of the β-structure formed. As this melts with increasing temperature, many intermolecular hydrogen bonds might remain in a significantly less ordered intermediate structure. These could give rise to the lower energy (1630-cm^{-1}) amide I bands seen. Some oligomers would eventually lose all memory of the original β-structure and could be in a randomly coiled conformation. Such a two-step structural transition would result in faster loss of VCD (correlated to inter-amide coupling) than absorption at 1630 cm^{-1} (correlated to β-type hydrogen bonds) as we have observed. In this view, the melting is *not* a cooperative phase transition, which is consistent with the observed gradual loss of $\Delta\epsilon/\epsilon$. Thus, the IR absorption frequency alone is *not* a good measure of the nature of the secondary structure in these compounds. In light of our results, over reliance on IR frequency measurements is probably, in general, unwise for conformational studies.

The deviation of IR and VCD spectra implies that the solution phase could also be viewed as having three structural types; β-structure, random coil, and one or more intermediate phases in contradistinction to our first view (above) of the data. The similarity of these to the film structural types discussed above is open to question.

It should be noted that in solution we probe the conformations of only one oligomer while in the solid state we attempt to compare conformations of several different oligomers. Hence, the usefulness of such a comparison is probably limited. At this time we cannot state to what extent mixed structures occur in each film sample. We can only propose that there appear to be three structural types. These VCD data also may be strongly affected by chain length and thus not all relevant to the $(L\text{-Val})_7$ and $(D\text{-Ala})_7$ solution cases. However, the consistency of the solution and film data suggests that the ideas proposed in the previous paragraphs are not totally irrelevant.

In summary, our results indicate that combining VCD with IR and CD results can lead to new insight into polypeptide and oligopeptide structural transitions. It is clear from the above discussion that the traditional method of using IR frequencies to determine secondary structure is limited in information content. In particular, one typically wants to know whether an extended β-structure has formed or not. This appears to be most detectable by VCD or CD techniques. The IR frequencies alone appear to sample the much more local phenomenon of interchain hydrogen bonding and, as such, can lead to premature conclusions as to structural integrity. From the results presented in this paper, added structural complexity beyond that previously proposed is evident in both the solid and solution phases of the oligomers studied. It is hoped that our work will stimulate future studies to be more carefully analyzed with respect to peptide conformation.

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Resonance Raman Study of Oxyhemerythrin and Hydroxomethemerythrin. Evidence for Hydrogen Bonding of Ligands to the Fe-O-Fe Center

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Abstract: Hydrogen bonding in the ligand binding site of the respiratory protein hemerythrin has been investigated by resonance Raman spectroscopy. Evidence for hydrogen-bond interactions between the oxo bridge of the Fe-O-Fe moiety and the exogenous ligand has been found for both oxy- and hydroxomethemerythrin. In the latter case peaks are observed at 492 and 506 cm^{-1} that shift upon ^{18}O bridge substitution to 477 and 491 cm^{-1}, respectively. These are assigned as ν_s(Fe-O-Fe) modes of two distinct Fe-OH conformations: the cis conformer nas an intramolecular hydrogen bond between the bound hydroxide and the oxo bridge that is lacking in the trans conformer. This proposal is supported by the observation of a temperature-dependent equilibrium between the conformers, with the cis conformer becoming more prevalent at low temperature as indicated by the increased intensity at 492 cm^{-1} relative to 506 cm^{-1}. The variation in the intensity as a function of temperature yields a ΔH° of −0.4 kcal/mol for the trans to cis conversion, consistent with the formation of a weak intramolecular hydrogen bond. The low frequency of ν_s(Fe-O-Fe) for *cis*-hydroxomethemerythrin relative to that of other methemerythrins is caused by weakening of the bridge bonds when the oxo group acts as a hydrogen-bond acceptor. A similarly low ν_s(Fe-O-Fe) frequency of 486 cm^{-1} is observed for oxyhemerythrin, indicating that the bound hydroperoxide ligand also has the ability to hydrogen bond to the oxo bridge. This hydrogen bond is considerably stronger than that of the hydroxide adduct such that only a single ν(Fe-O-Fe) peak is observed for oxyhemerythrin between 90 and 300 K. This peak undergoes a shift of +4 cm^{-1} in D_2O, an effect specific to oxyhemerythrin, owing to a weakening of the hydrogen bond upon deuterium exchange. An effect of deuterium exchange is also observed for *cis*-hydroxomethemerythrin, although in this case the shift is due to coupling between ν_s(Fe-O-Fe) and the Fe-O-D bending vibration. An additional peak located at 565 cm^{-1} in hydroxomethemerythrin is assigned as the Fe-OH stretch on the basis of its shift to 538 cm^{-1} in $^{18}OH_2$. The relative intensity of this peak is independent of temperature, indicating that hydrogen bonding has little or no effect on the Fe-OH vibration.

Hemerythrin is a nonheme iron-containing respiratory protein found in many marine invertebrates. The coelomic protein from the sipunculid *Phascolopsis gouldii* has an octameric quaternary structure, as does the protein from most other sources.[1] Each protein monomer contains one dioxygen binding site involving two iron atoms that, in the deoxy form of hemerythrin, are in the ferrous state.[1,2] After the dioxygen is bound, the iron atoms are oxidized to the ferric state with concomitant reduction of O_2 to peroxide, resulting in the formation of oxyhemerythrin.[2-5] An

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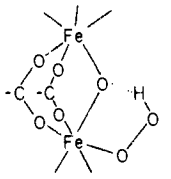

Figure 1. Proposed structure of the ligand binding site of oxyhemerythrin.[8,13] The hydroperoxide ligand is derived from O_2 and the oxo bridge from solvent; the carboxylate bridges are from Asp and Glu, and the remaining ligands are N(His).

additional form of the protein, methemerythrin, also contains ferric iron but does not bind O_2. Instead, methemerythrin avidly binds small anions such as N_3^-, SCN^-, OCN^-, CN^-, and Cl^-.[3]

Crystallographic studies of methemerythrin, azidomethemerythrin, azidometmyohemerythrin, and oxyhemerythrin have shown that the dioxygen (and anion) binding site contains a μ-oxo-bridged binuclear iron cluster.[6-8] The two face-sharing octahedral iron atoms are also bridged by carboxyl groups of aspartate and glutamate (Figure 1), with the remaining coordination sites being filled by imidazole nitrogens of histidine side chains and the exogenous ligand. This oxo-bridged iron cluster is responsible for many of the noteworthy spectroscopic and magnetic properties of the protein, including the strong antiferromagnetic coupling of the ferric iron atoms,[9] the unusually high intensity of the iron ligand field transitions,[2,10] the near-UV electronic transitions,[3,10,11] and the resonance-enhanced Raman spectra of active-site vibrational modes.[4,5,12] On the basis of comparisons with model compounds, the near-UV bands in the electronic spectrum of hemerythrin have long been suspected to be $O^{2-} \rightarrow Fe^{3+}$ charge-transfer transitions.[3,11] Direct evidence for this assignment is the strong, selective enhancement of symmetric (ν_s) and asymmetric (ν_{as}) Fe-O-Fe vibrations in the hemerythrin resonance Raman (RR) spectra obtained with excitation into these near-UV transitions.[13] We have recently discovered that Fe-O-Fe vibrational modes are quite sensitive to exogenous ligand binding, and this has afforded an opportunity for a more detailed study of oxy- and hydroxomethemerythrin.

