Resonance Raman Study of the μ-Oxo-Bridged Binuclear Iron Center in Oxyhemerythrin

Andrew K. Shiemke, Thomas M. Loehr, and Joann Sanders-Loehr*

Contribution from the Department of Chemistry and Biochemical Sciences, Oregon Graduate Center, Beaverton, Oregon 97006. *Received February 29, 1984*

Abstract: Oxyhemerythrin, the respiratory protein of the marine sipunculid *Phascolopsis gouldii*, was investigated by resonance Raman spectroscopy. The presence of a μ-oxo-bridged binuclear iron center in oxyhemerythrin was established by finding ν_s(Fe–O–Fe) at 486 cm^{-1} with ultraviolet excitation. In $H_2{}^{18}O$ solvent, the 486-cm^{-1} vibrational mode shifts to ≈475 cm^{-1}, a shift similar in magnitude to that observed for other μ-oxo-bridged Fe(III) proteins, such as several methemerythrins and ribonucleotide reductase. A study of the enhancement profile of the ν_s(Fe–O–Fe) mode in oxyhemerythrin indicates that resonance occurs by coupling to the ≈360-nm transition and serves to assign this band to an $O^{2-} \rightarrow$ Fe(III) charge-transfer process. By contrast, although the ν_s(Fe–O–Fe) mode in azidomethemerythrin at 507 cm^{-1} is also strongly enhanced in the UV, this mode is observable with visible-light excitation. Its enhancement profile is complex, showing apparent maxima at ≈525, 430, and <350 nm. These data indicate that significant differences exist in the electronic structures of the two chromophores. Further new findings in the present study are the observation of spectral lines at ≈753 cm^{-1} in oxyhemerythrin and 768 cm^{-1} in azidomethemerythrin that are assigned to the asymmetric vibration of the Fe–O–Fe bridge atoms. These frequencies also undergo isotope shifts in $H_2{}^{18}O$ by ≈35 cm^{-1} to lower energy, as expected from model complexes where the infrared active ν_{as}(Fe–O–Fe) have been well documented. In addition, the Fe–O–Fe deformation has been identified in azidomethemerythrin where it occurs at 292 cm^{-1} in $H_2{}^{16}O$ and 286 cm^{-1} in $H_2{}^{18}O$. The new data provide a firm ground for the resonance Raman spectroscopic characterization of this structural unit in metalloproteins. D_2O solvent effects were also investigated; whereas azidomethemerythrin shows no deuterium-sensitive vibrational modes, both ν(O–O) at 844 cm^{-1} and ν(Fe–O_2) at 503 cm^{-1} in oxyhemerythrin were seen to shift in D_2O by +4 and −3 cm^{-1}, respectively. Since the deuterium isotope effect appears to be specific for oxyhemerythrin, it is likely that the bound peroxide is protonated. The ability of the hydroperoxide moiety to undergo internal hydrogen bonding with the μ-oxo bridge oxygen may be the key to electronic structural differences in oxyhemerythrin relative to methemerythrin and ligated methemerythrins.

Hemerythrin is the respiratory protein of several marine invertebrates. The oxygen binding site of hemerythrin contains two non-heme irons which reversibly bind one molecule of dioxygen.[1-3] The deoxy form of the protein contains high-spin Fe(II) with octahedral coordination.[2,4] Upon binding dioxygen, charge is transferred from both irons to the dioxygen to give a peroxide complex, $(Fe^{III})_2$–O_2^{2-}. This representation of the active site of oxyhemerythrin is supported by spectroscopic studies which have shown both irons to be high-spin Fe(III) with effectively octahedral symmetry[4,5] and by resonance Raman studies which have located the dioxygen stretching vibration at 844 cm^{-1}, a frequency characteristic of peroxide.[6,7] Whereas deoxyhemerythrin is magnetically dilute, the oxy form contains antiferromagnetically coupled irons with an exchange coupling constant ($-J$) of 77 cm^{-1}.[8] The structural feature responsible for this coupling has been proposed to be a μ-oxo bridge by analogy to the behavior of model complexes and methemerythrins.[5,8] Model compounds that closely reproduce the bridging geometry as well as the optical and magnetic properties of methemerythrins have recently been synthesized.[9]

In methemerythrin, both iron atoms are irreversibly oxidized to Fe(III) and the binuclear iron center exhibits a high affinity for small anions (e.g., N_3^-, CN^-, SCN^-, OCN^-).[5] The crystal structures of methemerythrins from *Themiste dyscrita* and *Phascolopsis gouldii* show that the two iron atoms are bound to amino acid side chains within four nearly parallel α-helical segments of a single polypeptide chain.[10,11] In the 2.2-Å resolution structure of azidomethemerythrin the active site contains two face-sharing octahedral iron atoms bridged by the carboxyl groups of aspartate and glutamate as well as by a μ-oxo group.[12] The presence of the μ-oxo bridge in methemerythrins is in agreement with the optical properties,[4] the strong antiferromagnetic coupling ($-J$ = 134 cm^{-1}),[8] and, in particular, the Fe–O–Fe vibrational mode at ≈510 cm^{-1}, which undergoes a spectral shift upon isotopic exchange with the aqueous solvent.[13]

Preliminary X-ray crystallographic data for oxyhemerythrin[14] indicate that its active-site structure is very similar to that of azidomethemerythrin, in which the peroxide and azide ions bind at the same site and the three bridging ligands remain intact. Further evidence that the structure of the binuclear iron center of methemerythrins is maintained in oxyhemerythrin comes from the similarity of their electronic[4,5,15] and X-ray absorption spectra,[16] as well as the magnitude of their antiferromagnetic coupling. EXAFS data[16] indicate that two short Fe–O bonds are present in both azidomethemerythrin (Fe–O = 1.80 Å) and oxyhemerythrin (Fe–O = 1.83 Å), as expected for a μ-oxo bridge. Resonance Raman experiments using mixed isotopes suggest that both

