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Magnetic Susceptibility Evidence for a Binuclear Iron Complex in Hemerythrin*

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ABSTRACT: Magnetic susceptibilities have been determined for oxy, deoxy, and several met derivatives of hemerythrin. Below 205°K all the methemerythrin derivatives were very clearly diamagnetic.

These findings directly support long-standing ideas that the two iron atoms per hemerythrin monomer are sufficiently closely linked to form an antiferromagnetically coupled pair.

The iron ions in most of the common iron porphyrin proteins appear to be perfect paramagnets, in the sense that their magnetic properties can be explained in terms of spins isolated completely from one another in a diamagnetic protein matrix. Recent electron spin resonance (Tsibris *et al.*, 1968; Beinert and Orme-Johnson, 1969), magnetic susceptibility (Moss *et al.*, 1969; Moleski *et al.*, 1970), and Mössbauer measurements (Moss *et al.*, 1968) have demonstrated, however, that another large class of iron proteins, that of the non-heme iron sulfur proteins (ferredoxins etc.), exhibits exactly the opposite property. In these, magnetic ions are grouped in clusters with spins closely coupled. Hemerythrin, the oxygen-transport protein of sipunculid worms and certain other invertabrae, is neither a heme protein nor a member of the ferredoxin class (Klotz and Keresztes-Nagy, 1963). However, on the basis of absorption spectra, and the fact that the protein binds O_2 and anionic ligands in the ratio of 1:2 Fe atoms (Keresztes-Nagy and Klotz, 1965), it has been proposed that the irons are linked in a bridged structure, in which any unpaired ionic spins could be coupled antiferromagnetically. Supporting this viewpoint, recent approximate room temperature magnetic susceptibility measurements indicated (Okamura *et al.*, 1969) a decrease in susceptibility on converting deoxyiron(II) hemerythrin into an iron(III) form. The magnitude of the decrease compared to experimental error was such as to allow either low-spin ($S = {}^1/_2$) or diamagnetically coupled ion pairs in the iron(III) form, but, in addition, Mössbauer measurements failed to detect any magnetic hyperfine broadening at 4.2°K. Such broadening is normally observed in applied fields if there are unpaired electrons associated with the iron ions. The combination of Mössbauer and single temperature susceptibility measurements led these authors as well to conclude that the iron atoms formed a coupled antiferromagnetic structure. By measuring susceptibilities at low temperatures, as a function of temperature, we are able to measure with high accuracy the absolute, rather than relative, paramagnetism of a series of hemerythrin compounds. We present here data which put a very low limit on the maximum paramagnetism of the iron(III) derivatives and their individual subunits, excluding completely the $S = {}^1/_2$ case, and confirming directly the antiferromagnetic coupling of the iron atoms into diamagnetic pairs in the subunits of oxidized hemerythrin.

Experimental Section

The method of purification of hemerythrin was essentially that previously described (Florkin, 1933). The worms were cut with scissors and the contents squeezed out and filtered

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through cheesecloth. The red cells were washed free of white cells and a brown coagulum by repeated centrifugation and resuspension in fresh sea water. To the packed red cells was added an equal volume of cold distilled water. The cells were then laked by two cycles of freezing and thawing. The resulting mixture was clarified by centrifugation at 100,000*g* for 1 hr. The supernatant was crystallized from a 20% ethanol–water mixture at 4°. Three such crystallizations were carried out with intermediate solvation in 0.5 M sodium phosphate buffer (pH 7.0). The iron to protein ratio of material prepared in this manner could not be improved by chromatography on Sephadex G-200 or DEAE-Sephadex, and it ran as a single band on both columns.

The material prepared from either ethanol crystallization or Sephadex chromatography had an iron content of 0.81% and an extinction coefficient of 3400 cm l. mole^{-1} at 330 nm. These values are identical with those reported by others (Keresztes-Nagy and Klotz, 1965; Klotz *et al.*, 1957) for oxyhemerythrin. The 280/330 ratio was 5.64 as previously reported (Love, 1957).

The methemerythrin and its derivatives were prepared by passing the purified oxyhemerythrin through a 2 × 10 cm column of Dowex 1X-8 which had been previously equilibrated with the sodium salt of the anion whose methemerythrin complex was desired. Concerted oxidation and complex formation occurred on the column. The desired complex was eluted from the column with distilled water and crystallized from 20% aqueous ethanol. For the susceptibility determination, the crystalline methemerythrin was dissolved, to a final concentration of 3–5 μmoles of iron/ml, in a solution consisting of 0.1 M phosphate buffer (pH 8.0), 0.01 M in EDTA and 0.05 M in the appropriate anion.

