X-ray absorption spectroscopy of the dimeric iron site in azidomethemerythrin from *Phascolopsis gouldii*

(extended x-ray absorption fine structure/absorption edge/hemerythrin/iron protein)

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ABSTRACT X-ray absorption spectroscopy has been used to study the dimeric iron center in azidomethemerythrin from *Phascolopsis gouldii*. Absorption edge data confirm that the two iron atoms are present as Fe(III) and suggest a hexa-coordination site for each of the iron atoms. The extended x-ray absorption fine structure (EXAFS) analysis provides direct structural evidence of a μ -oxo bridge between the two iron atoms at an average Fe—O distance of 1.71–1.76 Å. Analysis using a multiple-scattering formalism calculates upper limits of 165° for the Fe—O—Fe bridging angle and 3.38 Å for the Fe—Fe distance. This result agrees with current crystallographic models being determined by refinement of structures of two azidomethemerythrins.

Hemerythrin is an oxygen-carrying protein that occurs in certain invertebrate animals. Unlike hemoglobin, its mammalian counterpart, this blood protein does not have heme as a prosthetic group. Rather, the site of reversible oxygen binding in hemerythrin is a dimeric iron center coordinated directly by side chains from the protein. Hemerythrin subunits have molecular weights of about 13,500 and each subunit has an active center of two iron atoms that bind one oxygen molecule.

Hemerythrin has been studied by a variety of techniques (1–8) in attempts at elucidating the structure of the active center and understanding the mechanism of reversible oxygenation. Mössbauer, magnetic susceptibility, CD, and optical electronic spectroscopy and x-ray crystallography have provided much detail about the iron environment. In particular, the two irons are antiferromagnetically coupled in oxy- and methemerythrins (7). Crystallographic studies show the coordinating groups to be histidine and carboxylate. Resonance Raman spectroscopy has shown that oxygen is bound as the peroxide (1) and has suggested the presence of OH[−], H₂O, or a μ -oxo bridge in oxy- and methemerythrin (5).

Three crystal structures of azidomethemerythrins have been determined at the Naval Research Laboratory: monomeric myohemerythrin from *Themiste zostericola* at 5.5-Å resolution (9) with model building and partial refinement to 2.5-Å resolution (3), low-resolution structures of octameric hemerythrin from *Phascolopsis gouldii* (10), and trimeric hemerythrin from *Siphonosoma funafuti* (unpublished results). The structures of the hydroxylmet and azidomet forms of the octameric hemerythrin from *T. dyscrita* have been determined and refined to 2.2-Å resolution by Stenkamp *et al.* (11, 12) at the University of Washington. Initially, there were substantial disagreements between the two models concerning the Fe—Fe distance and the presence of a μ -oxo bridge. More recently, through a series of revisions and refinements at higher resolution, a common model has emerged for the azidomethemerythrin active center. It consists of two hexa-coordinate μ -oxo-bridged iron atoms li-

gated to the protein by five histidyl residues and two bidentate carboxylate bridges.

These structures have greatly enhanced our understanding of the hemerythrin active center. Much desired detail is, however, still missing. Electronic information such as the oxidation state is not directly accessible from the crystal structure. Definitive evidence for the presence of a μ -oxo bridge is important, as is verification of the Fe—Fe distance. In addition, structures of the active oxy and deoxy forms (as well as of other met states) have yet to be determined crystallographically. X-ray absorption spectroscopy (XAS) has proven useful for investigation of the metal active center in metalloproteins and metalloenzymes (13–15). We have used XAS to examine five derivatives (including the oxy and deoxy) of hemerythrin from *P. gouldii* and report here the result of our study on the most stable state of the protein, azidomethemerythrin. First, we examine the iron K-absorption edge for information about oxidation state and coordination geometry. Then, we analyze the extended x-ray absorption fine structure (EXAFS) with an emphasis on verifying the presence of a μ -oxo bridge and determining the Fe—Fe separation.

