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Semi-met Oxidation Level of Chalcogenide Derivatives of Methemerythrin

MÖSSBAUER AND EPR STUDIES*

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Conclusive evidence is presented for an $S = 1/2$ spin-coupled pair of high spin ferric and ferrous ions in the major reaction product of sulfide with the met form of the non-heme iron oxygen-carrying protein hemerythrin. Evidence for an analogous selenide derivative is also reported. Mössbauer and EPR spectroscopy establish (*a*) the charge and spin states of the individual iron atoms in sulfidehemerythrin as Fe(III), $S = 5/2$, and Fe(II), $S = 2$, and (*b*) the existence of an antiferromagnetic exchange interaction that couples the two spins to a resultant spin $S = 1/2$. The combined Mössbauer and EPR data confirm the correctness of the formulation first proposed for semi-methemerythrin by Harrington, P. C., deWaal, D.J. A., and Wilkins, R. G. ((1978) *Arch. Biochem. Biophys. 191,* 444-451) and furthermore show that a majority of the iron centers in the protein can be stabilized at this oxidation level. The results also demonstrate a new route to semi-methemerythrin. A titration of methemerythrin with selenide indicates that this derivative forms by a two step process consisting of first, reduction to the semi-met oxidation level by selenide and second, binding of selenide to either one or both irons.

The non-heme iron oxygen-carrying protein, Hr[1] is known to contain an antiferromagnetically coupled pair of high spin ferric irons at the oxygen binding site in both oxy and met forms (1). Recently, evidence has been presented for an intermediate Fe(II)-Fe(III) semi-met oxidation level of Hr, whose EPR spectra suggest an $S = 1/2$ ground state reminiscent of the reduced [2Fe-2S] iron-sulfur proteins (2-5). Also, the resonance Raman spectrum of a purple colored sulfide derivative of metHr (hereafter referred to as sulfideHr) shows a single peak whose frequency does not correlate with those of any known iron-sulfide complexes (6). These results prompted us to examine the chalcogenide derivatives of metHr in more detail. We report here the results of our preliminary studies of these derivatives using Mössbauer and EPR spectroscopies.

MATERIALS AND METHODS

Preparation of Protein Samples—OxyHr was isolated from the coelomic fluid of worms of the species *Phascolopsis gouldii* (obtained live from the Marine Biological Laboratory, Woods Hole, MA) according to a standard procedure (7). MetHr was prepared by oxidation with $Fe(CN)_6^{-3}$ and was crystallized by dialysis at 4 °C against 80:20 (v/v) aqueous buffer:ethanol. Aqueous buffer is 5 mM Tris-perchlorate, pH 8. Sulfide Hr was obtained as purple crystals by anaerobic dialysis of metHr against sodium sulfide at pH 8 followed by dialysis against 80:20 (v/v) aqueous buffer:ethanol all at 4 °C (8). For addition of selenide, anaerobic solutions of metHr in 50 mM Bis/tris/sulfate, pH 6.1, were prepared in septum capped vials. The metHr concentration (expressed as monomer) was determined from the optical spectrum of the azide derivative (1). Aliquots of a 0.074 M stock solution of Na_2Se (purchased from Alfa Products, 95% pure) prepared under Ar in 0.5 M Bis/tris/sulfate, pH 6.1, were added via gas tight syringe. The samples for EPR (see below) were frozen in liquid nitrogen 5-10 min after addition of selenide.

Mössbauer Spectra—Crystals of Hr were centrifuged into half-inch diameter cylindrical cups made of nylon and immediately frozen in liquid nitrogen. Spectra were recorded on a constant acceleration spectrometer equipped with a variable temperature cryostat. All velocities are given relative to metallic iron at 300 K.

EPR Spectra—A crystalline suspension of sulfideHr or solutions of selenideHr were injected anaerobically into 3-mm (outer diameter) quartz tubes equipped with rubber septa. After freezing in liquid nitrogen, the tubes were evacuated and flame sealed. Spectra were obtained on either a Bruker ER200 spectrometer equipped with an Oxford ESR 10 helium flow system or a Bruker ER220D spectrometer similarly equipped.