In the case of oxyhemerythrin our previous RR studies indicated that dioxygen is coordinated as the hydroperoxide anion.[13] Furthermore, we proposed the existence of a hydrogen bond between the bound hydroperoxide ligand and the oxo bridge, as shown in Figure 1. Support for this structure came from an X-ray crystallographic study of oxyhemerythrin that showed that the hydroperoxide ligand is nearer to the iron-oxo bridge vector than is the azide ligand in azidomethemerythrin.[8] However, in our earlier Raman work we failed to observe any effect of D_2O on the oxo bridge vibrations.[13] We have now found that the ν_s(Fe-O-Fe) of oxyhemerythrin is sensitive to deuterium isotope substitution in a manner consistent with the oxo bridge acting as a hydrogen-bond acceptor.

Methemerythrin, which has no bound exogenous ligand, undergoes a pH-dependent transformation between acid and base forms, with a pK_a of 7.6.[14-16] The high-pH form, hydroxomethemerythrin, is derived by hydroxide ion binding to methemerythrin through a simple associative mechanism.[17] As shown in the present study the RR spectrum of hydroxomethemerythrin is unique in that it exhibits two Fe-O-Fe vibrational modes at 492 and 506 cm^{-1}, whereas only a single such mode appears in this region in all other ligated methemerythrins.[12] The occurrence of one of the Fe-O-Fe modes of hydroxomethemerythrin at a frequency considerably lower than that for all other methemerythrins, as well as the temperature dependence of the relative intensity of the two hydroxomethemerythrin modes, leads us to propose the existence of two conformers in hydroxomethemerythrin, with structures illustrated in Figure 2. The two conformers are distinguished by the presence of a hydrogen bond between the bound hydroxide ligand and the oxo bridge in the cis conformer and the absence of this hydrogen bond in the trans conformer. The 492-cm^{-1} feature was previously misassigned as an Fe-OH stretching mode on the basis of its marked, but somewhat anomalous, shift to higher energy when the protein was prepared in D_2O.[17] We have now obtained evidence that this deuterium-induced shift arises from a coupling between the Fe-O-Fe stretching mode and the Fe-O-D bending mode of at least one of the hydroxomethemerythrin conformers. In addition, a new peak has been located at 565 cm^{-1} and identified as the Fe-OH stretching mode of the bound hydroxide ligand.

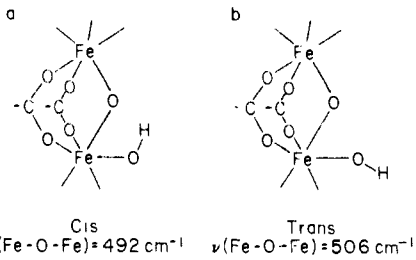

Figure 2. Proposed structures of the ligand binding site of hydroxomethemerythrin in the (a) cis conformation and the (b) trans conformation. The hydroxide ligand is derived from solvent; other ligands are identical with those in Figure 1.

Experimental Section

Hemerythrin. *P. gouldii* marine worms were obtained form the Marine Biological Laboratory, Woods Hole, MA. Hemerythrin was isolated from the coelomic fluid by the procedure of Klotz et al.[18] The protein (predominantly in the oxy form) was purified by crystallization via dialysis against 15% (v/v) ethanol in water. The crystals were dissolved in Raman buffer comprised of 0.05 M Tris and 0.21 M sulfate ion (pH 8.0), and the solution was stored as a sterile solution at 5 °C. The isolated protein was converted to hydroxomethemerythrin by addition of solid potassium ferricyanide in a 4-fold molar excess per hemerythrin monomer, followed by extensive dialysis against Raman buffer (pH 9.8). Preparation of samples with $6.2 < pH < 9.8$ was accomplished by dialysis of methemerythrin against either 0.05 M MES (pH 6-7) or 0.05 M Tris (pH 7-10) containing 0.2 M sodium sulfate and adjusted to the desired pH with H_2SO_4.

Deoxyhemerythrin was prepared by anaerobic dialysis of methemerythrin against a 4-fold molar excess of sodium dithionite (British Drug Houses) and equimolar potassium thiocyanate. Excess reagents were removed by further anaerobic dialysis against Raman buffer (pH 8.0). Raman spectra of oxyhemerythrin were obtained within 2 days of oxygenation of the deoxy protein to minimize spectral contributions of methemerythrin. Anion adducts of methemerythrin were prepared by adding solid potassium or sodium salts of the various anions to methemerythrin to a final concentration of 0.1-0.5 M, with the following exceptions. The formate adduct of methemerythrin was prepared by addition of solid sodium formate to methemerythrin in Raman buffer (pH 6.0) until no further changes occurred in the optical spectrum. The following extinction coefficients (in M^{-1} cm^{-1} per monomer) were de-

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termined: ϵ_{480} = 600, ϵ_{362} = 6000, ϵ_{331} = 6200. An ~1500-fold molar excess of formate was required for complete conversion. Deuterated sodium formate (99 atom % D) was obtained from Merck Sharp & Dohme, Montreal. Cyanomethemerythrin was prepared by adding solid KCN to hydroxomethemerythrin (pH 9.8) to a final concentration of 0.3 M. Protein concentrations were determined spectrophotometrically by using published extinction coefficients.[3,19]

$^{18}OH_2$ and $^{18}OD_2$ Buffer Exchange. $^{18}OH_2$ buffer was prepared by addition of 6.0 mg of Tris base and 28.4 mg of Na_2SO_4 to 1.0 mL of $^{18}OH_2$ (95 atom %; Monsanto Co., Miamisburg, OH) to give 0.05 M Tris–0.2 M sodium sulfate. The solution was titrated with concentrated sulfuric acid to pH 10. Crystalline methemerythrin (obtained by dialysis against 15% ethanol) was dissolved in the $^{18}OH_2$ buffer to a final concentration of 1.6 mM protein monomer. Solid Tris base (~2.0 mg) was then added to raise the pH to 9.8.