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peroxide and azide are bound in an end-on fashion at the active site.[17]

The most notable exceptions to the spectral similarities between met- and oxyhemerythrin come from Mössbauer and resonance Raman spectroscopy. Mössbauer spectra of oxyhemerythrins show a well-resolved pair of quadrupole-split doublets indicating the presence of two inequivalent iron atoms. In azidomethemerythrins, on the other hand, the two irons are more nearly chemically equivalent as judged by their Mössbauer parameters.[5,18,19] Although the differences in the Mössbauer spectra are not well understood, the similarities of the electronic spectra, EXAFS, and preliminary crystal structure would lead one to expect only a minor structural difference between azidomet- and oxyhemerythrin. In resonance Raman spectra obtained with visible-light excitation, all methemerythrins exhibit a resonance-enhanced peak between 507 and 516 cm^{-1} assigned to the symmetric stretch of a bent Fe–O–Fe moiety.[13,17] No such vibrational mode was observed for oxyhemeythrin. We have now resolved this ambiguity and obtained direct evidence for the μ-oxo bridge in oxyhemerythrin by finding that both the symmetric and asymmetric Fe–O–Fe vibrational modes are detectable using ultraviolet excitation. However, differences in the Fe–O–Fe frequencies and enhancement profiles between oxy- and methemerythrins again indicate a structural variation in their active sites. Deuterium isotope exchange experiments point to protonation of the bound peroxide, as had been postulated earlier to explain anomalous Mössbauer results.[5]

Experimental Section

Hemerythrin. *Phascolopsis gouldii* marine worms were obtained from the Marine Biological Laboratory, Woods Hole, MA. Oxyhemerythrin was prepared from the coelomic fluid of the worms using a procedure similar to that published by Klotz et al.[20] The protein was purified by crystallization via dialysis against 15% ethanol in water, then dissolved in sample buffer: 0.05 M Tris, 0.12 M sulfate (pH 8.0). The protein was stored as a sterile solution at 5 °C. For resonance Raman spectral measurements, samples were dialyzed extensively against Raman buffer: 0.05 M Tris–sulfate (pH 8.0) containing 0.3 M SO_4^{2-} as an internal intensity standard. Azidomethemerythrin was prepared by dialyzing oxyhemerythrin vs. Raman buffer containing 0.01 M N_3^-.

Oxygen Exchange. Raman buffer was prepared by adding 6.0 mg of solid Tris base and 42.6 mg of Na_2SO_4 to 1.0 mL of $H_2{}^{18}O$ (95.4 atom %, Monsanto Co.) to give 0.05 M Tris and 0.3 M sulfate. This was titrated with concentrated H_2SO_4 to a pH of 8.0. Oxygen exchange into oxyhemerythrin was accomplished as follows: (Thiocyanato)methemerythrin (≈2 mM in hemerythrin monomer, 0.1 M in thiocyanate) was reduced to deoxyhemerythrin by dialysis against an anaerobic solution of dithionite (≈8 mM) in sample buffer. Excess dithionite and thiocyanate were removed by extensive anaerobic dialysis against sample buffer. The deoxyhemerythrin was then crystallized by anaerobic dialysis against 20% ethanol in water. The deoxy crystals were collected under N_2 and dissolved in 1.0 mL of $H_2{}^{18}O$ Raman buffer which had been degassed with dry N_2. The solution was placed in a sealed vial and stored at 4 °C for 2 days. The protein was then converted to oxyhemerythrin by exposure to air for 6 h and used immediately for Raman experiments. Optical spectra indicated that the samples contained >90% oxyhemerythrin.

For the preparation of azidomethemerythrin,[13] oxyhemerythrin was first crystallized by dialysis against 15% ethanol in water. The crystals were then dissolved in 1.0 mL of $H_2{}^{18}O$ Raman buffer. Solid NaN_3 was added to give a final concentration of 0.01 M and, thereby, convert the oxyhemerythrin to azidomethemerythrin with concomitant bridge exchange. Hydroxomethemerythrins and (anion-free) methemerythrins were prepared from ^{18}O-bridged oxyhemerythrin, which was prepared as described above. The oxyhemerythrin was oxidized by addition of a 5-fold excess of $K_3Fe(CN)_6$ and then dialyzed vs. 0.05 M 2-(*N*-morpholino)ethanesulfonate/0.3 M sulfate (pH 6.0) in H_2O to give ^{18}O-bridged methemerythrin or vs. 0.05 M Tris/0.3 M sulfate (pH 9.0) in H_2O to give ^{18}O-bridged hydroxomethemerythrin. (Thiocyanato)methemerythrin was prepared from ^{18}O-bridged methemerythrin by the addition of 50X KSCN. When the optical spectrum showed that conversion was complete, the sample was dialyzed vs. 0.05 M Tris/0.3 M sulfate/0.025 M SCN^- (pH 8.0) in H_2O.