MATERIALS AND METHODS

Hemerythrin Preparation. *P. gouldii* sipunculan worms were purchased from the Marine Biological Laboratory (Woods Hole, MA), and the coelomic fluid from 100 animals was pooled. Hemerythrin was purified as described by Klotz *et al.* (16) with modifications as in Subramanian *et al.* (17) and omitting the crystallization step. Aquomethemerythrin was produced by treating the purified oxyhemerythrin with K₃Fe(CN)₆ at 2.2 mol/mol of protein subunit. Azidomethemerythrin was formed by dialysis against 5 mM NaN₃. Samples were prepared for XAS by pressure concentration to 17 mM in hemerythrin subunits.

Data Collection. X-ray absorption data for azidomethemerythrin were collected by using synchrotron radiation at the Stanford Synchrotron Radiation Laboratory on beam line IV-2 using a Si[220] two-crystal monochromator. An EXAFS cell (40 × 4 × 1 mm) with Mylar windows was filled with protein solution. X-ray absorption data were collected at room temperature as fluorescence excitation spectra, using an array of 20 NaI(Tl) scintillation detectors. The energy region of the iron K-absorption edge (6,800–8,300 eV) was scanned 20 times at about 30 min per scan. At the end of the data-collection period, the sample retained its characteristic orange color. Electronic absorption spectra taken before and after data collection ensured that the integrity of the protein sample was maintained during data acquisition.

Data Analysis. Data-reduction procedures have been de-

scribed in detail (13, 18). Energy was calibrated with respect to an iron foil by assigning 7,111.2 eV to the first inflection on the absorption edge. E_0 is defined to be 7,130 eV. A polynomial was fit to the pre-edge region and subtracted from the data. The relative modulation of the absorption coefficient above the edge was fit with a smooth spline that was subsequently subtracted from it and normalized by a theoretically calculated μ_0 , as would be observed for the free atom, to obtain the final EXAFS spectrum given by

$$\chi(k) = \frac{\mu - \mu_s}{\mu_0} = \frac{1}{k} \sum_s \frac{|f_s(\pi, k)|}{R_{as}^2} \sin[2kR_{as} + \alpha_{as}(k)] e^{-\sigma_{as}^2 k^2} e^{-2\lambda R_{as}}, \quad [1]$$

where the symbols used are identical to those defined in ref. 13. For data analysis, the observed EXAFS was modeled by a sum of sine waves contributed by each absorber-scatterer pair such that

$$\chi(k) \approx \sum \frac{c_0 e^{-c_1 k^2}}{k^{c_2}} \sin(a_0 + a_1 k + a_2 k^2). \quad [2]$$

The structure-dependent parameters c_0 and a_1 were optimized in a least-squares fit of Eq. 2 to the observed EXAFS to provide information about number of scatterers and absorber-scatterer distances. The structure-independent parameters for light atoms were obtained from fits to the EXAFS of a series of iron model compounds of known structure (19). Parameters for Fe were derived from a set of oxo-bridged models. Fourier filtering was used to isolate a particular shell of scatterers for fitting to the observed EXAFS. The EXAFS spectrum from $k = 4$ to $k = 16 \text{ \AA}^{-1}$ was Fourier transformed and filtered through a square window to isolate a peak of interest. The isolated peak was back-transformed into k space, where models of the selected shell of scatterers were fit to the EXAFS as described above.

EXAFS analysis becomes complicated beyond the first coordination shell because of interference from atoms in the absorber-to-scatterer path. The intervening atom effects will be described in detail elsewhere (20) and are only briefly outlined here. A series of oxygen-bridged dimeric iron model compounds of known structure (including dihydroxo- and dialkoxo-bridged dimers and μ -oxo-bridged dimers and trimers) were examined by EXAFS. In the analyses of outer-shell iron backscatterers, when the Fe—O—Fe angle deviates less than about 30° from linearity, the backscattering wave is dominated by the multiple-scattering pathway through which the photoelectron is scattered by the intervening oxygen atom twice, once on its way from the absorbing iron to the scattering iron and once on its way back (i.e., the pathway along Fe—O—Fe bonds). The results indicate that an intervening atom in the absorber-to-scatterer path amplifies the backscattering wave and also causes additional phase shifts as a result of scattering. The magnitude of this effect varies with the bridging angle due to the angular dependence of forward scattering by the intervening atom. Angle determination by EXAFS analysis has recently been suggested by Teo (21) to be possible by using a multiple-scattering formalism. For determination of the bridging angle and the iron-iron distance in an Fe—O—Fe system, the Fourier-filtered iron backscattering EXAFS is least-squares fit to Eq. 2 to evaluate the backscattering amplitude, which is related to the bridging angle. The calculation of iron-iron distance is less straightforward. As determined in the study of model compounds, a linear phase shift of $0.21 \text{ \AA}$ (caused by the photoelectron twice entering the potential of the bridging oxygen atom) should be added to the fitting result. This gives the length of the multiple-scattering pathway—i.e., the Fe—O—Fe distance. The direct iron-iron distance can then be calculated.