RESULTS AND DISCUSSION

Fig. 1 shows ^{57}Fe Mössbauer data for the purple derivative obtained when *P. gouldii* metHr is dialyzed anaerobically at pH 8 against sodium sulfide and then crystallized by dialysis against 80:20 (v/v) aqueous buffer:ethanol. The spectra recorded at $T > 60$ K can be resolved into four Lorentzians of roughly equal areas and a minor doublet accounting for 11 ± 2% of the total area, as illustrated for the 200 K data in Fig. 1*a*. The four major components can be assigned to two quadrupole doublets, *A* and *B*, on the basis of the temperature dependence of their energies (see Fig. 1*a*, *inset*). Our crystalline sample of aquometHr (high spin ferric) gave the quadrupole splittings Δ (isomer shifts δ_{Fe}) in mm/s of 1.61 (0.48) at 4.2 K, 1.62 (0.47) at 100 K, and 1.62 (0.43) at 200 K. Published values for deoxyHr (high spin ferrous) are 2.89 (1.20) at 4.2 K, 2.81 (1.19) at 77 K, and 2.75 (1.11) at 195 K (9-11). By comparison with these data, the two doublets, *A* and *B* in Fig. 1 can be identified as arising from Fe(III), $S_A = 5/2$, and Fe(II), $S_B = 2$, respectively. A solution obtained by addition of 0.1 M Na_2S to crystals of metHr yielded Mössbauer spectra at 200 K very similar to those of Fig. 1, including the contribution of the minor doublet.

In contrast to all previously published Mössbauer spectra of Hr derivatives (9-12), those of sulfideHr recorded at 4.2 K show resolved magnetic hyperfine splittings. The zero field spectrum, Fig. 1*b*, resembles the corresponding spectra of the $S = 1/2$ spin-coupled [2Fe-2S] iron-sulfur proteins in the reduced state (13). MetHr and oxyHr contain a diagmagnetic, spin-coupled pair of high spin ferric ions and thus show no magnetic splitting of the Mössbauer spectra. The minor com-

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[1] The abbreviations used are: Hr, hemerythrin; Bis/tris/sulfate, bis(2-hydroxyethyl)imino-tris(hydroxymethyl)methane adjusted to the indicated pH with H_2SO_4.