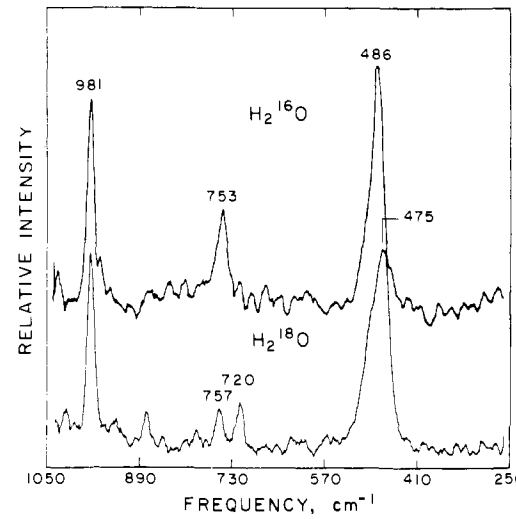

Figure 1. Resonance Raman spectrum of oxyhemerythrin in $H_2{}^{16}O$ (upper) and $H_2{}^{18}O$ (lower). Protein concentration ≈1.2 mM in monomer; 363.8-nm excitation, 20 mW; scan rate, 1.0 cm^{-1}/s; slit width, 8.0 cm^{-1}. The peak at 981 cm^{-1} is due to 0.3 M SO_4^{2-} used as an internal standard.

Deuterium Exchange. D_2O buffer was prepared 6-fold concentrated (1.8 M SO_4^{2-}) by adding 0.75 mL of concentrated H_2SO_4 to 7.5 mL of D_2O. Solid Tris base was added to give a pD of 8.55. The solvent was removed with a rotary evaporator, and the remaining solid was redissolved in 45 mL of D_2O to give 0.3 M SO_4^{2-}–Tris (pD 8.55). The D_2O buffer exchange was accomplished in either of two different ways: (1) protein was dialyzed against the D_2O buffer for ≈2 days; (2) concentrated protein (≈6 mM) was applied to a Sephadex G-25 column (≈2.5-mL column volume) which had been saturated with D_2O buffer and was then eluted with D_2O buffer. The second method was much faster, being complete in ≈30 min, though both methods gave identical results. Azidomethemerythrin in D_2O buffer was prepared by adding solid NaN_3 to oxyhemerythrin in D_2O buffer, to give a final azide concentration of 0.01 M.

Spectroscopy. Resonance Raman spectra were collected on a computer-interfaced Jarrell-Ash spectrophotometer[21] equipped with Spectra-Physics 164-05 (Ar) and 164-01 (Kr) ion lasers, an RCA C31034 photomultiplier tube, and an ORTEC Model 9302 amplifier/discriminator. Both lasers are equipped with ultra-high-field magnets to enhance ultraviolet output. Typical sample concentrations were 1–1.5 mM in hemerythrin monomer. Sample decomposition was minimized through use of a flow cell. A reservoir of 2–3 mL of protein solution was placed in a constant-temperature bath maintained at 3–5 °C. The sample was pumped through 0.8-mm i.d. tygon tubing and a 1.6-mm o.d. quartz capillary tube (situated transverse to the incident laser beam) in a recirculating system with a flow rate of ≈1.5 mL/min. In all cases a 90°-scattering geometry was used, and multiple scans were collected to enhance the signal-to-noise ratio. Sample integrity was monitored by obtaining optical spectra (Cary 16) both before and after exposure to the laser beam. Only upon prolonged exposure of oxyhemerythrin to UV laser light was there any appreciable sample degradation; however, even these samples typically retained >80% activity as measured by the A_{500}/A_{326} ratio, using extinction coefficients reported by Dunn et al.[7] For the resonance Raman enhancement profiles, 0.3 M SO_4^{2-} (ν_1 = 981 cm^{-1}) was used as the internal intensity standard. Corrections for self-absorption were made[22] but found to be insignificant compared to the uncertainty inherent in the measurement.

Results and Discussion

Fe–O–Fe Vibrations. The resonance Raman spectrum of oxyhemerythrin obtained with 363.8-nm excitation is shown in Figure 1. In $H_2{}^{16}O$, the protein shows a strongly enhanced feature at 486 cm^{-1} and a weaker feature at ≈753 cm^{-1}. When the protein

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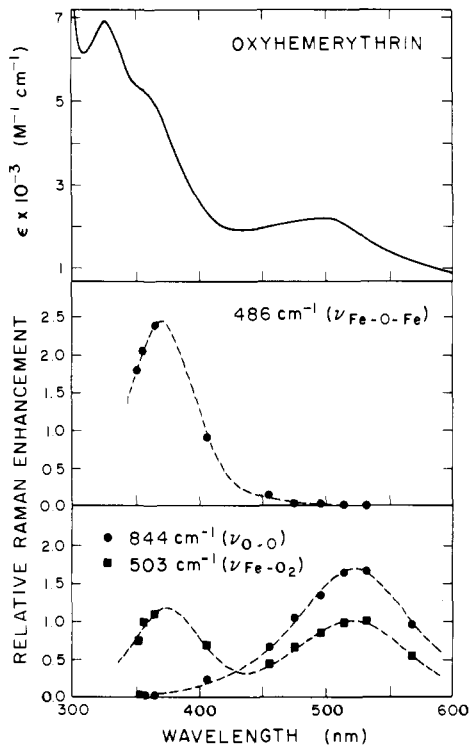

Figure 2. Resonance Raman enhancement profiles for oxyhemerythrin. (Upper) Electronic absorption spectrum of oxyhemerythrin. (Middle) Enhancement profile for the symmetric Fe–O–Fe vibration at 486 cm^{-1}. (Lower) Enhancement profiles for the vibrations of the bound peroxide: O–O stretch at 844 cm^{-1}; Fe–O_2 stretch at 503 cm^{-1}. Enhancement measured as the height of the Raman peak relative to the height of ν_1 of 0.3 M SO_4^{2-} at 981 cm^{-1} and normalized to the ν(Fe–O_2) height obtained with 530.9-nm excitation.

