonds indicated in Table II were included in the calculation. For the Fe–S–Fe and Fe–O–Fe vibrations, the isotopic frequency ratios were insensitive to the choice of force constants, coupling constants, bond lengths, and bond angles. The calculated shift for a S–S vibration is from 444 to 431 cm^{-1}; the shift for an Fe–S–Fe bridge is from 444 to 436 cm^{-1}. The observed shift is to 438 cm^{-1}, much closer to that calculated for Fe–S–Fe than to that calculated for S–S.

For iron–oxygen complexes, the calculated mass dependence of the vibrational frequencies agrees quite well with the observed values (see Table II). Dunn et al. (1973) found the observed mass dependence of the O–O stretching frequency

Table II: Calculated and Observed Mass Dependence of Vibrational Frequencies for Iron–Sulfur and Iron–Oxygen Complexes

	$\frac{\nu_{34}}{\nu_{32}}$		$\frac{\nu_{18}}{\nu_{16}}$
	Calculated[a]		
S-S	0.970	O-O	0.943
Fe-S	0.981	Fe-O	0.956
Fe-SH[b]	0.982	Fe-OH[b]	0.959
	0.981–0.983		0.955–0.959
	Observed		
sulfidomethemerythrin	0.986 ± 0.003	azidomethemerythrin[d] ($\nu_{Fe-O-Fe}$)	0.968 ± 0.002
		oxyhemerythrin[e] (ν_{O-O})	0.945

[a] The simple valence force field approximation (Wilson et al., 1955) was used for all calculations. [b] The SH (or OH) group was treated as a single point mass in the diatomic oscillator approximation. [c] The range given includes symmetric stretching frequencies for a variety of force constants, coupling constants, bond lengths, and bond angles. Although the vibrational frequencies are very sensitive to these parameters, the isotopic frequency shift is not. [d] Kurtz et al. (1977). [e] Dunn et al. (1973).

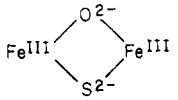
agrees with that predicted for a diatomic oscillator. Similarly, Kurtz et al. (1977) found the observed shift of the $Fe^{III}-O^{2-}-Fe^{III}$ vibration only slightly less than that calculated by using the valence force field approximation. Because we expect similar agreement between calculated and observed vibrational frequencies in the Fe–S complexes, we have assigned the 444-cm^{-1} vibration in sulfidomethemerythrin to an Fe–S vibration.

If the 444-cm^{-1} band were due to an Fe–SH vibration, one would expect a shift to 440 cm^{-1} when $-SD^-$ replaces $-SH^-$. The fact that there is no shift of the 444-cm^{-1} vibration when D_2S is added to a solution of methemerythrin in D_2O suggests that S^{2-} is the ligand involved rather than HS^-. Since S^{2-} is

not known to coordinate to a single iron, it seems likely S^{2-} is bridging the two irons.

The Fe–S–Fe complex should give rise to two iron–sulfur vibrations. The symmetric combination should be Raman active and the observed 444-cm^{-1} band is assigned to this vibration. The higher frequency asymmetric stretching vibration is not expected to be strongly Raman active and is not observed.

Valence force field calculations (Wilson et al., 1955) predict substitution of O^{2-} with S^{2-} in Fe^{III}–O–Fe^{III} would shift the symmetric stretching frequency from 507 cm^{-1} to about 400 cm^{-1} rather than to the observed value of 444 cm^{-1}. The calculation assumes the geometry, force constants, and coupling constants are the same in the two molecules. This is most likely not the case. The frequency is very sensitive to the Fe–O–Fe bond angle which could change significantly when sulfur replaces oxygen. It is also possible sulfidomethemerythrin contains both a μ-sulfido and a μ-oxo bridge:



This dibridged structure could account for the unusually high Fe–S vibrational frequency.

Two binuclear iron compounds containing a single μ-sulfido bridge have been reported. The first, μ-sulfido-bis[[*N*-(hydroxyethyl)ethylenediaminetriacetato]iron(III)] [$(Fe^{III}HEDTA)_2S^{2-}$], has an absorption maximum at 490 nm (Philip & Brooks, 1974). The second, μ-sulfido-bis[[*N*,*N*′-ethylenebis(salicylideniminato)]iron(III)] [$(Fe^{III}Salen)_2S^{2-}$], has an absorption maximum at 510 nm (Mitchell & Parker, 1973; Floriani & Fachinetti, 1973). The similarity between these absorption spectra and that found for sulfidomethemerythrin points to a μ-sulfido bridge in sulfidomethemerythrin also. Raman spectroscopy of these compounds and their μ-oxo bridged counterparts should aid in establishing the 444-cm^{-1} vibration as that of a μ-sulfido bridged binuclear iron(III) structure.

Assuming sulfidomethemerythrin has an active-site structure similar to that proposed for azidomethemerythrin (Hendrickson, 1978), there are two sites where the sulfide could be bound. It could replace either azide or O^{2-}. With visible excitation frequencies, there is no resonantly enhanced Raman vibration in sulfidomethemerythrin near 500 cm^{-1}. This suggests that, unlike other methemerythrins, sulfidomethemerythrin contains no μ-oxo bridge. The Fe^{III}–O^{2-}–Fe^{III} bridge has presumably been replaced by an Fe^{III}–S^{2-}–Fe^{III} bridge.

We were unable, however, to simultaneously bind azide and sulfide to methemerythrin. If sulfide is bound in place of O^{2-}, its larger size or its electronic structure may sufficiently perturb the active-site geometry so that azide can no longer be bound at the active site.