A similar procedure was used to prepare $^{18}OD_2$ buffer. The buffer was prepared by adding 7.2 mg of Tris and 34.1 mg of Na_2SO_4 to 1.2 mL of $^{18}OD_2$ (97 atom % ^{18}O, 98 atom % D; KOR Isotopes, Cambridge, MA) to give 0.05 M Tris–0.2 M Na_2SO_4, and concentrated sulfuric acid was added to obtain a solution pH of 10.2. Hydroxomethemerythrin (in H_2O) was concentrated to ~0.1 mL, 15 mM in protein monomer, by using a Centricon 10 filtration device (Amicon). To this protein solution was added 0.4 mL of $^{18}OD_2$ buffer. This concentration–dilution procedure was repeated three times with the $^{18}OD_2$ buffer, and the final solution was concentrated to ~0.2 mL, 7 mM in protein monomer.

^{18}O Bridge Exchange. Substitution of ^{18}O into the oxo bridge position of hemerythrin was carried out by our previously published procedure,[13] but with a slight modification. Because the protein crystals contain ~50% water, dissolution in $^{18}OH_2$ buffer causes dilution of the label. To mitigate this problem, the deoxyhemerythrin crystals were centrifuged and suspended in 0.5 mL of unbuffered $^{18}OH_2$. Very little of the protein dissolved. This suspension was tightly stoppered and stored at 5 °C overnight. This procedure was repeated a second time before the deoxyhemerythrin crystals were finally dissolved in 1.0 mL of $^{18}OH_2$ buffer (pH 8.0); the solution was then stored for 2 days at 5 °C. All manipulations of deoxyhemerythrin were performed anaerobically. Deoxyhemerythrin was oxygenated by exposing it to the atmosphere for 6 h while in the $^{18}OH_2$ buffer. The sample was then dialyzed vs. $^{16}OH_2$ Raman buffer (pH 8.0) for 20 h followed immediately by acquisition of the oxyhemerythrin Raman spectrum. This sample of oxyhemerythrin containing an ^{18}O bridge was later converted to hydroxomethemerythrin by addition of solid potassium ferricyanide followed by dialysis in Raman buffer (pH 9.8).

Deuterium Exchange. D_2O buffer was prepared by addition of solid Na_2SO_4 and solid Tris to D_2O (99.7 atom %; Merck Sharpe & Dohme, Montreal) to give 0.05 M Tris–0.2 M Na_2SO_4. The D_2O solution was titrated with concentrated sulfuric acid to a pD of 8.4 or 10.2 for oxy- and hydroxomethemerythrin, respectively. Deuterium exchange of the proteins was achieved by successive cycles of concentration and dilution. Crystalline protein (either met- or oxyhemerythrin) was dissolved in 2–3 mL of the appropriate D_2O buffer. The solutions were concentrated to ~0.2 mL with a Centricon 10, then diluted to ~2 mL with D_2O buffer. This procedure was repeated at least three times, and the samples were left overnight in the D_2O buffer at 5 °C prior to obtaining Raman spectra.

Spectroscopy. Resonance Raman spectra were collected on a computer-interfaced Jarrell-Ash spectrophotometer[20] equipped with Spectra-Physics 164-05 (Ar) and 164-01 (Kr) lasers, an RCA C31034A photomultiplier tube, and an ORTEC Model 9302 amplifier/discriminator. Both lasers are equipped with ultra-high-field magnets to enhance ultraviolet output. Spectra of hemerythrin at low temperature were obtained by backscattering from a capillary sample tube held in either a copper cold finger and Dewar[21] cooled with liquid N_2 (sample temperature 90 K) or a Varian E-4540 variable-temperature controller using a stream of cold N_2 gas to maintain the sample temperature (100–250 K). Sample temperatures for the variable-temperature experiment were measured with an iron-constantan thermocouple and a Rubicon Instruments potentiometer. The voltages were corrected relative to 0 °C, and the temperature was determined from standard tables of thermoelectric voltage vs. temperature. Solution spectra were measured with a flow cell[13] in a 90° scattering geometry with a sample reservoir of 2–3 mL held at −2 or +35 °C. Sample concentrations were typically 1.5–2 mM in hemerythrin monomer for the solution spectra and 5–8 mM for the frozen samples. Spectra of isotopically labeled samples intended for comparison were run consecutively, on the same day, with identical instrumental and sample conditions. In all cases ν_1 of sulfate (981 cm^{-1} in solution, 990 cm^{-1} when frozen) was used as an internal frequency standard. Reported peak positions are accurate within 1 cm^{-1}. The frequency of isolated peaks was determined by comparison to the internal standard. Where overlapping peaks occur, positions were determined from a computer-generated fit of the spectrum. Unless otherwise stated, fits were performed with a 90% Lorentzian–10% Gaussian peak shape and an 18-cm^{-1} bandwidth (full width at half-height). Absorption spectra were obtained on a Perkin-Elmer Lambda 9 spectrophotometer.

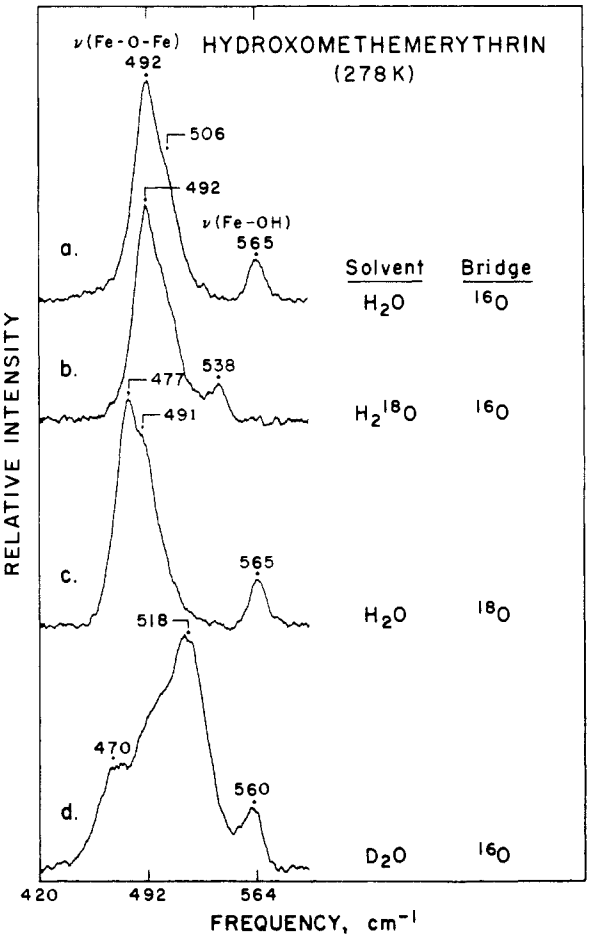

Figure 3. Resonance Raman spectra of hydroxomethemerythrin at 278 K (excitation at 363.8 nm, 20 mW at the sample, 90° scattering geometry, protein concentration of 1.5–2.0 mM in monomer). Each spectrum represents the accumulation of 10–15 scans with a slit width of 7 cm^{-1} and scan rate of 0.5 cm^{-1}/s. Isotopic composition of the bridge oxygen and the solvent is listed to the right of each spectrum. Data have been submitted to a 17-point Savitsky–Golay smoothing routine.