Table I. Vibrational Frequencies (cm^{-1}) for the Stretching and Deformation Modes of the Fe–O–Fe Cluster in Hemerythrins and Model Compounds

sample	ν_s(Fe–O–Fe)[a]		ν_{as}(Fe–O–Fe)[e]		δ(Fe–O–Fe)[a]	
	$H_2{}^{16}O$	$H_2{}^{18}O$	$H_2{}^{16}O$	$H_2{}^{18}O$	$H_2{}^{16}O$	$H_2{}^{18}O$
hemerythrin						
oxy	486	475	~753	720	n.o.	n.o.
azidomet	507	493	768	733	292	286
thiocyanatomet	514	498	780	742	~290	~285
met	510	496	~750	~715	n.o.	n.d.
hydroxomet	508	n.d.	780	~750	n.o.	n.d.
cyanatomet	509	493[f]	782	n.d.	n.o.	n.d.
cyanomet	512	499[f]	780	n.d.	n.o.	n.d.
model compound						
$Fe_2O(CH_3COO)_2$-$(HB(pz)_3)_2$[b]	528	511	751	721	283	269
$[Fe_2O(phen)_4$-$(H_2O)_2]^{4+}$[c]	395	390	827	788	n.o.	n.o.
$[Fe_2OCl_6]^{2-}$[d]	458	440	870	826	203	198

[a] Values obtained by Raman spectroscopy (n.d. = not determined; n.o. = not observed). The ν_s(Fe–O–Fe) values for the methemerythrins in H_2O are similar (±1 cm^{-1}) to those reported previously.[13] [b] Reference 27. [c] Reference 29. [d] Reference 26. [e] Values for hemerythrin obtained by Raman spectroscopy. Values for model compounds obtained by IR spectroscopy. Uncertainty in frequencies (indicated by ~) due to the interference of other protein peaks. [f] Reference 13.

is prepared in $H_2{}^{18}O$, these two peaks shift to lower energy by 11 and 33 cm^{-1}, respectively (Figure 1 and Table I). The isotope dependence identifies these vibrations as Fe–O modes and the exchangeability with solvent oxygen implies that they are associated with an Fe–O–Fe moiety in oxyhemerythrin. The 486-cm^{-1} peak can be assigned to the symmetric stretching vibration on the basis of its intensity, frequency, depolarization ratio, and the magnitude of its O-isotope shift (see below). The symmetric Fe–O–Fe mode has been observed previously in a number of methemerythrin species by using visible-light excitation, but never

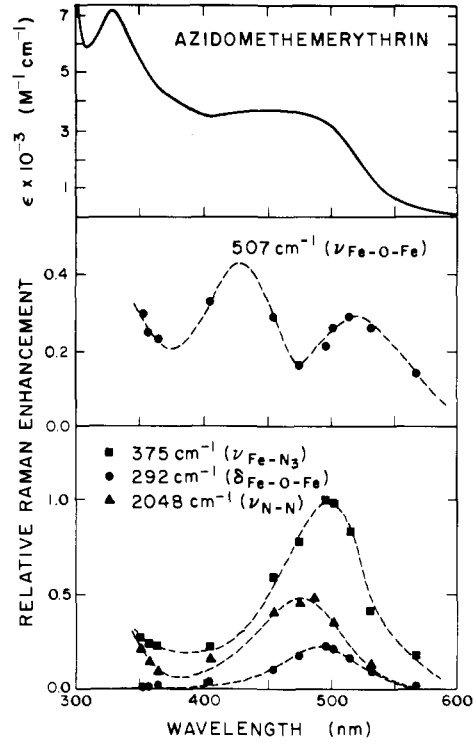

Figure 3. Resonance Raman enhancement profiles for azidomethemerythrin. (Upper) Electronic spectrum of azidomethemerythrin. (Middle) Enhancement profile for the symmetric Fe–O–Fe vibration at 507 cm^{-1}. (Lower) Enhancement profiles for the Fe–N(azide) stretch at 375 cm^{-1}, N–N(azide) stretch at 2048 cm^{-1}, and Fe–O–Fe deformation at 292 cm^{-1}. Enhancement measured as in Figure 3 but normalized to the ν(Fe–N_3) height obtained with 496.5-nm excitation.

in oxyhemerythrin.[13,17] The failure to detect this vibrational mode of oxyhemerythrin was due to the fact that, in contrast to the methemerythrins, it is only enhanced with near-UV excitation (Figure 2). Thus, the present findings provide the first direct evidence for the existence of a μ-oxo bridge in the binuclear iron site of oxyhemerythrin.

Our ability to exchange the μ-oxo-bridge oxygen of oxyhemerythrin is also a new result. In earlier experiments Kurtz et al.[17] and Freier et al.[13] were unable to observe ^{18}O-exchange during the conversion of deoxy to oxyhemerythrin. In their work, crystals of oxyhemerythrin were dissolved in $H_2{}^{18}O$ buffer, reduced with dithionite, and then immediately reoxygenated.[13,17] The only obvious procedural differences are that our deoxyhemerythrin was prepared by dithionite reduction of (thiocyanato)methemerythrin and that the deoxyhemerythrin (with or without crystallization) was equilibrated in $H_2{}^{18}O$ buffer for 2 days prior to oxygenation. The longer period of exposure of deoxyhemerythrin to $H_2{}^{18}O$ is the most likely explanation for the exchange observed in our experiments. Alternatively, it is possible that we achieved a conformation of deoxyhemerythrin that is more susceptible to bridge exchange by having performed the reduction on the thiocyanato (as opposed to the oxy) form of the protein. Kinetic studies have shown that (thiocyanato)methemerythrin undergoes more facile reduction with dithionite than other forms of methemerythrin.[23] Moreover, the mixed valence Fe(II),Fe(III) semi-methemerythrins are believed to exist in several different conformations.[24]