RESULTS

Absorption Edge Structure. Absorption edge position and edge structure can be used as indicators of the oxidation state and coordination geometry of the x-ray absorbing atom. By simple electrostatics, more energy is required to remove a core electron as the positive charge on the iron is increased. Thus, the absolute position of the continuum edge is observed to increase by about 3 eV for each successive one-electron oxidation (22). Indeed, studies on a series of iron complexes indicate that the inflection on the edge occurs near 7,121 eV for Fe(II) and near 7,124 eV for Fe(III) (unpublished results).

The pre-edge structure in the Fe(III) models particularly reflects coordination site symmetry. The transition at *ca.* 7,114 eV is far more intense for penta-coordinate models such as in Fig. 1, curve b, than it is for hexa-coordinate models such as in curves c and d. The *N*-(2-hydroxyethylenediamine)triacetate-containing structure, in which the coordination angles are distorted by as much as 15° from octahedral symmetry, has the most intense pre-edge transition among the hexa-coordinate models.

Examination of the absorption edge position of azidomethemerythrin (Fig. 1, curve a) shows an inflection point at 7,124 eV, consistent with the iron atoms being in the +3 oxidation state in this protein. The pre-edge structure shows only a weak 7,114 eV transition, very similar to those shown by the hexa-coordinate compounds. This evidence suggests an approximately centrosymmetric configuration (for example, octahedral coordination) at each iron atom in the active center. This is consistent with the assignments made from a recent near-IR spectroscopic study of the protein (6).

Direct Evidence for a μ -oxo Bridge. The hemerythrin Fe EXAFS spectrum is an average spectrum of two absorbing iron atoms. As a consequence, the results reported here give average values per absorbing atom. The x-ray crystallographic work de-

FIG. 1. Fe K-absorption edges for azidomethemerythrin from *P. gouldii* (curve a), $[\text{Fe}(\text{salen})]_2\text{O}$ (curve b), $[\text{Fe}(\text{HEDTA})]_2\text{O}$ (curve c), and $[\text{Fe}(\text{dipic})(\text{H}_2\text{O})]_2$ (curve d), where salen = *N,N'*-ethylenedibis(salicylideneimine), HEDTA = *N*-(2-hydroxyethylenediamine)triacetate, and dipic = 2,6-pyridinedicarboxylate. The relative weaknesses of the pre-edge transition at 7,114 eV and the absorption edge position at 7,124 eV suggest that both iron atoms are hexa-coordinate Fe(III).

scribed above has shown that the coordinated ligands for both iron atoms are rather similar. Here, we describe our analysis of the EXAFS data to obtain structural information about the first coordination shell of the absorbing iron atoms.

The Fourier transform from $k = 4$ to $k = 16 \text{ \AA}^{-1}$ of the Fe EXAFS of azidomethemerythrin is shown in Fig. 2. Several distinct features are apparent. The first major peak is at 1.6 $\text{\AA}$, uncorrected for phase shifts in the transform. A second large peak occurs near 3 $\text{\AA}$. This suggests the presence of rigidly held high-Z backscatterers and will be discussed in more detail below. (The low- R peak near 0.8 $\text{\AA}$, which is also observed in model complexes, is ascribed to an artifact arising from residual background. It is too near-in to represent an interatomic distance, but it is sensitive to variation in background parameters.) The transform peak at 1.6 $\text{\AA}$ is analyzed for structural information about the first coordination shell of the iron atoms.

