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A Magnetic Susceptibility Study of Hemerythrin Using an Ultrasensitive Magnetometer*

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ABSTRACT: The magnetic susceptibilities of oxy- and metaquohemerythrin in the range 3–200°K have been determined using an ultrasensitive superconducting quantum magnetometer, and for the first time their antiferromagnetic components have been conclusively resolved. The exchange coupling constants, J, between the two high-spin iron(III) atoms in each subunit are -77 and -134 cm^{-1}, respectively. The electronic spectra of these two proteins in deuterium oxide have been measured from 800 to 1200 nm but no additional bands were obtained. After comparison to magnetic and spectroscopic data obtained previously for ferric dimer model complexes, it is concluded that the two iron(III) atoms in subunits of both oxy- and metaquohemerythrin are oxo bridged.

Hemerythrin is the oxygen-carrying nonheme iron protein found in the invertebrate phyla: sipunculids, brachiopods, polychaetes, and priapulids. The nature of the iron in the oxy, deoxy, and met (oxidized) forms of this protein from the sipunculid *Golfingia gouldii* has been studied by physical methods such as Mössbauer spectroscopy (Okamura *et al.*, 1969; York and Bearden, 1970; Garbett *et al.*, 1971), electronic spectroscopy (Garbett *et al.*, 1969; Gray, 1971) and magnetic susceptibility determinations (G. Gunther, 1969, personal communication; S. Simon and G. R. Rossman, 1969, unpublished data; Okamura *et al.*, 1969; York and Bearden, 1970; Moss *et al.*, 1971; Gray, 1971).

The hemerythrin (mol wt 108,000) from *G. gouldii* consists of eight subunits, each of which contains two iron atoms capable of binding one oxygen molecule (Klotz and Keresztes-Nagy, 1963). As the molecular weight of each subunit is 13,500, conventional susceptibility determinations (Earnshaw, 1968; Figgis and Lewis, 1965) are of little value in determining the magnitude of any possible magnetic interactions between the two iron atoms. Even a superconducting

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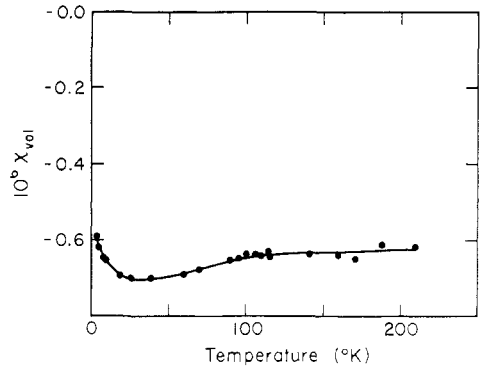


FIGURE 1: The temperature dependence of the total diamagnetism of oxyhemerythrin, measured as a 20% ethanol suspension (160 μl containing 460 μg of iron) at 47 G.

coil vibrating sample magnetometer could not resolve changes in susceptibility with temperature for oxy- and several methemerythrins (Moss *et al.*, 1971). Consequently, Mössbauer spectroscopy has been a more valuable technique for confirming the presence (but not the magnitude) of magnetic interaction between the iron atoms in oxy- and methemerythrin (Okamura *et al.*, 1969; Garbett *et al.*, 1971).

In this paper we use a new ultrasensitive magnetometer to examine the temperature variation of the magnetic susceptibility of oxy- and metaquohemerythrin. As a result we can discuss in more detail the relationship between the two iron atoms in each hemerythrin subunit. In addition, we have extended the range of the electronic spectra of these two proteins out to 1200 nm by using deuterium oxide solutions.

Experimental Section

Preparation of Hemerythrin. Crystalline oxyhemerythrin was isolated from the coelomic fluid of the marine worm *G. gouldii* as described previously (Klotz *et al.*, 1957). Metaquohemerythrin was prepared by the method of Garbett *et al.* (1969), and crystallized from 20% aqueous ethanol.

Magnetic Susceptibility Measurements. The magnetic data were obtained with a magnetometer system which employs a new principle of high-sensitivity measurement based upon quantum superconductivity. A full description of this system will be published elsewhere (Hoenig *et al.*, 1972). For the study of the hemerythrins, a sample "infinitely" long (2.5 cm) relative to the two pickup coils of the superconducting dc flux transformer is placed in a weak axial magnetic field, provided by the superconducting solenoid. The two pickup coils are so constructed that they automatically compensate for the effect of the sample holder, which extends through both coils.