The ν_s(Fe–O–Fe) assignment in oxyhemerythrin is compatible with analogous vibrations which have been observed in the Raman spectra of other oxo-bridged compounds.[25-29] Systems that have

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been shown to contain a nonlinear Fe–O–Fe structural unit and give rise to a resonance-enhanced symmetric Fe–O–Fe vibration near 500 cm^{-1} are ribonucleotide reductase,[28] $Fe_2O(CH_3COO)_2(HB(pz)_3)_2$,[27] $[Fe_2O(phen)_4]^{4+}$,[29] and all forms of methemerythrin[13] (other than sulfidomethemerythrin). The corresponding isotope shifts for ν_s(Fe–O–Fe) with ^{18}O are −15 cm^{-1} for ribonucleotide reductase,[28] −5 to −18 cm^{-1} for the model compounds (Table I), and −10 to −16 cm^{-1} for the methemerythrins (Table I). The −11-cm^{-1} shift for oxyhemerythrin is well within this range. Furthermore, all of these systems appear to show maximal resonance enhancement with UV excitation. Although the ν_s(Fe–O–Fe) mode in methemerythrins was originally observed in resonance with visible electronic transitions,[7,13] we have now found additional enhancement with UV excitation for the N_3^- (Figure 3, middle), CN^-, CNO^-, SCN^-, OH^-, Cl^-, ClO_4^-, and met (anion-free) forms of the protein. Thus, the 486-cm^{-1} peak in the Raman spectrum of oxyhemerythrin shows the characteristics expected for the symmetric stretching vibration of a bent Fe–O–Fe moiety.

The additional oxygen-isotope sensitive vibrational mode at ≈753 cm^{-1} in oxyhemerythrin can be assigned to the asymmetric Fe–O–Fe vibration on the basis of its frequency, its low intensity relative to ν_s(Fe–O–Fe), and its isotope shift. An analogous oxygen isotope-sensitive peak was observed in every form of methemerythrin we investigated (Table I). In all cases, the ν_{as}(Fe–O–Fe) was maximally enhanced with UV excitation. For oxyhemerythrin and azidomethemerythrin, the UV enhancement profiles of ν_{as}(Fe–O–Fe) appeared to parallel those of ν_s(Fe–O–Fe). The intensity of the asymmetric vibration was difficult to quantitate due to the presence of an additional spectral feature at ≈757 cm^{-1} which becomes apparent in $H_2{}^{18}O$ (Figure 1). Since this feature was also discernible in all of the methemerythrin samples studied in both $H_2{}^{16}O$ and $H_2{}^{18}O$ and is not attributable to the Raman buffer at this concentration, it is most likely due to a protein moiety such as tryptophan.[30] Although accurate depolarization ratios could not be obtained for the 750–780-cm^{-1} feature, it appears to be depolarized as expected for an asymmetric vibration.

The 750–780-cm^{-1} frequencies and the −33 to −38 cm^{-1} isotope shifts of the asymmetric bridge modes in hemerythrins are comparable to the behavior of μ-oxo-bridged model complexes (Table I). The closest agreement is with the pyrazolylborate complex,[9a,27] which has a similar small Fe–O–Fe bridging angle of 123.5° compared to 135° in azidomethemerythrin.[31] The bond angles in the other two model compounds are ≈155° and, thus, are expected to have their asymmetric vibrational modes at higher energy.[32] In model compounds ν_{as}(Fe–O–Fe) is generally observed by IR spectroscopy. The phenanthroline and chloride complexes also give weak Raman bands at corresponding frequencies, but only the protein shows clear-cut resonance enhancement of this vibrational mode. It has been suggested that the protein band is a $\nu_s + \delta$ combination band as in the pyrazolylborate complex.[27] This seems unlikely since the protein peak at ≈750 cm^{-1} exhibits a much greater isotope shift than would be expected for a combination band from a $\Delta\nu_s$ of −11 to −16 and a $\Delta\delta$ of −6 cm^{-1} (see below).

A third $H_2{}^{18}O$-sensitive vibration has been detected in several methemerythrins. In azidomethemerythrin, the peak at 292 cm^{-1} shifts to 286 cm^{-1} in the ^{18}O-substituted protein (Figure 4). This vibrational mode had previously been assigned to ν(Fe–histidine).[7,13] However, it is more appropriately described as the deformation mode of the Fe–O–Fe moiety. Analogous vibrations have been detected in the Raman spectra of two of the oxo-bridged model compounds (Δ = −5 to −14 cm^{-1} in $H_2{}^{18}O$) and probably also in (thiocyanato)methemerythrin (Table I). However, no δ(Fe–O–Fe) frequencies have yet been observed in either oxyhemerythrin or the hydroxo, cyanato, cyano, and ligand-free forms of methemerythrin.

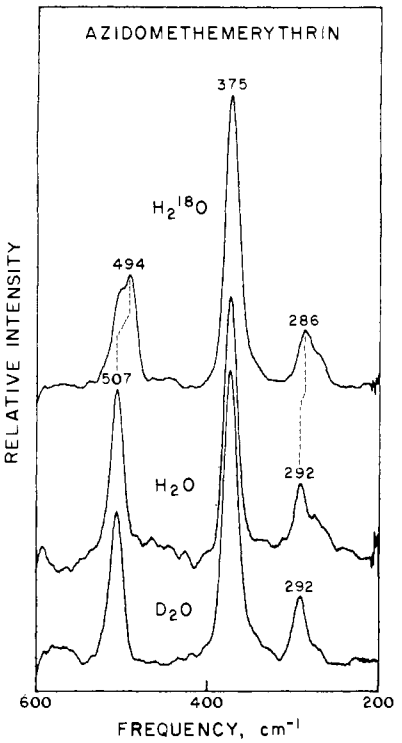

Figure 4. Resonance Raman spectrum of azidomethemerythrin in $H_2{}^{18}O$ (upper), H_2O (middle), and D_2O (lower). Experimental conditions: 530.9-nm excitation, 40 mW; scan rate 0.5 cm^{-1}/s; slit width, 6.5 cm^{-1}; ~1 mM in hemerythrin monomer.