Results and Discussion

Temperature Dependence of Fe–O–Fe Vibrations in Hydroxomethemerythrin. The RR spectra of most ligated methemerythrins exhibit a single strong peak between 507 and 516 cm^{-1} (Table I), which has been assigned to the symmetric stretching frequency of the Fe–O–Fe cluster.[5,12] In contrast to this straightforward behavior, the RR spectrum of hydroxomethemerythrin exhibits two features in this region (Figure 3a).[12,17] Curve resolution of the spectrum in Figure 3a indicates that these two peaks are centered at 492 and 506 cm^{-1} with an intensity ratio of ~3:1. When the oxo bridge is replaced by ^{18}O, the 492- and 506-cm^{-1} features shift to 477 and 491 cm^{-1}, respectively (Figure 3c), thus proving that both of these peaks arise from ν_s(Fe–O–Fe) vibrations. The components at 477 and 491 cm^{-1} also show a 3:1 intensity ratio. The contribution of residual peaks at 492 and 506 cm^{-1} to the spectrum in Figure 3c is consistent with the presence of 15% ^{16}O-bridged hydroxomethemerythrin in this sample. The latter was verified by converting this sample to the thiocyanate form and observing that its ν_s(Fe–^{18}O–Fe) and ν_s(Fe–^{16}O–Fe)

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Table I. Vibrational Frequencies for the Symmetric Fe–O–Fe Vibration in Various Forms of Hemerythrin[a]

ligand	ν_s(Fe–O–Fe) H_2O	ν_s(Fe–O–Fe) $H_2{}^{18}O$	shift in D_2O
	Non-H-Bonded		
N_3^-	507	493	0
SCN^-	514	498	0
CN^-	512	498	0
OCN^-	509	497	0
Cl^-	510	496	0
none (met)	512	496	0
none (ClO_4^-)	512	496	0
$HNCN^-$	508	495	0
$HCOO^-$	513	497	0[b]
OH^- (trans)	506	491	0[c]
	H-bonded		
OH^- (cis)	492	477	+26[d]
OOH^- (oxy)	486	472	+4

[a] All spectra taken on liquid samples in flow cell at 278 K with near-UV excitation; frequencies in cm^{-1}. [b] Hemerythrin derivative prepared from deuterated sodium formate ($DCOO^-$) as well as in D_2O. [c] Curve fitting of spectrum in Figure 3d indicates that a peak near 506 cm^{-1} is still present in the deuterated protein. [d] Upward shift of most intense spectral feature due to coupling of ν_s(Fe–O–Fe) with δ(Fe–O–D).

spectral features at 498 and 514 cm^{-1}, respectively, were present in an intensity ratio of 5.5 to 1.

The two observed ν_s(Fe–O–Fe) modes (Figure 3a) must arise from two different conformations of hydroxomethemerythrin since their relative intensities are independent of pH. The ratio of the 492 to 506 cm^{-1} peak areas remains constant between pH 9.2 and 10.0, despite a 6.3-fold increase in OH^- concentration. The ν_s(Fe–O–Fe) of ligand-free methemerythrin at 512 cm^{-1} appears only with decreasing pH, corresponding to a pK_a of ~7.9, in agreement with the acid/base equilibrium constant determined by other methods.[14-16] Thus, any contribution of methemerythrin to the 506-cm^{-1} peak may be excluded, and both peaks must be due to vibrational modes of the hydroxide adduct. In addition, the effect of pH on the RR spectra of various hemerythrin derivatives was found to be insignificant, indicating that pH-induced alterations of protein structure (if any occur) are not important to the existence of two ν_s(Fe–O–Fe) modes in hydroxomethemerythrin.

The assignment of the two Fe–O–Fe modes to distinct conformations of hydroxomethemerythrin is further supported by the temperature dependence of the relative intensities of the two peaks. When the temperature is lowered to 90 K, there is a sharpening of all spectral peaks and an ~2-cm^{-1} decrease in frequencies (Figure 4a). The intensity of the 504-cm^{-1} peak is only $^1/_{12}$ that of the 490-cm^{-1} peak at 90 K compared to $^1/_3$ at 278 K. The observed temperature dependence is in the direction expected for the proposed hydrogen-bond interaction. A hydrogen bond between the bound hydroxide ion and the oxo group generates the cis form, and disruption of this hydrogen bond generates the trans form (Figure 2). Accordingly, the 492-cm^{-1} Fe–O–Fe mode arises from the cis conformer that is more prevalent at low temperature owing to the stabilizing influence of the hydrogen bond, whereas the 506-cm^{-1} mode arises from the trans conformer whose concentration increases at higher temperatures due to the disruption of this hydrogen bond.

The energy of the hydrogen bond in hydroxomethemerythrin can be quantitated by using the temperature dependence of the equilibrium between the two conformers according to the relationship

$$\ln K_{eq} = -\Delta H^\circ/RT + \Delta S^\circ/R \quad (1)$$

The concentrations of the cis and trans conformers are proportional to the integrated intensities of the 492- and 506-cm^{-1} peaks respectively