Two types of susceptibility measurements were performed. One involves the determination of the total volume susceptibility by moving the sample from one pickup coil into the other. A second and more sensitive mode of operation is used to determine changes in susceptibility as a function of temperature by placing the sample in one of the pickup coils.

The induced current in a superconducting sensor due to the sample-related flux is used to drive a feedback loop so that the compensation current depends linearly on the flux change arising either from sample movement or from change of sample temperature. In the second mode of operation, the sensitivity to changes in susceptibility is independent of the large-background diamagnetic susceptibility. This mode can be used up to about 120°K; at higher temperatures the total susceptibility must be determined.

The magnetic susceptibility runs were performed in two parts. First, a series of total susceptibility determinations were made over the range 3.2–200°K. Then, in the case of oxyhemerythrin, a series of relative susceptibility runs were made. A sizable paramagnetic component shows up at low temperatures, probably due to the presence of impurities or decomposition products (0.91 μg of high-spin Fe(III) in the sample of oxyhemerythrin could produce the observed paramagnetic rise below 20°K). This is subtracted out of the data assuming the Curie law behavior $\chi = C/T$. The resulting component, presumably the antiferromagnetic contribution of the hemerythrin sample, is then analyzed by isolating the temperature-dependent contribution of the iron from the assumed temperature-independent contribution of the remaining protein and solvent. This is accomplished by assigning the most diamagnetic susceptibility in the Curie-law impurity corrected data to 0.0 (at 18.5°K in oxyhemerythrin and 21°K in the methemerythrin) and presenting all other data as paramagnetism relative to this zero.

The samples used for the magnetic measurements were crystalline oxy- and metaquohemerythrin in the form of slurries in 20% aqueous ethanol centrifuged into a synthetic SiO_2 tube which was sealed off under helium gas with epoxy resin. The oxyhemerythrin sample had a volume of 160 μl, contained a total of 460 μg of iron, and was run at 47-G field strength. The metaquohemerythrin had a volume of 122 μl, contained 356 μg of Fe, and was run at 18.7-G field strength. All the sample manipulation was carried out in such a manner that the sample temperature never exceeded 5° at any time before the wet chemical analysis at the conclusion of the susceptibility determinations.

Electronic spectra of oxy- and metaquohemerythrin were measured on a Cary 14RI spectrophotometer, using 1-cm cells cooled to 5° in a quartz dewar. In order to extend the range of measurement to 1200 nm, D_2O solutions of hemerythrin were studied. The hemerythrins were recrystallized several times using a 20% C_2H_5OD–D_2O mixture at 4°; the C_2H_5OD was prepared from sodium ethoxide and D_2O. The hemerythrins were examined in 0.1 M deuterated Tris-cacodylate buffer (pD 7.0). Deuterated Tris and cacodylic acid were prepared by evaporating a D_2O solution of each compound to dryness, and repeating this procedure twice.

Iron Analysis. The iron contents of the samples used for susceptibility work were determined spectrophotometrically by the *o*-phenanthroline method, after they had been digested with perchloric acid. The concentrations of iron in the solutions employed for electronic spectral studies were obtained using a Perkin-Elmer Model 303 atomic absorption spectrophotometer.

Results

The temperature dependence of the total diamagnetism of the oxyhemerythrin sample is presented in Figure 1. The sample, whose diamagnetism is close to that of water, becomes slightly more diamagnetic as the temperature is lowered until below 40°K where there is an indication of a paramagnetic component. A similar curve was obtained for the methemerythrin but with a larger paramagnetic component at low temperatures. An expansion of the temperature dependence of oxyhemerythrin obtained by the higher sensitivity relative susceptibility method is presented in Figure 2. Above 120°K additional points from the total susceptibility determinations

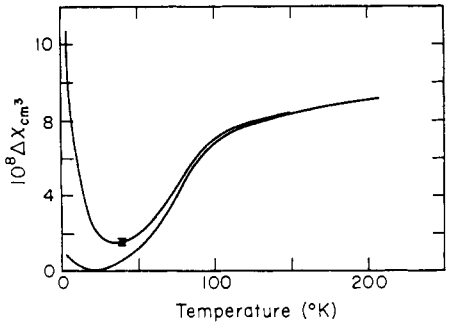


FIGURE 2: The temperature dependence of the paramagnetic component to the susceptibility of oxyhemerythrin, measured as a 20% ethanol suspension (160 μl containing 460 μg of iron) at 47 G. The lower curve is obtained by subtracting the contribution assumed to arise from paramagnetic impurities, with a Curie constant $C = 4.94 \times 10^{-7}$ °K emu/cm^3.