Enhancement Profiles: Exogenous Ligands. Analysis of Raman spectral intensities as a function of excitation wavelength gives information about the nature of the electronic transitions responsible for the vibronic coupling. The electronic spectra for oxyhemerythrin and azidomethemerythrin are shown in the upper panels of Figures 2 and 3, respectively. The lower panels illustrate enhancement profiles for the vibrations due to the exogenous ligands, O_2^{2-} and N_3^-. The data for the visible region are in good agreement with previously published profiles.[7,13] The intensity of $\nu(Fe–O_2)$ of oxyhemerythrin maximizes at 525 nm whereas the intensities of $\nu(Fe–N_3)$ and $\nu(N–N)$ of azidomethemerythrin maximize at 490 and 480 nm, respectively. We have extended these profiles into the near-UV region of the electronic spectrum. The O–O stretching vibration at 844 cm^{-1} in oxyhemerythrin steadily decreases in intensity with increasing frequency of the incident light, vanishing with near-UV excitation. This is not the case for the stretching modes due to $Fe–N_3$ at 375 cm^{-1} and N–N at 2048 cm^{-1} in the azidomethemerythrin spectrum or $Fe–O_2$ at 503 cm^{-1} in the oxyhemerythrin spectrum. All three of these vibrations show enhancement into the near-UV; the $Fe–O_2$ stretching mode appears to be coupled to the 360-nm transition in oxyhemerythrin, whereas the $Fe–N_3$ and N–N stretching frequencies appear to be coupled to the 325-nm transition in azidomethemerythrin.

The enhancement profiles for $\nu(Fe–O_2)$ and $\nu(Fe–N_3)$ are in excellent agreement with the single-crystal spectroscopic studies by Solomon et al.[15,33] These authors observed electronic transitions that were polarized *perpendicular* (⊥) to the Fe–Fe axis and assigned them to charge transfer resulting from end-on binding

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of peroxide or azide to a single iron atom. The ⊥-polarized transitions at ≈500 and ≈360 nm in oxyhemerythrin are presumably responsible for the Raman enhancement maxima of $\nu(Fe-O_2)$ at 525 and ≈360 nm. Similarly, the ⊥-polarized transitions at ≈490 and ≈325 nm in azidomethemerythrin are consistent with the observed Raman enhancement maximum of $\nu(Fe-N_3)$ at 490 nm and an additional maximum <350 nm.

Enhancement Profiles: μ-Oxo Bridge. The symmetric Fe–O–Fe vibrations of azidomethemerythrin and oxyhemerythrin (Figures 2 and 3, middle) both show the strongest resonance enhancement by coupling with electronic transitions in the 300–400-nm region. These electronic transitions were originally assigned as μ-oxo → Fe(III) charge-transfer bands, on the basis of comparison to the electronic spectra of μ-oxo-bridged iron model complexes.[5] This assignment is consistent with the single-crystal studies in which absorption *parallel* to the iron–iron axis was found to dominate the UV spectra of oxy- and azidomethemerythrin.[15,33] However, Schugar et al.[34] have suggested that these near-UV bands in the model complexes (particularly the $[(Fe-HEDTA)_2O]^{2-}$ complex) are due to simultaneous pair electronic (SPE) transitions, and that the same mechanism may be responsible for the near-UV bands in hemerythrin. It has been pointed out that the nature of the temperature dependence of the intensity of the hemerythrin near-UV bands is inconsistent with an SPE assignment.[33] Our enhancement profiles show that excitation in the near-UV region selectively enhances Fe–O–Fe vibrations. For this reason, we would also assign these electronic bands as charge-transfer bands.

In a theoretical analysis of μ-oxo-bridged binuclear Cr(III) complexes,[35] several electronic bands at ≈350 nm were assigned to transitions from a metal d orbital to M–O–M bridge orbitals (combinations of d orbitals from both metals and p orbitals from the bridging μ-oxo atom[36]). This type of electronic transition would be particularly favorable for the resonance Raman enhancement of Fe–O–Fe vibrational modes by including contributions from all three atoms of the bridge to the terminal molecular orbital for the electronic transition. We suggest that a similar mechanism prevails in the case of the strong enhancement of the symmetric Fe–O–Fe vibrational mode in hemerythrin.

The interpretation of the ν(Fe–O–Fe) excitation profiles in the visible region is less straightforward than for the UV region. Whereas oxyhemerythrin shows no enhancement in the visible region, azidomethemerythrin shows that its ν_s(Fe–O–Fe) is coupled to electronic transitions at both ≈525 and ≈430 nm. This striking difference in the visible enhancement profiles indicates that there must be significant differences in the electronic structures and/or vibronic coupling in the Fe–O–Fe moieties in oxyhemerythrin and azidomethemerythrin. The electronic transitions at ≈525 and ≈430 nm in azidomethemerythrin are probably due to additional charge transfer interactions between various iron d orbitals and Fe–O–Fe bridge orbitals. In the case of the oxo-bridged chromium complexes, multiple charge-transfer transitions have also been ascribed to the M–O–M moiety.[35] Although the 430–530-nm region is not well-resolved in the single-crystal spectra of azidomethemerythrin, a distinct shoulder is observed at ≈500 nm in the spectrum polarized parallel to the Fe–Fe axis[33] indicating the presence of an O^{2-} → Fe(III) charge-transfer band at ≈500 nm. All of the other methemerythrins are likely to have similar Fe–oxo charge-transfer bands in the visible region, as they all exhibit resonance-enhanced Fe–O–Fe vibrations with excitation between 430 and 530 nm.[13]