The EXAFS spectrum was filtered with a square window from 1.1 $\text{\AA}$ to 2.1 $\text{\AA}$ about the peak at 1.6 $\text{\AA}$ (marked by the bars in Fig. 2). One-, two-, and three-wave fits for oxygen and nitrogen ligands starting at several values for scattering amplitude(s) and distance(s) were calculated; the results of some of these fits are shown in Table 1. The quality of each model is judged by the minimization function value and by its chemical feasibility. Single-wave Fe—O and Fe—N fits result in high function values and negative or unreasonably low coordination numbers. Two-wave fits with both Fe—O and Fe—N waves offer little improvement. Addition of a short Fe—O' distance to the single Fe—O(N) distances significantly improves the fits. The best fit is obtained when Fe—O, Fe—N, and Fe—O' waves are all included. The final Fe—(O,N,O') distances in our analysis of azidomethemerythrin are 2.13 $\text{\AA}$, 2.08 $\text{\AA}$, and 1.76 $\text{\AA}$, respectively, with corresponding coordination numbers of 2.1, 2.5, and 1.0. The Fe—N and Fe—O distances of 2.08 $\text{\AA}$ and 2.13 $\text{\AA}$ should be considered as not significantly resolved because of possible correlations among shells of atoms at similar distances. This three-wave fit, however, gives the most reasonable total coordination number for the iron absorber. The absolute necessity of a short Fe—O' distance for an acceptable fit to our experimental data is direct evidence for a μ -oxo bridge between the iron atoms of azidomethemerythrin.

The sensitivity of the fitting procedure to addition of a short Fe—O bond is illustrated by results with the μ -oxo-bridged tetraphenylporphyrin, $[\text{Fe}(\text{TPP})_2\text{O}]$, where the Fe—O distance is known to be 1.76 $\text{\AA}$ (22). A similar analysis is performed for

FIG. 2. Fourier transform of Fe EXAFS for azidomethemerythrin from *P. gouldii*. The transform is taken for $k = 4$ – $16 \text{ \AA}^{-1}$, with k^3 weighting. The large peak at 3.0 $\text{\AA}$ has been analyzed and found to be an Fe backscattering peak.

Table 1. Results from one-, two-, and three-wave fits to the Fourier-filtered EXAFS for the first coordination shell peak of azidomethemerythrin from *P. gouldii*

	Function value*	Fe—O		Fe—N		Fe—O'	
		R, $\text{\AA}$	No.	R, $\text{\AA}$	No.	R, $\text{\AA}$	No.
1	1.121	1.73	0.6				
2	0.743	1.89	−1.8				
3	0.596	2.08	2.4				
4	1.011	2.28	−1.8				
5	1.062			1.79	1.0		
6	0.644			1.95	−2.2		
7	0.706			2.13	2.5		
8	1.065			2.34	−1.6		
9	0.563	1.87	2.8	1.94	−5.0		
10	0.454	2.06	1.5	1.96	−1.2		
11	0.585	2.07	2.6	2.28	0.4		
12	0.421	2.27	−1.4	1.95	−2.0		
13	0.402	2.07	2.6			1.76	0.8
14	0.549			2.13	2.6	1.75	0.8
15	0.375	2.13	2.1	2.08	2.5	1.76	1.0

* Fits were done by minimizing the difference between the observed and calculated EXAFS at each point in k space, squared, and then weighted by k^6 . The minimization function value F is given by $F^2 = \sum [k^6(\text{data} - \text{fit})^2]/N$.

the first major Fourier-filtered peak from the EXAFS spectrum. Curve-fitting results indicate that (i) a one-wave fit yields 3.5 nitrogen atoms at 2.07 $\text{\AA}$, the function value being 0.736, and (ii) a two-wave fit yields 4.0 nitrogen atoms at 2.07 $\text{\AA}$ and 1.0 oxygen atom at 1.75 $\text{\AA}$, with the function value reduced to 0.518.

The three-wave fit to the major transform peak of azidomethemerythrin derives a total of 5.6 atoms coordinated at each iron atom. Because there is an appreciable uncertainty in predicting coordination numbers, this result could be consistent with five or six ligands at either or both of the iron atoms. However, the relatively weak pre-edge feature for azidomethemerythrin (indicating a local center of symmetry at each iron atom) coupled with the EXAFS coordination number of 5.6 strongly suggests that both iron atoms are hexa-coordinate.