Before the susceptibility measurements, oxidized hemerythrin derivatives were treated with 5 μl of 1 mM glucose oxidase and 20 μl of glucose to remove dissolved oxygen. Oxygenated protein samples were measured without these additions, and the susceptibility of a buffer solution equilibrated in air was subtracted from these results as a correction for dissolved oxygen. Optical spectra were taken of these samples after susceptibility measurements and the characteristic (Keresztes-Nagy and Klotz, 1965) spectra were observed. Deoxygenated protein solutions were obtained either by adding the above glucose oxidase system or a 5-fold excess of sodium dithionite to oxygenated samples. Difficulty was experienced, however, in obtaining colorless solutions of the concentrated protein solutions by either method.

The preparation of oxidized hemerythrin (methemerythrin) subunits was made by adding a 40-fold molar excess (relative to the subunit molarity) of cyanogen bromide and a 100-fold molar excess (relative to the protein iron) of sodium azide to an oxygenated hemerythrin sample. The deep red color of the oxygenated material faded within a few minutes to the orange color of the methemerythrin azide. To ensure completion, the reaction mixture was allowed to stand at room temperature for 30 min before freezing for the susceptibility measurement. Although the reaction products were not analyzed for monomers in this particular experiment, we feel confident that a good yield of monomers was obtained, since in identical experiments analysis of the reaction products on Sephadex G-200 indicated quantitative conversion of octomers to monomers. In order to measure the magnetic moment of the iron released from any protein configuration which could be responsible for iron–iron interactions, the subunit preparation was subsequently made 5 N in HCl and the susceptibility measurements were repeated.

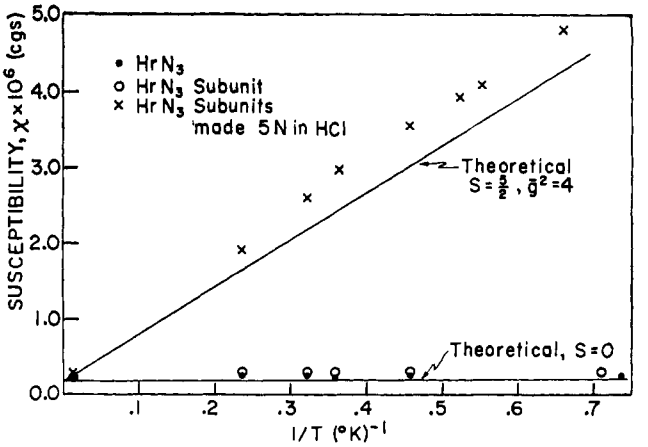

FIGURE 1: The temperature dependence of the susceptibility of methemerythrin (Hr) azide, methemerythrin azide subunits, and methemerythrin azide subunits made 5 N in HCl.

To check for magnetic heterogenity and the nature of possible magnetic contaminants all samples were examined by electron spin resonance, some down to temperatures of 20°K. A variety of trace signals typical of iron, with *g* values around 2, 4, and 6, trace copper signals, and in the deoxygenated samples, narrow signals like those of free radicals, were observed. For the oxidized and oxygenated samples, approximate double integrations of observed signals and comparison to standards indicated that they represented quantities of spins much less than stoichiometric with the hemerythrin iron concentration. It is important to note, however, that a variety of magnetic contaminants can exist in the protein and, unless quantitated, could appear deceptively important against the background of the diamagnetic protein. This situation is aggravated in the deoxygenated forms where relatively strong signals due to the glucose oxidase system or sodium dithionite addition can be observed.

Variable-temperature magnetic susceptibility measurements were made as described previously (Moss *et al.*, 1969) with a superconducting coil vibrating sample magnetometer designed and constructed by Dr. A. Redfield of IBM Watson Laboratories.

Results and Discussion

Figure 1 shows a typical experimental result with measured susceptibility points for iron(III)HrN_3, iron(III)HrN_3 subunits, and the HrN_3 subunit preparation made 5 N in HCl. Table I lists all the derivatives observed with the upper limits of their magnetic moments as determined by the experimental error. All of the met (oxidized) derivatives were very accurately diamagnetic. Since the iron(III), $3d^5$, configuration has an odd number of electrons the most obvious conclusion which can be drawn is that the unpaired spins are coupled antiferromagnetically to form a complex with net spin zero. The diamagnetism of the subunit preparation shows that this coupling must be by pairs of metal atoms within the subunits. Only under the very strong denaturing conditions provided by 5 N HCl were the spin-coupled pairs broken so that the magnetic properties of the individual ions could be detected (Figure 1). The maximum experimental error in the measured diamagnetism enables us to put a lower limit on the strength of exchange coupling between two $S = 1/2$ ions in the complex, expressed in terms of the energy splitting