The excitation profile of δ(Fe–O–Fe) in Figure 3 is surprisingly different from that of ν(Fe–O–Fe). As has been observed previously,[7] the 292-cm^{-1} peak is maximally enhanced with 490-nm excitation, in parallel with the 375-cm^{-1} $\nu(Fe-N_3)$ of the exogenous azide ligand. However, δ(Fe–O–Fe) does not appear to be enhanced with UV excitation and is missing entirely in the resonance Raman spectrum of oxyhemerythrin. These results suggest that

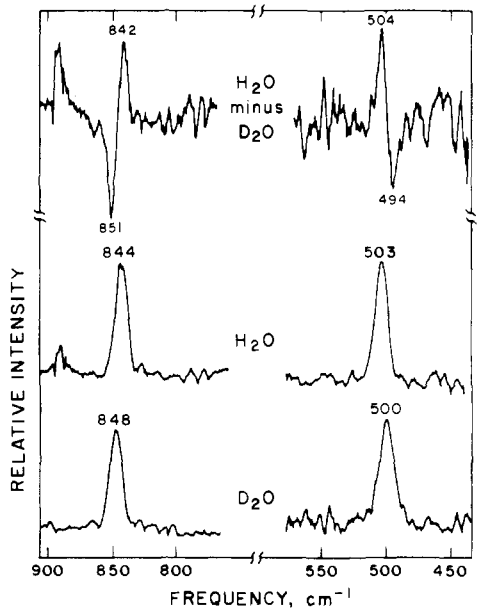

Figure 5. Resonance Raman spectrum of oxyhemerythrin in H_2O (middle) and in D_2O (lower). Upper curve: computer-generated difference spectrum of middle minus lower spectrum. Frequencies of the Raman peaks (±1 cm^{-1}) are reported relative to ν_1 of SO_4^{2-} at 981 cm^{-1}. Experimental conditions: 530.9-nm excitation, 25 mW; scan rate, 0.2 cm^{-1}/s; slit width, 8 cm^{-1}; ≈1 mM in hemerythrin monomer.

the δ(Fe–O–Fe) motion is coupled to an N_3^- → Fe(III) electronic transition. The only other δ(Fe–O–Fe) mode that we observed was in the spectrum of (thiocyanato)methemerythrin (Table I). These two complexes have markedly more intense electronic absorption bands[5] in the visible region (ϵ = 3700 and 5100 M^{-1} cm^{-1} for N_3^- and SCN^-, respectively) than oxyhemerythrin (ϵ = 2200 M^{-1} cm^{-1}) or the other methemerythrins ($\epsilon \leq 1200$ M^{-1} cm^{-1}). Thus, they may be more effective in promoting vibronic coupling of δ(Fe–O–Fe) with a L → Fe(III) charge-transfer process.

D_2O Substitution. Additional information about the structure of the binuclear iron center in oxyhemerythrin has been obtained by examining the resonance Raman spectrum of the deuterated protein (Figure 5). In H_2O solution, ν(O–O) of the bound peroxide is observed at 844 cm^{-1} and $\nu(Fe-O_2)$ at 503 cm^{-1}. However, in D_2O solution, ν(O–O) is shifted to 848 cm^{-1} and $\nu(Fe-O_2)$ is shifted to 500 cm^{-1}. The computer-generated difference spectra (upper panel) show that these shifts, though small, are readily detectable. The 4-cm^{-1} increase in ν(O–O) and the 3-cm^{-1} decrease in $\nu(Fe-O_2)$ agree with the values of +2 and −3 cm^{-1} reported previously for oxyhemerythrin in D_2O.[37]

The isotopic shifts in deuterated oxyhemerythrin appear to be specific to peroxide binding rather than a general change in protein conformation affecting the structure of the active site. Addition of 0.01 M NaN_3 to the oxyhemerythrin samples used to obtain the data in Figure 5 results in indistinguishable azidomethemerythrins in H_2O and D_2O (Figure 4). This absence of a deuterium isotope effect in azidomethemerythrin was also reported by Kurtz et al.[17] Since neither the 507- and 292-cm^{-1} Fe–O–Fe vibrations nor the 375-cm^{-1} $Fe-N_3$ vibration are affected, there seem to be no deuterium substitution-induced conformational effects on the vibrations in the ligand binding site of the protein. Furthermore, neither the symmetric nor the asymmetric Fe–O–Fe vibration observed in oxyhemerythrin with UV excitation is affected by deuteration. Thus, the $\nu(Fe-O_2)$ and ν(O–O) shifts seen upon deuteration appear to be due to effects of the isotope substitution specific to the bound peroxide.