Iron-Iron Distance and Bridging Angle Determinations. The large peak near 3 $\text{\AA}$ in the Fourier-transformed EXAFS spectrum of azidomethemerythrin (Fig. 2) suggests the presence of a rigidly held heavy scatterer beyond the first coordination shell. The 3- $\text{\AA}$ peak in the transform was filtered through a square window from 2.5 $\text{\AA}$ to 3.4 $\text{\AA}$ and back-transformed into k space for analysis. The resulting Fourier-filtered EXAFS spectrum is a sine curve with a maximum near $9 \text{ \AA}^{-1}$ (Fig. 3, curve b). Least-squares fits to this curve were done with parameters for various atom types (C, N, O, S, and Fe); only iron parameters gave a satisfactory fit. The filtered EXAFS from the peaks at 3.5 $\text{\AA}$ and 3.9 $\text{\AA}$ in the Fourier transform does not show this backscattering envelope characteristic of iron. Because of differential falloff in scattering amplitude, the use of data past $k = 12 \text{ \AA}^{-1}$ was important in identifying and isolating a heavy backscatterer at a large distance [scattering amplitudes at $k = 4 \text{ \AA}^{-1}$ are 0.36 for C and 0.43 for Fe and at $k = 13 \text{ \AA}^{-1}$ are 0.03 for C and 0.25 for Fe (24)].

Structural results from the least-squares fit to the isolated iron-iron EXAFS show an apparent number of 2.0 iron atoms at 3.20 $\text{\AA}$ from each absorber. However, there is only one iron backscatterer for each absorbing iron atom in the hemerythrin subunit. The high apparent scattering amplitude, together with our evidence for a μ -oxo bridge from the first-shell fit, indicates that the oxide ion intervenes in the iron-iron scattering path. From a polynomial fit to our results from the series of μ -oxo-bridged dimers, an apparent number of 2.0 is found to correspond to a bridging angle of 165° . Thus, the pathway through

FIG. 3. (a) EXAFS spectrum (—) obtained by Fourier filtering the major frequency components from 1.1 Å to 3.4 Å in the transform in Fig. 2; (·····), a six-wave fit with the results summarized in Table 2. (b) Fourier-filtered spectrum (—) of the 3.0 Å backscattering peak in the transform in Fig. 2; (·····), fit. Each filtered back-transform was first deconvoluted to yield $\chi(k)$ for analysis and is then displayed here as $k^2 \cdot \chi(k)$.

the intervening atom is expected to dominate and the increment of 0.21 Å determined from iron-dimer models must be added to the curve-fitting results of 3.20 Å to account for phase shifts caused by scattering at the intervening oxygen atom. This yields a pathlength of 3.41 Å or an average Fe—O distance of 1.71 Å. Simple geometric calculation then gives a direct Fe—Fe distance of 3.38 Å.

The estimation of errors for bonding parameters in the Fe—O—Fe system of hemerythrin is complicated by the multiple-scattering analysis. The standard error for each factor in the analysis is estimated from distributions of agreement between EXAFS values and crystal-structure parameters for model compounds. These component errors are then propagated through the analysis to yield estimated SDs for final parameters. The error in curve-fitted Fe—Fe distance (0.03 Å) is taken by analogy with the distributions for a series of copper models and that for the scatterer number is taken as 20% (13). The error associated with the scatterer increment (0.02 Å) and those for the polynomial coefficients come from the dimeric iron models. After propagation, these lead to estimated SDs of 0.02 Å for the Fe—O distance, 0.05 Å for the Fe—Fe distance, and 8° for the Fe—O—Fe bridging angle. Unaccounted-for systematic error may of course exist.

Analysis of the Iron Site by Multiple-Shell Fitting. To obtain more structural detail about the iron site, all the major frequency components in the EXAFS spectrum were examined. A fairly wide square window (from 1.1 to 3.4 Å) was used to filter the Fourier-transformed spectrum, which was then back-transformed into k space for least-squares fitting analysis. This process neglects the contribution of scatterers more than about 3.7

Å from the absorbing iron atom and filters out the residual background subtraction and instrumental noise. The Fourier-filtered EXAFS spectrum is shown in Fig. 3 (curve a). Multiple-shell fits made to this spectrum are summarized in Table 2.