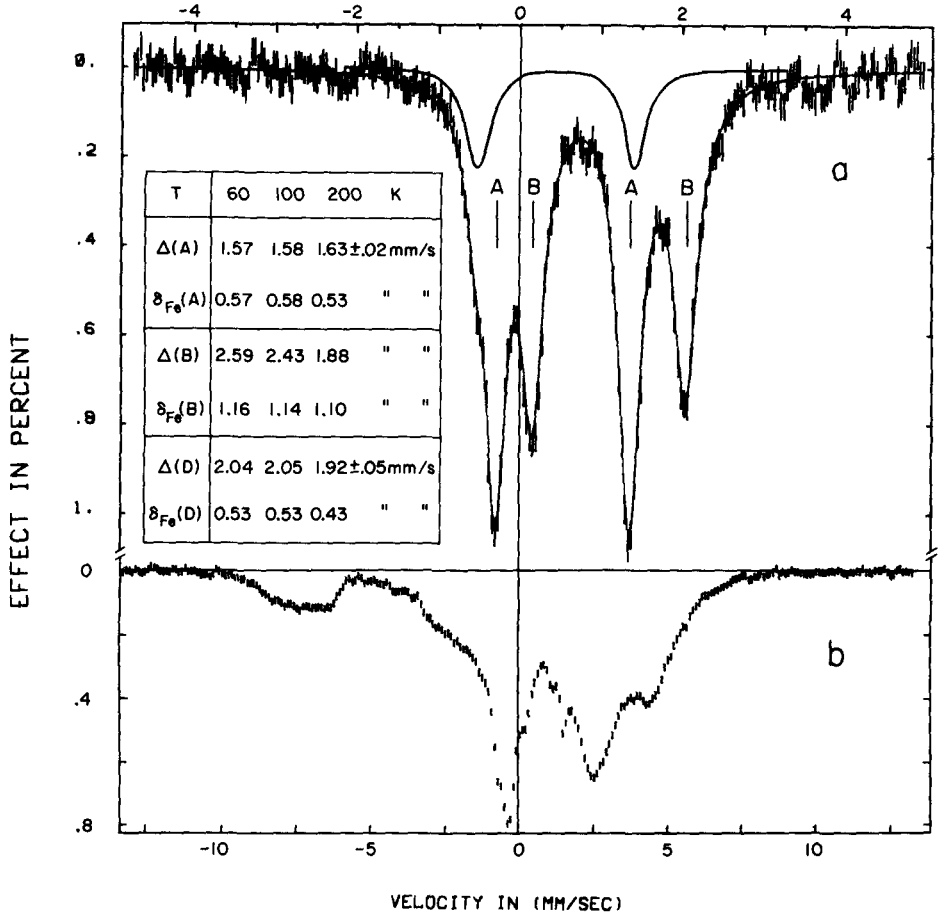

FIG. 1. **Mössbauer spectra of crystalline sulfideHr.** Spectra were taken in zero field at 200 K (*a*) and 4.2 K (*b*). The *solid line* in *a* which traces the observed spectrum is a simulation assuming one minor and two major quadrupole doublets *D*, *A*, and *B* accounting for 12.5, 45, and 42% of the total area, respectively. The *solid line* in *a* above the observed spectrum depicts the minor doublet, *D*. The average width of the Lorentzian lines is 0.33 mm/s. The *inset* lists the quadrupole splittings, Δ, and isomer shifts, δ_{Fe}, of the three components *A*, *B*, and *D* at 60 K, 100 K, and 200 K. The much larger splitting in spectrum *b* is evidence for magnetic hyperfine interactions at both major iron sites. In *b*, a sharp quadrupole doublet at 0.49 and 1.55 mm/s, due to the minor diamagnetic species, *D* accounting for 12% of the total area, has been subtracted.

ponent *D* of Fig. 1*a* must be of this type too, as it remains a sharp quadrupole doublet in all magnetically split spectra. However, $\Delta(D)$ and $\delta_{Fe}(D)$ are different from those of metHr and oxyHr (9). Mössbauer spectra of sulfide Hr measured in weak applied fields (~350 G) at 4.2 K show overall splittings of ~7.5 mm/s and vary substantially with the direction of the field; such field dependence is a strong indication of an EPR active center. At temperatures of $T < 30$ K, sulfide Hr indeed exhibits an EPR spectrum with the absorption derivative illustrated in Fig. 2. Saturation studies show that the signal arises from more than one species, the major component having a maximum at $g = 1.87 \pm 0.01$, zero crossing at $g = 1.71 \pm 0.01$, and a minimum at $g = 1.40 \pm 0.01$. These values are close to those of the azide adduct of semi-metHr (1.90, 1.81, 1.49) (4) and also resemble those of binuclear iron complexes in the reduced (pink) form of purple acid phosphatases (1.92, 1.77, 1.63) (14). According to the standard spin coupling model based on strong isotropic exchange (15), using a Hamiltonian $H = -J\,\tilde{S}_A \cdot \tilde{S}_B$ with $J < 0$, the ground state has net spin $S = 1/2$ and *g*-tensor $\tilde{g} = \frac{7}{3}\tilde{g}_A - \frac{4}{3}\tilde{g}_B$, where $\tilde{g}_A$ and $\tilde{g}_B$ are the intrinsic *g*-tensors of the high spin ferric, $S_A = 5/2$, and ferrous, $S_B = 2$, ions, respectively. If one substitutes the spin only value $g_A = 2$ for the high spin ferric *g*-tensor, the model predicts g_B values of 2.10, 2.22, and 2.45 for the ferrous site, which indicate unexpectedly large contributions to the magnetic moment from spin-orbit interaction among the t_{2g} orbitals. Relatively low lying orbital states of the ferrous ion can be inferred also from the strong temperature dependence of the quadrupole splitting $\Delta(B)$ (See Fig. 1*a*, *inset*).

Our combined Mössbauer and EPR data establish (*a*) the charge and spin states of the individual iron atoms in sulfide Hr as Fe(III), $S_A = 5/2$, and Fe(II), $S_B = 2$, and (*b*) the existence of an antiferromagnetic exchange interaction that couples the two spins to a resultant spin of $S = 1/2$. These results confirm the correctness of the formulation first proposed for semi-metHr by Harrington *et al.* (2) and furthermore show that a majority of the iron centers in the protein can be stabilized at this oxidation level. Definite charge and spin states can be assigned to the two iron atoms in sulfide Hr, and

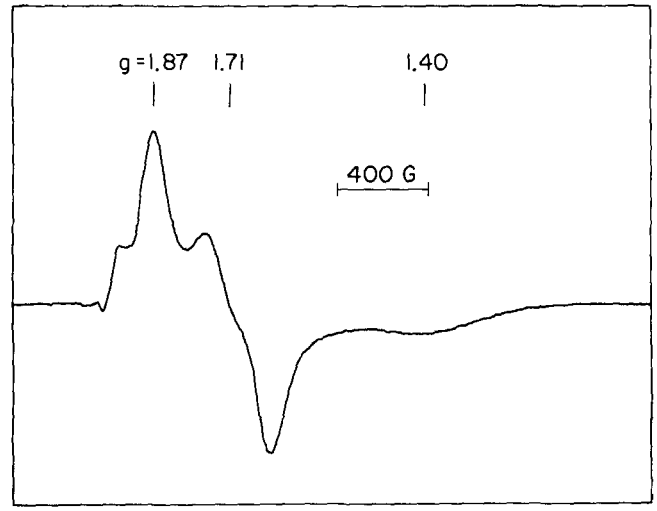

FIG. 2. **Derivative EPR spectrum of sulfide Hr.** This spectrum is of a portion of the same sample used to obtain the Mössbauer spectra in Fig. 1. Conditions: temperature, 3.1 K; frequency, 9.39 GHz; power, 100 microwatt; modulation, 10 G at 100 kHz; time constant, 0.5 s; receiver gain, 2.5×10^5.