$$[\text{cis}] = I_{492}/S_c \quad (2)$$

$$[\text{trans}] = I_{506}/S_t \quad (3)$$

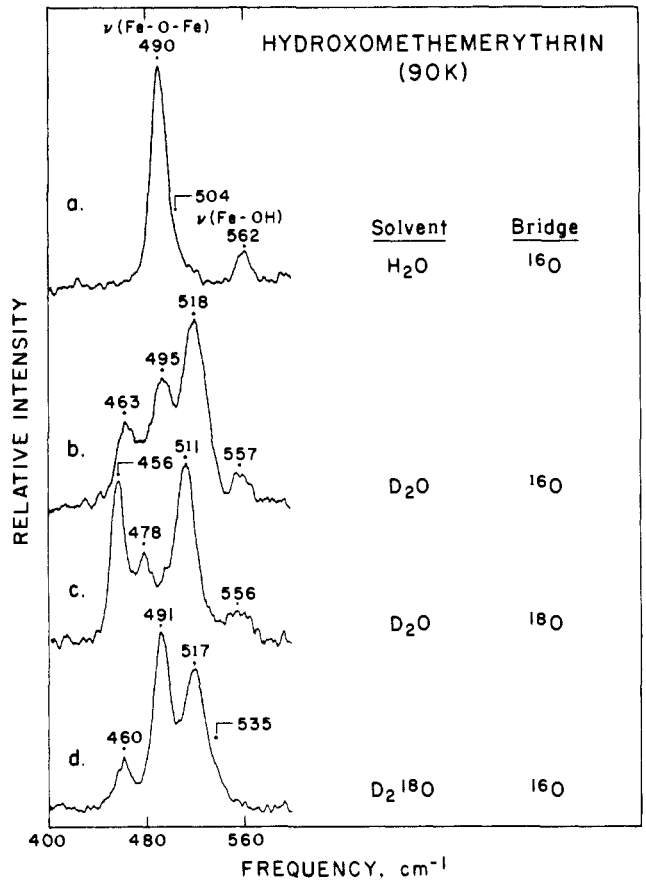

Figure 4. Resonance Raman spectra of hydroxomethemerythrin at 90 K (excitation at 406.7 nm, 17 mW at the sample, 5–10 mM protein concentration, 180° scattering geometry). Spectra are the accumulation of 15–20 scans with a slit width of 8 cm^{-1} and scan rate of 1 cm^{-1}/s. A 17-point smoothing routine was used.

where S_c and S_t are the molar scattering coefficients of *cis*- and *trans*-hydroxomethemerythrin, respectively. For the conversion of trans to cis

$$K_{eq} = [\text{cis}]/[\text{trans}] = \{I_{492}/I_{506}\}\{S_t/S_c\} \quad (4)$$

Combining eq 1 and 4 yields

$$\ln \{I_{492}/I_{506}\} = -\Delta H^\circ/RT + \Delta S^\circ/R - \ln \{S_t/S_c\} \quad (5)$$

The variation in the intensity ratio (5) over a 90–300 K temperature range is plotted in Figure 5. The linear relationship (correlation coefficient 0.98) indicates that ΔH° corresponds to a conversion between two discrete states. The slope of the line yields an enthalpy change of –0.4 kcal/mol for the formation of the hydrogen bond between the hydroxide ligand and the oxo bridge. The presence of molar scattering coefficients in eq 5 precludes the determination of ΔS°. If the molar scattering coefficients of the two conformers were equivalent, ΔS° could be determined from the ordinate intercept in Figure 5. However, the extent of resonance enhancement of the two conformers is not identical. Freier et al.[12] observed 492- and 506-cm^{-1} peaks of nearly equal intensity with 457.9-nm excitation (at 276 K) whereas with 363.8-nm excitation (Figure 3a) their intensity ratio is ~3:1. Since temperature restrictions prevent the determination of the relative scattering coefficient for either conformer, the ΔS° for the formation of the hydrogen bond must remain unknown.

Fe–OH Vibration in Hydroxomethemerythrin. We have now identified the Fe–OH stretching mode as a weak feature at 565 cm^{-1} (Figure 3a) that shifts to 538 cm^{-1} upon [^{18}O]hydroxide substitution (Figure 3b). The frequency observed in $^{18}OH_2$ is very close to the 540 cm^{-1} expected for an Fe–^{18}OH stretching vibration calculated from a simple harmonic oscillator model. The 5-cm^{-1} shift in D_2O is rather small compared to other metal–hydroxide complexes whose M–OH vibrations typically shift 10–25 cm^{-1} upon deuteration.[22] This explains our previous failure to assign

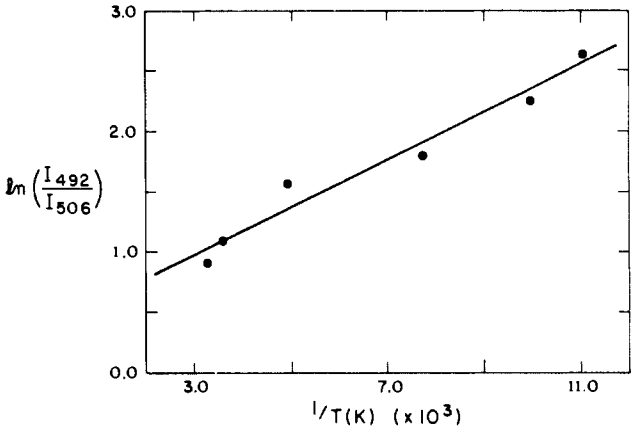

Figure 5. Temperature dependence of the relative intensity of the two ν_s(Fe–O–Fe) modes of hydroxomethemerythrin. Each data point represents the average of two separate determinations (the average of three at 90 K) using conditions as in Figure 4.

the 565-cm^{-1} peak in hydroxomethemerythrin to the Fe–OH stretch.[17]

It is of interest that only a single Fe–OH stretching frequency is observed, even in samples containing a substantial amount of trans conformer (e.g., Figure 3a). Furthermore, the intensity of the 565-cm^{-1} peak remains constant at 13% (±2%) of the combined intensities of the ν_s(Fe–O–Fe) peaks between 90 and 300 K. This indicates that the 565-cm^{-1} peak contains contributions from both the cis and trans conformers and that ν(Fe–OH) is not sensitive to the hydrogen bond to the oxo bridge. In view of the unusually small deuterium isotope dependence of this vibration, its lack of sensitivity to hydrogen bonding is not surprising.

Deuterium Effects on Fe–O–Fe Vibrations in Hydroxomethemerythrin. Given the presence of the hydrogen bond in the cis conformer of hydroxomethemerythrin, one might expect the 492-cm^{-1} peak to exhibit a deuterium isotope effect, whereas the 506-cm^{-1} peak should be unaffected. As shown in Figure 3d and Table I, deuterium exchange has a marked influence on the RR spectrum of hydroxomethemerythrin. When the spectral data for the H_2O solution are compared with those of the D_2O solution, the major peak at 492 cm^{-1} appears to have shifted to 518 cm^{-1}, a shoulder near 500 cm^{-1} remains that may be derived from the original 506-cm^{-1} peak, and a new unresolved feature appears at ~465 cm^{-1}. The poor resolution in Figure 3d (due to extensive overlap of bands) makes it difficult to determine the exact number of components in this spectral envelope. The spectrum of hydroxomethemerythrin in D_2O solution becomes much more distinct when the temperature is reduced to 90 K (Figure 4b). This spectrum has three well-resolved features centered at 518, 495, and 463 cm^{-1}, with bandwidths of 19, 17, and 16 cm^{-1}, respectively. The improved resolution of this spectrum stems from the narrower bandwidths and the reduced contribution of the trans conformer at this temperature.