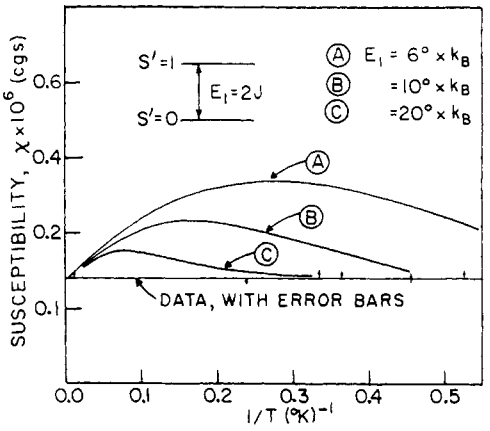

FIGURE 2: The temperature dependence of the susceptibilities of an exchange-coupled pair of low-spin iron(III) ions compared to the observed data for methemerythrin cyanate.

to the excited state ($S' = 1$) where the spins are parallel. This is shown graphically in Figure 2; the value is $2J \gtrsim 20$°K for coupled spins $^1/_2$; if the coupled spins were $S = {}^5/_2$ it would be much higher, $2J \gtrsim 60$°K. These are comparatively large values typical of binuclear complexes (Figgis and Lewis, 1964). Moreover, they are only *lower* limits, so that the calculation should not be viewed as defining an exact coupling but instead as suggesting that the electronic structure of the metal pair can be viewed as a system with at least some electrons fully shared by the iron atoms and delocalized over the pair and any bridging ligands. This kind of situation makes considerations of the formal valence of the individual irons rather difficult and suggests that the oxidation of the protein *complex* be viewed as a removal of electrons from an overall system of the two irons and probable bridging ligands rather than a change in charge localized on an individual iron atom and describable in formal valence terms.

Because of our difficulties in obtaining a completely colorless deoxygenated reduced protein in the concentrated samples, and the well-known trouble in producing an oxygenated reduced complex without some formation of iron(III) methemerythrin (Klotz *et al.*, 1957; Okamura *et al.*, 1969), we report the measurements on the reduced protein only as a reference for future studies.

In agreement with earlier work we have found the oxygenated form of the protein to be diamagnetic. In apparent contrast to past work we have found diamagnetism from 1.5 to 4°K in the deoxygenated protein as well, though a small increase in paramagnetism was measured at 77 and 201°K. Other authors have detected at room temperature changes of moments on deoxygenation approximating that for changes from low-spin ($S = 0$) to high-spin ($S = 2$) iron(II) ions (Kubo, 1953; Okamura *et al.*, 1969). Our present low temperature data and these observations are not incompatible, however, as the S_{z^2} states of the $S = 2$ ion can be split by spin–orbit coupling so that the nonmagnetic $S_{z^2} = 0$ lies far below the magnetic states $S_{z^2} = 1$ or $S_{z^2} = 4$. If this is the case, our data indicate that this zero-field splitting must be $\gtrsim 20$°K. Our low-temperature apparatus is not designed to study this splitting in detail *via* variable-temperature studies above 4°K, so we report only that our new data are not in actual contradiction to past work. It should be noted that a high susceptibility for the deoxygenated form would imply that coupling between the iron spins is broken in the absence of a bridging ligand.

TABLE I: Magnetic Moment of Hemerythrin Derivatives.

Derivative	Magnetic Momement[a] (μ_{eff}^2)
Met-HrF$^-$	<0.2
Met-HrN$_3^-$	<0.06
Met-HrOCN$^-$	<0.08
Met-HeSCN$^-$	<0.06
Met-HrCN$^-$	<0.08
HrO$_2$	<0.1
Deoxygenated Hr[b]	<0.08

[a] $\mu_{eff}^2 = [3k/N\mu_\beta^2][dx_m/d(1/T)]$, where N = Avogadro's number, χ_m = molar susceptability, μ_B = Bohr magneton, and k = Boltzmann's constant. $d\chi_m/d(1/T)$ is measured from 77 to 1.5°K in these experiments. For $S = 0$ the theoretical value of $\mu_{eff}^2 = 0$ and for a single unpaired electron, $S = {}^1/_2$, μ_{eff}^2 should be about 3.0. [b] A slight increase in susceptibility relative to the low-temperature points was observed at 200°K. This could imply population of a magnetic state at higher temperatures (see Results and Discussion).

The close coupling of two or more iron atoms to form a functional unit seems now to be an ubiquitous feature of metalloprotein construction. With the experimental evidence in hand, it is natural to ask what special biological advantage the configurations confer. For the non-heme iron–sulfur electron-transfer proteins the answer may lie in a greater flexibility of oxidation–reduction potential associated with a binuclear complex. In this oxygen-carrying protein, however, there is no obvious explanation, and theoretical studies exploring the stability and reversibility of O_2 binding by bridged binuclear Fe compounds, such as those proposed by previous authors (Keresztes-Nagy and Klotz, 1965), are needed.

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