A model that includes only first-coordination-shell atoms follows the general profile of this spectrum but fails to fit any of the beats. Inclusion of an iron shell in this model greatly improves the function value (from 0.805 to 0.484) and reproduces the series of beats at high k as expected, since iron should be the only atom with appreciable scattering ability at high k . However, the saddle near $k = 7.5 \text{ Å}^{-1}$ is still poorly modeled. Further inclusion in the model of carbon atoms beyond the first coordination shell greatly improves the fit. The best fit is obtained by adding two shells of carbon atoms at 2.89 Å and 3.28 Å and is shown superimposed over the EXAFS spectrum in Fig. 3 (curve a). All the beats in the spectrum are matched, including the saddle at $k = 7.5 \text{ Å}^{-1}$. These carbon shells can reasonably be interpreted as second-shell distances for carboxylate and imidazole groups. The crystal structure of bis(L-histidinato)nickel(II) monohydrate (25) has both imidazole and carboxylate ligands with Ni—O (2.12 Å) and Ni—N (2.09 Å) average bond lengths similar to those observed here for azidomethemerythrin (Fe—O = 2.13 Å, Fe—N = 2.07 Å). The second-shell Ni—C distances are 3.07 Å and 3.12 Å to imidazole and 2.84 Å to carboxylate, very close to values calculated here from fits to the azidomethemerythrin EXAFS spectrum. Both the EXAFS-derived backscatterer numbers and distances for the second shell of carbon atoms might be systematically in error because multiple scattering effects by the first-shell atoms and the inherently larger static disorder are not taken into account in the analysis.

DISCUSSION

These results provide precise parameters for a model of the dimeric iron site. The average iron atom is coordinated to an oxygen atom at a distance of 1.71–1.76 Å, as well as to two or three nitrogen atoms at 2.07 Å and to two or three oxygen atoms at 2.13 Å. The short-distance oxygen is typical of a μ -oxo bridge between two iron atoms, which are separated by 3.38 ± 0.05 Å with a bridging angle of $165 \pm 8^\circ$. These results are in good agreement with the crystallographic model refined by Stenkamp *et al.* (12) and with our recent interpretation of a map of azidomethemerythrin based on anomalous-scattering phases (unpublished results). The nitrogen atoms can be identified with histidines-54 and -25 and the coordinated N_3^- at one of the iron atoms and with histidines-77, -73, and -101 at the other. The resultant Fe—N coordination number of less than 3 could be due to scatter in the distribution of bond distances, which tends to decrease the EXAFS amplitude. The oxygen atoms are consistent with carboxylate oxygens from glutamate-58 and aspartate-106 bridging the two iron atoms.

A μ -oxo bridge has been suggested to account for the antiferromagnetic coupling in oxy- and met-hemerythrin. A recent resonance Raman study has assigned a Raman band near 510

Table 2. Results of multiwave fits to EXAFS of azidomethemerythrin from *P. gouldii*

<i>F</i>	Fe—O'		Fe—O		Fe—N		Fe—Fe		Fe—C		Fe—C'	
	<i>R</i> , Å	No.	<i>R</i> , Å	No.	<i>R</i> , Å	No.	<i>R</i> , Å	No.	<i>R</i> , Å	No.	<i>R</i> , Å	No.
0.805	1.76	1.0	2.11	2.3	2.06	1.9						
0.484	1.76	1.0	2.12	2.2	2.07	2.1	3.21	2.4				
0.453	1.76	1.0	2.12	2.2	2.07	2.0	3.21	2.4	2.89	1.5		
0.430	1.76	1.0	2.13	2.2	2.07	2.4	3.20	2.0	3.28	3.6		
0.403	1.76	1.0	2.13	2.2	2.07	2.3	3.20	2.0	2.89	1.3	3.28	3.3

EXAFS fitting range, $k = 4\text{--}16 \text{ Å}^{-1}$. Errors are estimated to be about ± 0.03 Å for distances and about 20% for coordination numbers. Each fit has two variable parameters per wave. Thus, the six-wave fit has 12 variables. Such necessarily elaborate models are justified by the extensive data range and meaningful improvement in the goodness-of-fit.

cm^{-1} to an Fe—O stretching vibration assigned to OH^- , H_2O , or a μ -oxo bridge between the iron atoms but cannot distinguish among them (5). It is also impossible to determine by crystallography whether a single oxygen atom, a hydroxide ion, or a water molecule is bridging the iron atoms. Since Fe—O distances for oxo-bridged iron complexes fall between 1.76 Å and 1.82 Å (26) and hydroxo-bridged iron complexes have a typical Fe—O bond length of 1.9–2.0 Å (27) (H_2O is usually coordinated at even longer distances), the unambiguous criterion for determination of the nature of the bridging oxygen in hemerythrin is the bond length.