The appearance of three intense spectral features at 518, 495, and 463 cm^{-1} in D_2O (Figure 4b) when only one was present at 490 cm^{-1} in H_2O (Figure 4a) is indicative of coupled vibrational modes in the deuterated protein. All three features must have Fe–O–Fe character because they shift to lower energy when the bridge is replaced by ^{18}O (Figure 4c). In addition, they must also have some Fe–OD character from the bound hydroxide ligand since they all shift to slightly lower energy and alter their relative intensities when the D_2O solvent is replaced by $D_2{}^{18}O$ (Figure 4d). In contrast, the Fe–OD stretch at 557 cm^{-1} is essentially unaffected by ^{18}O substitution in the bridge and is, therefore, unlikely to be an interactive mode. Fermi resonance coupling can arise when there is a coincidence in the symmetries and energies of fundamental vibrations.[23] The most likely candidate for a mode that involves a solvent-exchangeable hydrogen and that may occur near 500 cm^{-1} and, hence, be able to couple with ν_s(Fe–O–Fe) is the Fe–O–D bend.

The bending vibrations of metal hydroxides are typically found between 600 and 1200 cm^{-1} and are subject to shifts of several hundred wavenumbers upon deuteration.[24] Thus, the δ(Fe–O–D) could easily occur near 500 cm^{-1} in hydroxomethemerythrin. If this mode were to undergo a Fermi resonance with ν_s(Fe–O–Fe) at 490 cm^{-1}, the result would be the appearance of two resonance-enhanced peaks that have diverged in frequency.[23] This is the likely origin of the peaks at 463 and 518 cm^{-1} in D_2O (Figure 4b) and at 456 and 511 cm^{-1} for the ^{18}O-bridged sample in D_2O (Figure 4c). The variability in intensity with isotopic composition is indicative of differences in the relative energies in the fundamentals and, hence, the strength of the coupling interactions. The remaining weak peaks at 495 cm^{-1} (Figure 4b) and 478 cm^{-1} (Figure 4c) may well be due to the coupling of ν_s(Fe–O–Fe) of the trans conformer with δ(Fe–O–D). Such an occurrence would indicate that there is substantially more trans conformer present at 90 K in D_2O than in H_2O (Figure 4a), a finding that would imply that deuterium substitution weakens the hydrogen-bonding interaction of the bound hydroxide with the μ-oxo bridge.

Deuterium Effect on the Fe–O–Fe Vibration in Oxyhemerythrin. The ν_s(Fe–O–Fe) of oxyhemerythrin is observed at 486 cm^{-1} (Figure 6a) and shifts 14 cm^{-1} to lower energy when the bridge is replaced by ^{18}O (Figure 6b).[13] Comparison with the behavior of the ν_s(Fe–O–Fe) of hydroxomethemerythrin (Table I) indicates that the vibrational frequency in oxyhemerythrin is characteristic of a hydrogen-bonded Fe–O–Fe. Moreover, the spectrum of oxyhemerythrin shows no evidence for an additional ν_s(Fe–O–Fe) near 510 cm^{-1}, which would be expected for a non-hydrogen-bonded form of the protein (Table I). All of the intensity at 503 cm^{-1} can be accounted for as the Fe–O_2 stretch,[4,13] and no changes in relative peak intensities are observed when the sample is cooled to 90 K. Thus, the hydrogen bond between hydroperoxide and the oxo bridge in oxyhemerythrin must be considerably more stable than that between hydroxide and the oxo bridge in hydroxomethemerythrin.

Definitive evidence for this hydrogen bond in oxyhemerythrin comes from the effect of deuterium isotope exchange on ν_s(Fe–O–Fe) (Figure 6c). Curve resolution of the spectra reveals peaks centered at 486 and 503 cm^{-1} in H_2O solution (Figure 6a) that shift to 490 and 500 cm^{-1}, respectively, in D_2O solution (Figure 6c). The shift of ν(Fe–O_2) to 500 cm^{-1} in D_2O agrees with our previously published results.[13] However, the effect of D_2O on ν_s(Fe–O–Fe) was missed. Observation of this shift in the present experiments is due to the almost total exclusion of methemerythrin from the sample and more complete substitution of deuterium on the hydroperoxide ligand. The fit of the spectrum in Figure 6c indicates <5% contamination by the met forms of hemerythrin, whereas previous spectra showed close to 20%.[13]

The RR spectra of many derivatives of hemerythrin have been investigated in D_2O, with shifts of ν_s(Fe–O–Fe) being observed only for oxy- and hydroxomethemerythrin (Table I). This lack of an influence following general protein exchange indicates that the spectral shift in oxyhemerythrin is due specifically to perturbation of the hydrogen-bond interaction at the ligand binding site. Although the observed shift of ν_s(Fe–O–Fe) to higher energy in D_2O is opposed to the expected mass effect, this type of shift is not unusual in hydrogen-bonded systems. In cases where the deuterium bond is weaker than the corresponding hydrogen bond, the vibrational frequency of the hydrogen-bond acceptor, e.g. ν_s(Fe–O–Fe), will be less affected by deuterium than by hydrogen. A similar type of behavior occurs with 2-methoxyethanol, which contains an intramolecular hydrogen bond between the alcohol and ether moieties.[25] We observed by infrared spectroscopy that

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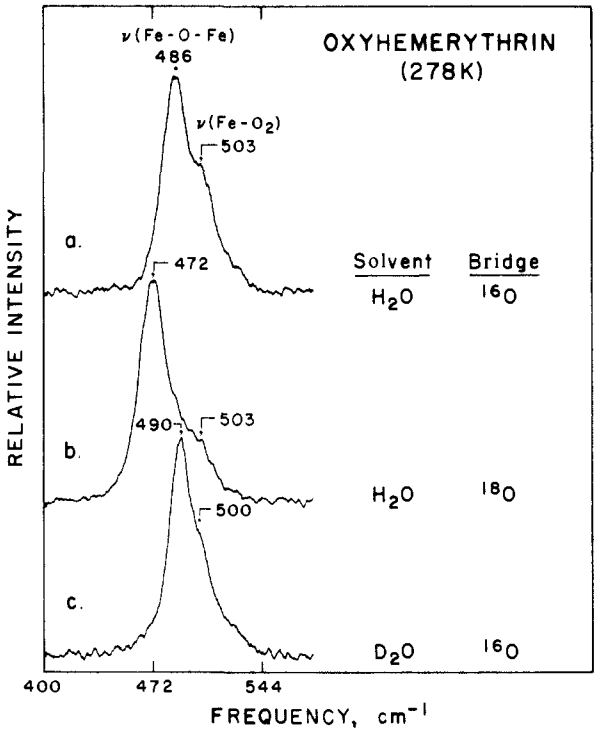