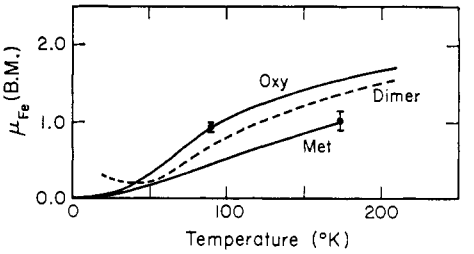


FIGURE 4: The temperature dependence of the magnetic moment per iron atom (corrected for assumed paramagnetic impurities) for oxy- and metaquohemerythrin. The curve denoted dimer represents the temperature variation of the magnetic moment of the oxo-bridged iron(III) dimer, $enH_2[(FeHEDTA)_2O]\cdot 6H_2O$ (Schugar *et al.*, 1972).

have been added. The lower curve shows the magnetism that remains after the assumed Curie-law contribution of the impurity has been subtracted from the raw data. Analogous curves for metaquohemerythrin are shown in Figure 3. They are qualitatively similar to those of oxyhemerythrin, but show a smaller temperature dependence at higher temperatures, and there is a larger paramagnetic component at the lowest temperatures. The data, corrected for paramagnetic impurities, have been plotted in terms of magnetic moment per iron atom *vs.* temperature in Figure 4. For comparison the temperature dependence of the magnetic moment (Schugar *et al.*, 1972) of the oxo-bridged iron(III) dimer, $enH_2[(FeHEDTA)_2O]\cdot 6H_2O$, has also been included.

If the two iron atoms in each subunit of oxy- and metaquohemerythrin are assumed to couple antiferromagnetically, the variation of magnetic moment with temperature (Figure 4) can be fitted by the standard spin–spin interaction model (Earnshaw, 1968). In this scheme the interaction Hamiltonian is $H = -2JS_1S_2$ where J is the exchange coupling constant. Assuming $g = 2.00$, $S_1 = S_2 = 5/2$, and the temperature-independent paramagnetism contribution is zero, it is found that $J = -77$ cm^{-1} for oxyhemerythrin and $J = -134$ cm^{-1} for metaquohemerythrin. The data in Figure 4 are not compatible with a low-spin ($S_1 = S_2 = 1/2$) iron(III) model.

The electronic spectra of oxy- and methemerythrins in the range 300–800 nm have recently been reviewed by Garbett *et al.* (1969). As D_2O has lower energy overtones than H_2O, we have extended the range to 1200 nm using deuterated oxy- and metaquohemerythrin. No additional bands could be resolved in the 800- to 1200-nm region, even though concentrated (1.6×10^{-2} M in Fe) hemerythrin solutions were used.

Discussion

The spectroscopic and magentic properties of several binuclear iron complexes have been examined previously, as some of these could be model systems for the two iron atoms in each hemerythrin subunit (Okamura *et al.*, 1969; Garbett *et al.*, 1969; Gray, 1971). A variety of binuclear iron(III) complexes are known in which the two iron atoms are coupled antiferromagnetically. The magnetic and structural properties of these compounds are summarized in Table I.

Mössbauer measurements (Okamura *et al.*, 1969; York and Bearden, 1970; Garbett *et al.*, 1971) have been used to show that the iron atoms in deoxyhemerythrin exist as high-spin iron(II) (*i.e.*, $S_1 = S_2 = 2$). The decrease in the paramagnetic contribution to the room-temperature susceptibility on comparison of deoxy- to oxy- or methemerythrin (Kubo, 1953; Okamura *et al.*, 1969; York and Bearden, 1970) could be due to antiferromagnetic interaction between pairs of high- or low-spin iron(III) atoms or to the formation of species containing isolated low-spin iron(III) atoms. The latter can be discounted as there is no evidence of magnetic hyperfine in-

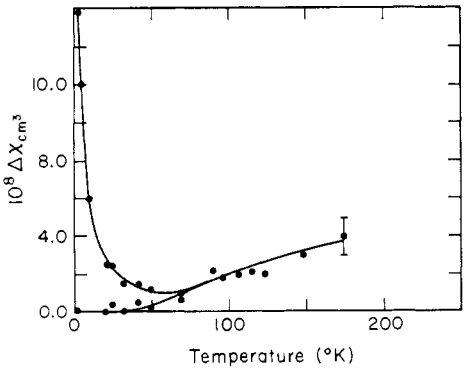


FIGURE 3: The temperature dependence of the paramagnetic component to the susceptibility of metaquohemerythrin, measured as a 20% ethanol suspension (122 μl containing 356 μg of iron) at 18.7 G. The lower curve is obtained by subtracting the contribution assumed to arise from paramagnetic impurities, with a Curie constant $C = 6.04 \times 10^{-7}$ °K emu/cm^3.