Further characterization of this complex, particularly Mössbauer spectroscopy, magnetic susceptibility, and kinetic measurements of complexation and exchange rates, should aid in understanding the chemistry at the μ-oxo bridge site in hemerythrin.

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Resonance Raman Examination of Axial Ligand Bonding and Spin-State Equilibria in Metmyoglobin Hydroxide and Other Heme Derivatives†

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ABSTRACT: Resonance Raman spectra and excitation profiles have been obtained within the 5700–6300-Å absorption band of purified sperm whale metmyoglobin hydroxide ($Mb^{III}OH$) solutions. A large enhancement occurs for a Raman peak at 490 cm^{-1} which is shown by isotopic substitution of ^{18}O for ^{16}O to be almost purely an Fe–O stretch. The Fe–O vibration in $Mb^{III}OH$ occurs 5 cm^{-1} to lower energy than the corresponding vibration at 495 cm^{-1} in human methemoglobin hydroxide ($Hb^{III}OH$) [Asher, S., Vickery, L., Schuster, T., & Sauer, K. (1977) *Biochemistry 16*, 5849], reflecting differences in ligand bonding between Mb(III) and Hb(III). A larger frequency difference (10 cm^{-1}) exists between $Mb^{III}F$ and $Hb^{III}F$ for the Fe–F stretch. We do not observe separate Fe–O or Fe–F stretches from the α and β chains of either $Hb^{III}OH$ or $Hb^{III}F$. Excitation profile measurements for $Mb^{III}OH$ indicate that the 5700–6300-Å absorption band is composed of two separate absorption bands which result from a high- and a low-spin form of $Mb^{III}OH$. The spin-state-sensitive Raman band at 1608 cm^{-1} reflects the high-spin species and has an excitation profile maximum at about 6000 Å while the low-spin Raman band occurs at 1644 cm^{-1} and shows an excitation profile maximum at 5800 Å. The Fe–O stretch at 490 cm^{-1} has an excitation profile maximum at about 6000 Å. The differences in frequency and Raman cross section between the Fe–X vibrations in $Mb^{III}X$ and $Hb^{III}X$ ($X = OH^-$, F^-) can be related to increases in the out-of-plane iron distance for the high-spin species of $Mb^{III}X$. The shift in the 1644-cm^{-1} $Mb^{III}OH$ low-spin-state Raman band indicative of the heme core size to 1636 cm^{-1} in $Hb^{III}OH$ indicates a larger heme core size in $Hb^{III}OH$. Raman frequency shifts are used to estimate differences in bond strain energies between $Mb^{III}X$ and $Hb^{III}X$ ($X = OH^-$, F^-). Previous resonance Raman excitation profile data can be interpreted in terms of separate contributions from different spin-state species.

Although the heme geometries and tertiary protein structures of the subunits of hemoglobin and of myoglobin are qualitatively similar, some important differences in functional properties result from differences in the detailed heme geometry. These differences in heme geometry are apparent from the recent X-ray results (Takano, 1977a,b; Ladner et al., 1977) indicating that the iron in metmyoglobin derivatives lies farther from the heme plane than in the corresponding methemoglobin derivatives. This may account for the lower ligand affinities of myoglobin compared to those of hemoglobin in the oxy quarternary form (Antonini & Brunori, 1971) and for the increased magnetic susceptibility of metmyoglobin derivatives compared to those of methemoglobin (George et al., 1964; Iizuka & Kotani, 1969a,b).

Some derivatives of metmyoglobin [Mb(III)][1] and methemoglobin [Hb(III)] such as the azide and hydroxide complexes have magnetic susceptibilities at room temperature intermediate between those of a high-spin iron and those of a low-spin iron (Smith & Williams, 1968; Beetlestone & George, 1964). From the temperature dependence of the susceptibilities, it appears that these derivatives are in a thermal spin-state equilibrium due to the small energy difference between the high- and low-spin species [for a review, see Iizuka & Yonetani (1970)]. The exact energy difference depends on the detailed protein structure. For example, small changes in the spin-state equilibrium of some methemoglobin derivatives can be induced by addition of allosteric effectors which switch the quaternary structure of the hemoglobin tetramer (Perutz et al., 1974, 1978; Messana et al., 1978). However, the differences in tertiary protein structure between myoglobin and hemoglobin result in considerably larger spin-state differences than those which are produced in hemoglobin by effectors. For example, the hydroxide derivative of metmyoglobin is 70% high spin at room temperature while the corresponding derivative of methemoglobin is only 45% high spin (Beetlestone & George, 1964; George et al., 1964; Yonetani et al., 1971).

Since resonance Raman spectroscopy permits the selective observation of those heme vibrations which are vibronically active in the resonant electronic transition [for recent reviews see Warshel (1977a) and Yu (1977)], we have used this method to correlate changes in protein structure with changes in the spin-state equilibrium and the heme geometry of various Mb(III) and Hb(III) derivatives (Asher & Schuster, 1979). The resonance Raman technique may be utilized to examine environmentally sensitive heme vibrations such as the iron–axial ligand stretches (Kincaid et al., 1979a,b; Desbois et al., 1978, 1979; Chottard & Mansuy, 1977; Asher et al., 1977; Asher, 1976; Brunner, 1974) and other vibrational modes

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[1] Abbreviations used: Mb(III), metmyoglobin; Hb(III), methemoglobin; $Fe^{III}POR$, ferric porphyrin; R_{ct-N}, distance between the center of the porphyrin and the pyrrole nitrogens; $Fe-N_\epsilon$, iron–proximal histidine bond; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; $InsP_6$, inositol hexaphosphate.

0006-2960/79/0418-5377$01.00/0