EXAFS analysis of the first coordination shell clearly identifies one oxygen atom at a distance of 1.76 Å. A distance of 1.71 Å is derived indirectly from the multiple-scattering analysis. These values differ at the 2σ level and at present we cannot fully reconcile the difference. The first-shell fit includes three waves and correlations may produce larger than usual errors. The distance of 1.71 Å from the Fe—O—Fe pathlength must be taken with caution because the multiple-scattering phenomenon is not completely understood. Also, an Fe—O distance this short is without precedent. Nevertheless, clearly the distance is shorter than 1.80 Å and thus the bridge must be of the μ -oxo type.

This work has also determined the Fe—Fe distance and the Fe—O—Fe bridging angle. Initially, crystallographic refinement of the iron positions against anomalous scattering data from azidomethemerythrin gave a distance of 3.44 ± 0.05 Å. A later refinement after improved scaling of the data gave a lower residual and a recalculated distance of 3.34 ± 0.07 Å. Structural refinement of hydroxomethemerythrin from *T. dyscrita* at 2.5-Å resolution gave an average Fe—Fe distance of 3.05 Å (4). The Fe—Fe distance for the refined structure of azidomethemerythrin from *T. dyscrita* is currently 3.26 Å (R. E. Stenkamp, personal communication). The limited extent of the diffraction data will make it difficult to extract accurate metrical details, such as the Fe—O—Fe bond lengths and bridging angle, from these crystal structures. The EXAFS work thus provides detail to complement the information obtained from the crystallographic studies. The Fe—Fe distance of 3.38 Å and the Fe—O—Fe bridging angle of 165° are probably upper limits for these values. The bridging angle is determined from the backscattering amplitude of the iron backscatterer, derived from a study of a series of iron complexes having a μ -oxo atom as the only bridge between the two iron atoms. In azidomethemerythrin, the fact that the iron atoms are further bridged by two carboxylate groups could increase the number of multiple-scattering pathways, amplify the backscattering amplitude, and thus lead to overestimation of the bridging angle and the Fe—Fe distance. However, this overestimation should not cause significant errors in the analysis because such multiple-scattering processes will occur at larger scattering angles (smaller bridging angles), where scattering will be relatively ineffective compared with the multiple-scattering path through the μ -oxo atom with a small scattering angle (large bridging angle). For example, assuming that multiple-scattering pathways other than the one through the μ -oxo atom account for 20% of the observed amplitude, the analysis gives an Fe—O—Fe bridging angle of 155° and an Fe—Fe distance of 3.34 Å.

Finally, although this multiple-scattering analysis of hemerythrin EXAFS has been made in reference to the series of model compounds, it must be noted that none of these is really an adequate model for hemerythrin EXAFS. Each of the eight μ -oxo-bridged dimers gives an uncorrected Fe—Fe distance of 3.34 ± 0.02 Å, as compared with known Fe—O—Fe pathlengths of 3.55 ± 0.02 Å. By contrast, the uncorrected distance in hemerythrin is 3.20 Å but the large scattering amplitude clearly distinguishes it from the doubly bridged compounds.

Moreover, the first-shell fitting requires the short Fe—O distance as for the μ -oxo bridged models.

In summary, this EXAFS study has provided a detailed picture of the iron site of azidomethemerythrin to complement the information already available from crystallographic work. Direct structural evidence for the presence of a μ -oxo bridge between the two iron atoms is observed. Our best estimate using multiple-scattering analysis predicts an Fe—O—Fe angle of 165° and an Fe—Fe distance of 3.38 Å for azidomethemerythrin. The active center of the two native forms of hemerythrin (oxy and deoxy) is not yet known.

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