Figure 6. Resonance Raman spectra of oxyhemerythrin in solution at 278 K. Conditions are the same as those for Figure 3.

while deuteration causes ν(C–OH) to shift from 1060 to 1041 cm^{-1}, a 19-cm^{-1} decrease, ν_{as}(C–O–C) shifts from 1126 to 1129 cm^{-1}, a 3-cm^{-1} increase. In contrast, the weak asymmetric Fe–O–Fe stretches of oxyhemerythrin and hydroxomethemerythrin at 753 and 782 cm^{-1}, respectively, do not appear to shift upon deuteration despite the fact that they drop by $\simeq$35 cm^{-1} upon substitution of the oxo bridge with ^{18}O.[13]

The 55% decrease in the intensity of ν(Fe–O_2) when ν_s(Fe–O–Fe) shifts to lower energy upon ^{18}O-bridge substitution (Figure 6a,b) is suggestive of coupling between these modes. The absence of an effect on the frequency of the 503-cm^{-1} peak indicates little or no mixing of the excited-state wave functions such as occurs in Fermi resonance. Rather, the observed behavior is characteristic of an "intensity borrowing" type of coupling whereby the ν(Fe–O_2) peak gains intensity simply owing to its proximity to the strongly enhanced ν_s(Fe–O–Fe) mode. Similar coupling has been observed in the RR spectra of O_2 adducts of cobalt porphyrins[26] in which axial base modes borrow intensity from the strongly enhanced O–O stretch. In the latter case the shift of the O–O vibration, through substitution of $^{18}O_2$, causes a marked decrease in the intensity of the axial base vibrational mode but little effect on its frequency. The enhancement of ν(Fe–O_2) in oxyhemerythrin by intensity borrowing from ν(Fe–O–Fe) also explains why ν(Fe–O_2) shows an excitation profile maximum in the near UV region while the ν(O–O) at 844 cm^{-1} does not.[13]

Rationale for Fe–O–Fe Frequencies. The majority of hemerythrin derivatives have ν_s(Fe–O–Fe) frequencies between 506 and 516 cm^{-1} (Table I). The slight variation in these frequencies does not appear to be related to properties of the exogenous ligand (e.g., size, ligand field strength, or π-donating ability) and probably stems from minor variations in Fe–O–Fe angle, which is known to affect Fe–O–Fe vibrational frequencies.[27] Since it is unlikely that a hydroperoxide or hydroxide ligand would cause a significant increase in the bridge angle, the 20–30-cm^{-1} shift to lower energy for the ν_s(Fe–O–Fe) vibrations of oxyhemerythrin and *cis*-hydroxomethemerythrin is more readily ascribed to hydrogen bonding. The only other protonated anions that are known to bind to methemerythrin are formate ($HCOO^-$) and cyanamide ($NCNH^-$), but these adducts show no evidence of hydrogen bonding to the oxo bridge (Table I). Whereas the nonpolar character of its proton precludes hydrogen bonding by formate, the proton of cyanamide is reasonably polar (pK_a = 11).[28] However, it appears that cyanamide is coordinated to the iron via its trigonal, protonated nitrogen, because ν(Fe–N) of cyanamidomethemerythrin shifts 5 cm^{-1} to lower energy upon deuteration of the ligand.[29] In this case the proton could be oriented away from the oxo bridge and, thereby, be unavailable for hydrogen bonding.

Due to the electron withdrawal caused by hydrogen bonds, a significant decrease in vibrational frequency is the expected behavior for a group when acting as a hydrogen-bond acceptor. This phenomenon has been observed in the spectra of α-hydroxy ketones where the C=O stretch at $\sim$1700 cm^{-1} in the non-hydrogen-bonded derivatives shifts 10–15 cm^{-1} to lower energy in compounds exhibiting intramolecular hydrogen bonding.[30] In horseradish peroxidase (HRP) compound II, a hydrogen bond between a distal histidine and the Fe^{IV}=O group has been suggested to be responsible for the $\sim$10-cm^{-1} reduction in the frequency of the Fe^{IV}=O vibration at pH values below 7.[31] In addition, the O–O stretch of O_2 complexes of cobalt porphyrins occurs at $\sim$1150 cm^{-1},[26] which is $\sim$20 cm^{-1} higher than the analogous vibrations of cobalt-substituted hemoglobin and myoglobin.[32] The lower vibrational frequencies in the proteins could be due in part to hydrogen bonding between the distal histidine and the bound dioxygen as observed in crystal structures of oxyhemoglobin and oxymyoglobin.[33] Similarly, the frequency reduction of ν_s(Fe–O–Fe) in oxyhemerythrin and *cis*-hydroxomethemerythrin is indicative of a weakening of the Fe–O bonds as is expected for an oxo group acting as a hydrogen-bond acceptor. The decrease in Fe–O bond strength is probably also responsible for the reduced antiferromagnetic coupling of oxyhemerythrin ($-J$ = 77 cm^{-1}) compared to methemerythrin ($-J$ = 134 cm^{-1}).[9]

Although there is some uncertainty as to whether H or D forms stronger hydrogen bonds,[34] there are numerous examples of the bond to hydrogen being stronger than the analogous bond to deuterium.[34-36] In the latter case, the vibrational frequency of the hydrogen-bond acceptor will actually undergo a smaller decrease in energy upon hydrogen bonding to a deuterium donor than to a hydrogen donor. The result is that the deuterated species will exhibit a higher vibrational frequency than the protonated species because there is less withdrawal of electron density in bonding to D than to H. Such effects have been observed in a number of the systems mentioned above. For example, the O–O stretch of the bound dioxygen in cobalt-substituted myoglobin and hemoglobin shifts 2–5 cm^{-1} to higher frequency in D_2O.[32] Similarly, the hydrogen-bonded Fe^{IV}=O stretch of HRP compound II shifts from 775 cm^{-1} in H_2O to 779 cm^{-1} in D_2O.[31] Also, the ν_{as}(C–O–C) of 2-methoxyethanol shifts from 1126 to 1129 cm^{-1} upon deuteration of the intramolecularly hydrogen-bonded OH group. The shift of the ν_s(Fe–O–Fe) of oxyhemerythrin from 486 cm^{-1} in H_2O to 490 cm^{-1} in D_2O is certainly in line with this