TABLE I: Summary of Magnetic Susceptibility Data for Mono- and Binuclear Iron(III) Complexes.

Iron Coordination	μ_{eff} BM (295°K) per Iron Atom	$-J$ (cm^{-1})
Fe^{III} high-spin six coordinate	5.7–6.0	0
	4.9–5.4	7–11
Fe^{III} low-spin six coordinate	2.1–2.4	0
Fe–O–Fe[b]	1.2–2.0	85–110

[a] Wu *et al.* (1972), and references therein. [b] Schugar *et al.* (1972), and references therein.

TABLE II: Electronic Spectra (300–1200 nm) of Oxy- and Metaquohemerythrin in D_2O Buffered with 0.1 M Deuterated Tris–Cacodylate (pD 7.0).

Compound	λ (nm)	ϵ_{max} (per Fe Atom)
Oxyhemerythrin	770 sh[a]	100
	500	1200
	370 sh	2700
	328	3500
Metaquohemerythrin	600 sh	100
	490 sh	290
	355	3300

[a] sh = shoulder.

teraction in the Mössbauer spectra on application of a 5-kG magnetic field at 4°K (Okamura *et al.*, 1969), and also Moss *et al.* (1971) found oxy- and several methemerythrins to be essentially diamagnetic between 1.5 and 77°K.

Our data establish not only that pairs of iron(III) atoms in both oxyhemerythrin and metaquohemerythrin are antiferromagnetically coupled, but also that the magnitude of this interaction corresponds to $J = -77$ and -134 cm^{-1} ($S_1 = S_2 = 5/2$), respectively. It should be noted that the data cannot be accommodated by a low-spin ($S_1 = S_2 = 1/2$) model and that the J values fall close to the range (-85 to -110 cm^{-1}) found for oxo-bridged ferric dimers (Table I).

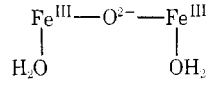
The electronic spectra of several oxo-bridged dimer models have been studied in some detail (Garbett *et al.*, 1969; Gray, 1971; Schugar *et al.*, 1972). The four ligand-field bands in the region 400–1000 nm have individual extinction coefficients (ϵ_{max}) in the range 3–120. The intensities of these transitions are considerably larger than the corresponding bands ($\epsilon_{max} \cong 0.1$) of monomeric, octahedral, high-spin iron(III) complexes (Hush and Hobbs, 1968; Holt and Dingle, 1968) owing to the antiferromagnetic interaction of the iron(III) atoms which leads to a partial relaxation of spin and/or orbital selection rules. Low symmetry components of the ligand field could also enhance the intensities. The ultraviolet spectra (200- to 400-nm region) of the oxo-bridged ferric dimers all contain at least four intense (ϵ_{max} 1–2 × 10^3) bands, which are characteristic of the Fe(III)–O^{2-}–Fe(III) system. Garbett *et al.* (1969) considered that these transitions have a charge-transfer origin, but we feel that they can be attributed to simultaneous pair electronic (spe) excitations (Schugar *et al.*, 1970, 1972; Gray, 1971). The latter explanation assumes that a single photon can simultaneously excite a ligand-field transition on each antiferromagnetically coupled iron(III) atom in the dimer. The resultant transition energy is thus the sum of the two one-center energies, to a good approximation.

The electronic spectra (Table II) of metaquo- and oxyhemerythrin in the range 300–800 nm are very similar to those recorded previously (Garbett *et al.*, 1969; Gray, 1971). Encouraged by the observation of bands at about 900 nm in the near-infrared spectra of oxo-bridged dimers, we examined this region carefully using D_2O solutions of metaquo- and oxyhemerythrin but were unable to discover any further transitions attributable to a binuclear iron site. It is possible, however, that the tail of the 750-nm band of oxyhemerythrin obscures a weak absorbance in the 800- to 1200-nm region.