A plausible explanation for the deuterium isotope effect is that the bound dioxygen species in oxyhemerythrin is protonated. The shift of the $Fe-O_2$ stretching mode from 503 to 500 cm^{-1} is close

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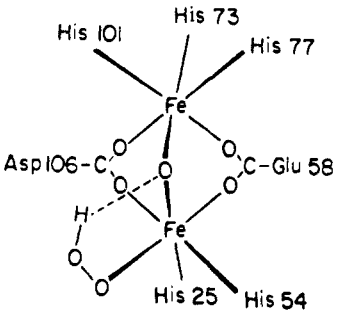

Figure 6. Proposed structure of the binuclear iron center of oxyhemerythrin based on X-ray absorption spectroscopy,[16] X-ray crystallography,[14] and the present investigation.

to that expected for the change in mass when D replaces H in a coordinated hydroperoxide (calculated value, 498 cm^{-1}). The 4-cm^{-1} increase in ν(O–O) is clearly not commensurate simply with a change in mass. However, electronic or structural alterations accompanying deuteration could increase the force constant for ν(O–O) and, thereby, outweigh the mass effect. The ν(O–O) of D_2O_2 similarly shows no mass effect.[38] It is of interest to note that for cobalt-substituted myoglobin and hemoglobin, in which the bound dioxygen is believed to be hydrogen bonded to the distal histidine, the ν(O–O) shifts from 1124 to 1136 cm^{-1} for oxyCoMb and from 1133 to 1138 cm^{-1} for oxyCoHb upon deuteration.[39]

Other unusual features associated with peroxide binding relative to azide binding are the following: (1) both ν_{as} and ν_s(Fe–O–Fe) are 20 cm^{-1} lower in oxyhemerythrin than in azidomethemerythrin (Table I); (2) ν_s(Fe–O–Fe) is only enhanced with UV excitation in oxyhemerythrin but with both visible and UV excitation in azidomethemerythrin. Since X-ray absorption spectroscopy and X-ray crystallography have indicated that the active-site geometries are very similar in these two forms of the protein,[14,16] the resonance Raman observations described here must be due to more subtle structural and electronic differences. Metal–ligand bond strengths are unlikely to be an important factor since methemerythrin complexes with a variety of exogenous ligands, including predominantly σ bonders (such as Cl^- and OH^-) and stronger π bonders (such as N_3^- and CN^-), all show resonance enhancement of ν_s(Fe–O–Fe) with visible excitation, and these vibrations occur in a remarkably narrow range from 507 to 516 cm^{-1},[13] some 20–30 cm^{-1} higher in frequency than observed for oxyhemerythrin.

An attractive proposal is that the hydroperoxide ligand in oxyhemerythrin is hydrogen bonded to the μ-oxo bridge (Figure 6). This structure would provide an explanation for the distinct lowering of the Fe–O–Fe symmetric stretching frequency in oxyhemerythrin relative to the methemerythrins. Support for the hydrogen-bonded model comes from X-ray crystallography where the electron density contours for the bound dioxygen in oxyhemerythrin appear to be located closer to the Fe–Fe axis than is the case for the bound azide in azidomethemerythrin.[14] An alternative explanation would be that steric restrictions at the exogenous ligand site force the Fe–O–Fe unit to adopt a slightly different conformation in the presence of a protonated peroxide relative to a variety of unprotonated ligands. This seems unlikely in view of the number of bulkier ligands such as linear, N-coordinated NCS^- and bent NO_2^-, which still have their Fe–O–Fe vibrations in the high-frequency range (Table I and ref 13). Moreover, if a change in the Fe–O–Fe angle had occurred in oxyhemerythrin relative to the methemerythrins, then the frequencies for the symmetric and asymmetric vibrations should have shifted in *opposite* directions[32] rather than both showing an $\approx$ 20-cm^{-1} decrease. Yet another possibility is that the lowered frequencies in oxyhemerythrin are due to incomplete oxidation of the iron atoms. However, the amount of residual Fe(II) is probably too small to account for the observed shifts in ν_s and ν_{as} in view of the characteristic high-spin Fe(III) isomer shift in the Mössbauer spectrum[5] and the peroxide vibrational frequency in the resonance Raman spectrum[17] of oxyhemerythrin.

Conclusion

The symmetric Fe–O–Fe vibration in oxyhemerythrin has now been located using an ultraviolet source for resonance Raman experiments. This finding provides direct evidence for the presence of a μ-oxo-bridged binuclear iron center in oxyhemerythrin, as had been suggested previously from spectroscopic and structural studies. We have also been able to detect the weaker asymmetric Fe–O–Fe vibration in oxyhemerythrin and in a number of methemerythrin–ligand complexes. Both ν_s and ν_{as} are $\approx$20 cm^{-1} lower for oxyhemerythrin than for the methemerythrins, indicating a difference in the electronic structure of the Fe–O–Fe moiety. This is corroborated by the fact that the μ-oxo bridge vibrations in oxyhemerythrin are only vibronically coupled with an ultraviolet electronic transition, whereas all the methemerythrins show vibronic coupling of the oxo-bridge vibrations with transitions both in the visible and in the UV region.

Analysis of the effects of deuterium substitution on the vibrations of the bound peroxide in oxyhemerythrin leads to the proposal that this group is protonated. Coordination of a protonated peroxide moiety at the active site is reasonable in view of the fact that no dinegative anions are known to bind to methemerythrins. Protonation serves to reduce the high charge of the end-on peroxy ligand and provides a mechanism for further stabilization of the bound peroxide by hydrogen bonding to the μ-oxo bridge. The presence of a H-bonded hydroperoxide species in oxyhemerythrin had been suggested previously as a possible explanation for the Mössbauer finding that the two irons are less chemically equivalent than in any of the met forms.[5] The involvement of the bridging oxo group as the H-bond acceptor would explain the unusual ν_s(Fe–O–Fe) frequency and resonance enhancement in oxyhemerythrin relative to the methemerythrins. Since several studies have shown that the oxygen affinity[40,41] and rate of oxygen binding[42] in hemerythrin are unaffected by changes in $[H^+]$ between pH 6 and 9, a proton attached to the bound peroxide would have to be derived from an appropriate donor in deoxyhemerythrin. A possible donor might be an Fe(II)-ligated hydroxo group[14,43] in the deoxygenated protein.

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Registry No. Iron, 7439-89-6; oxygen, 7782-44-7.

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