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behavior. Our previous observation of a 6-cm^{-1} increase in the ν_s(Fe–O–Fe) of ribonucleotide reductase in D_2O may also be due to hydrogen bonding of a protein functional group to the μ-oxo bridge.[21]

Rationale for Hydrogen-Bonded Structures. Although we have established that the oxo bridge acts as a hydrogen-bond acceptor in both oxy- and hydroxomethemerythrin, it is more difficult to definitively establish that the protonated ligands (Figures 1 and 2) are indeed the hydrogen-bond donors. In the case of oxyhemerythrin there is crystallographic evidence that the bound hydroperoxide is hydrogen bonded to the oxo bridge.[8] Furthermore, the shift in the O–O stretch of oxyhemerythrin from 844 cm^{-1} in H_2O to 848 cm^{-1} in D_2O is commensurate with a protonated peroxide.[13] Crystallographic data are not available for hydroxomethemerythrin, and the effects of deuterium substitution on ν(Fe–OH) are ambiguous. However, since hydrogen bonding is unique to oxy- and hydroxomethemerythrin, and since there are no other suitable bond donors in the vicinity of the ligand binding site of hemerythrin,[37] OH^- and OOH^- ligands are strongly implicated as the hydrogen-donating groups.

Crystallographic studies of ligand-free methemerythrin have revealed the presence of additional electron density near the oxo bridge, possibly from a nonligated water molecule.[38] However, this water molecule appears to be displaced upon perchlorate or exogenous ligand binding to methemerythrin as judged by both Raman[29] and crystallographic[37] results. It is, therefore, not likely to participate in the hydrogen bonding of oxyhemerythrin or hydroxomethemerythrin.

The strength of hydrogen bonds ($\Delta H°$) has been determined for many small molecules and has been found to vary depending on the type of hydrogen-bonding interaction.[39,40] Whereas $\Delta H°$ for most *inter*molecular hydrogen bonds ranges from −3 to −10 kcal/mol, $\Delta H°$ for *intra*molecular hydrogen bonds typically ranges from −1 to −4 kcal/mol and some have values as low as that for hydroxomethemerythrin ($\Delta H°$ = −0.4 kcal/mol). Examples of the latter are 2-nitroethanol ($\Delta H°$ = −0.40 kcal/mol), 2-fluoro-6-chlorophenol (−0.18 kcal/mol), ethanolamine (−0.70 kcal/mol), and 2-benzylphenol (−0.33 kcal/mol).[40] The bond energy determined for *cis*-hydroxomethemerythrin is thus commensurate with the existence of a very weak intramolecular hydrogen bond. This is similar to the situation in hemoglobin where the hydroxide ligand of hydroxomethemoglobin is hydrogen-bonded to the distal histidine.[41]

In oxyhemerythrin, only the cis conformation is observed. This indicates that the hydrogen bond in oxyhemerythrin is considerably stronger than in *cis*-hydroxomethemerythrin and is likely to be in the −1 to −4 kcal/mol range of the more typical intramolecular hydrogen bonds. For oxyhemerythrin, assuming reasonable angles and distances for the coordinate and covalent bonds,[42] we estimate a hydrogen-bond distance (μ-O···HOO) of 1.7 Å and a hydrogen-bond angle of 130°. The distance is that of a fairly strong hydrogen bond; the angle is within the range generally observed for intramolecular hydrogen bonds in proteins.[43] The bond angle for intramolecular hydrogen bonds in protein and small molecules can actually occur anywhere between 90 and 180° depending on the constraints of the covalent structure and, thus, is far less uniform than intermolecular hydrogen-bond angles, which tend to be closer to 180°.[43,30a] Since the cyclic hydrogen-bonded structure in *cis*-hydroxomethemerythrin contains only four atoms, it is certain to be a rather strained system. This is reflected in somewhat narrower covalent bond angles,[42] an estimated hydrogen-bond distance (μ-O···HO) of 2.3 Å, and a hydrogen-bond angle of 95°. These latter two values are close to the limits for hydrogen-bonded atoms in proteins[43] and, thus, are in agreement with the low enthalpy of the cis to trans interconversion in hydroxomethemerythrin.

The hydrogen bonding of the dioxygen ligand to the oxo bridge has some interesting implications for the chemistry of oxyhemerythrin. As previously proposed,[8,13] the structure in Figure 1 would be expected to facilitate the rapid conversion of deoxyhemerythrin to oxyhemerythrin, with the peroxide proton originating from the hydroxide bridge of deoxyhemerythrin. This model is consistent with the absence of a pH effect on the rate of oxygenation.[44] In contrast, the displacement of peroxide by other anions is acid-catalyzed,[45] reflecting the need for further protonation in achieving the release of H_2O_2. The fact that the release of peroxide from oxyhemerythrin (autooxidation) is slow[45] indicates that hydrogen bonding to the oxo bridge may exert a stabilizing influence on the peroxide complex. The copper-containing respiratory protein oxyhemocyanin is even more resistant to autooxidation because both ends of the peroxide are metal coordinated in the structure Cu–O–O–Cu.[46]

In addition to being stable toward autooxidation, the peroxide complex in oxyhemerythrin also does not produce intermediate redox states. This is in marked contrast to mononuclear complexes such as the peroxide adduct of Fe^{III}EDTA, which undergoes rapid decomposition due to the formation of Fe(II) and superoxide.[47] Presumably, a binuclear metal center can be of considerable help in this regard. A species that is more reminiscent of oxyhemerythrin in its stability and structure is a cyanide-bridged binuclear cobalt complex, Co^{III}–CN–Co^{III}–O_2^{2-}, with a side-on bonded peroxide.[48] As in oxyhemerythrin, this peroxide also results from an oxidative addition, utilizing the two-electron-transfer potential of the binuclear metal site. Side-on binding of peroxide as in the cobalt compound is the general rule in metal complexes,[49] and this also appears to be the case in the Fe^{III}EDTA peroxo complex.[50] Thus, the end-on binding observed in oxyhemerythrin is clearly facilitated by the hydrogen-bonding capability of the Fe–O–Fe site. In an analogous fashion, hydrogen bonding to the proton of a distal donor is believed to be an important factor in stabilizing the dioxygen complexes of hemoglobin and myoglobin[33] and of model systems containing iron and cobalt porphyrins.[51]

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Registry No. ^{18}O, 14797-71-8; D, 7782-39-0; OH^-, 14280-30-9; OOH^-, 14915-07-2; N_3^-, 14343-69-2; SCN^-, 302-04-5; CN^-, 57-12-5; OCN^-, 661-20-1; Cl^-, 16887-00-6; $HNCN^-$, 67131-47-9; $HCOO^-$, 71-47-6.

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