The visible absorption bands of metaquohemerythrin have positions (600 and 490 nm) and intensities consistent with their assignment to ligand-field transitions of an oxo-bridged ferric dimer. By comparison to the spectra of $enH_2[(FeHEDTA)_2O]\cdot 6H_2O$ and $Na_4[(FeEDTA)_2O]\cdot 12H_2O$ (Schugar *et al.*, 1972) the transition at 355 nm could be a spe excitation. Other methemerythrins (X^- = *e.g.*, OH^-, Cl^-, Br^-, CN^-, N_3^-, NCS^-, and NCO^-) have two bands between 300 and 400 nm. One of these could be a spe excitation whereas the second band is probably an $X \rightarrow Fe^{III}$ charge-transfer transition (Garbett *et al.*, 1969). In all hemerythrins higher energy bands (<300 nm) arising from the iron dimer unit are masked by amino acid absorption.

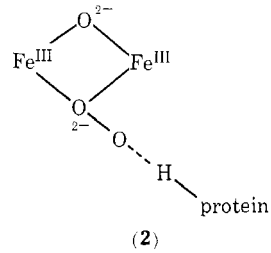
The electronic absorption spectrum of oxyhemerythrin has similar features to many of the methemerythrin spectra (Garbett *et al.*, 1969) except that the former has an intense band at 500 nm. A similar absorption band at 520 nm is found for the complex formed between $[Fe^{III}(EDTA)]^-$ and H_2O_2 in basic solution (Walling *et al.*, 1970). This band presumably arises from an $O_2^{2-} \rightarrow Fe^{III}$ or $HOO^- \rightarrow Fe^{III}$ charge-transfer transition. The 370- and 328-nm transitions could be due to spe excitations or, alternatively, to other charge-transfer transitions.

Our magnetic data confirm that oxo-bridged iron(III) dimers are good model systems for oxy- and metaquohemerythrin; the electronic spectral results also indicate the presence of Fe(III)–O^{2-}–Fe(III) units in these two proteins. The sulfhydryl group of cysteine cannot be a bridging ligand as blockage of this group has no effect on the electronic spectrum of hemerythrin (Keresztes-Nagy and Klotz, 1963). The two iron atoms could be bridged by a sulfur atom of methionine or the phenolic oxygen of tyrosine but the exchange coupling constants through such ligands would be expected to be much smaller than the values we have found experimentally. Consequently, the magnetic susceptibility and Mössbauer and electronic spectra of metaquohemerythrin are all consistent with the active-site representation (Garbett *et al.*, 1969)



(1)

The active site of oxyhemerythrin is more difficult to deduce as there are several ways in which an O_2^{2-} group can bind to a dimeric iron unit. The Mössbauer spectra (Okamura *et al.*, 1969; York and Bearden, 1970; Garbett *et al.*, 1971) indicate that there are two types of iron environment in each dimeric pair. Garbett *et al.* (1969) proposed that structures such as



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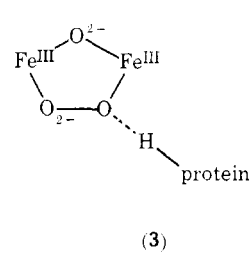
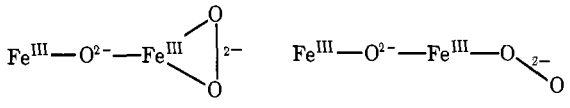


(3)

would be consistent with the Mössbauer spectra. We feel, however, that the antiferromagnetic coupling constants would

be substantially less than -77 cm^{-1} for double-bridged structures such as **2** and **3**. It seems reasonable, therefore, that the O_2^{2-} unit only binds to one iron atom resulting in the possible single-bridged structures



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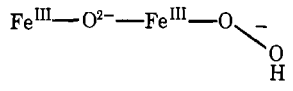

(6)

All of these representations would be consistent with our magnetic susceptibility data and also with the difference in Mössbauer quadrupole splittings (1.0 and 1.9 mm per sec) for the two iron atoms found by previous workers (Okamura *et al.*, 1969; York and Bearden, 1970; Garbett *et al.*, 1971).

In summary, susceptibility measurements using our ultrasensitive magnetometer have confirmed that oxo-bridged iron(III) dimers are useful model systems for the active sites of oxy- and metaquohemerythrin. We intend to apply this technique to the study of other metalloproteins containing more than one metal per subunit.

Acknowledgments

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