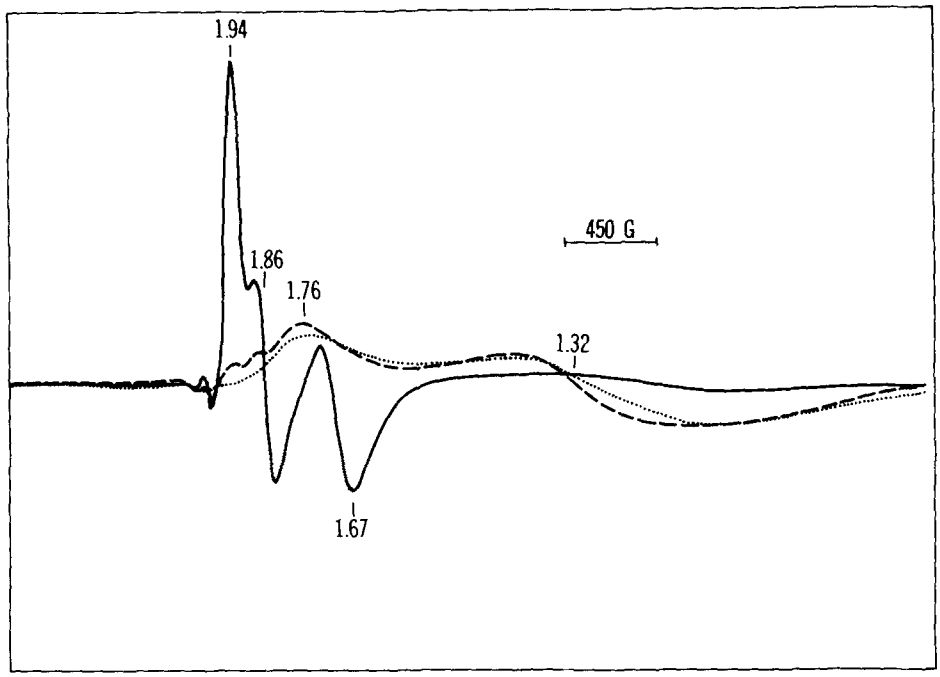

FIG. 3. **Derivative EPR spectra resulting from addition of Na_2Se to aquometHr.** Hr at 1.48 mM is in 50 mM Bis/tris/sulfate, pH 6.1. Conditions: temperature, 4 K; power, 2 milliwatt; modulation, 16 G at 100 kHz; field center, 4700 G; field range, ±2250 G; time constant, 0.1 s. Spectra are shown for; ~1 Se^{-2}:2Fe, receiver gain, 2×10^4 (——); ~1 Se^{-2}:1Fe, receiver gain, 3.2×10^4 (– – –); ~2 Se^{-2}:1Fe, receiver gain, 3.2×10^4 (·····).

the exchange interaction is antiferromagnetic as in met- and deoxyHrs.

Our results also demonstrate a new route to semi-metHr. An analogous selenide derivative can be prepared which exhibits an optical spectrum (not shown) very similar to that of sulfideHr but a distinctive EPR spectrum. Shown in Fig. 3 are EPR spectra resulting from a titration of aquometHr with selenide.The EPR spectrum resulting from addition of ~1 Se^{-2}/2 Fe shows *g* values (1.94, 1.86, 1.67) nearly identical to those reported for *P. gouldii* semi-metHr prepared by one electron reduction with $Na_2S_2O_4$ (1.93, 1.86, 1.68) (3). Selenide to iron molar ratios of ~1:1 and ~2:1 generate a much broader EPR spectrum with *g* values of 1.76 and 1.32 (zero crossing). Only the latter two solutions exhibit the purple color and optical spectra (not shown) resembling those of sulfideHr.

These results suggest that 2 chalcogenides/binuclear iron site are required to form the purple semi-met derivatives, one for reduction and one for ligation. For selenide, reduction occurs prior to ligation, while the optical titration of Freier *et al.* (6) indicates that for sulfide these two processes are not so well separated. The minor doublet in the Mössbauer spectrum of sulfideHr, which must arise from a diamagnetic site, might reflect a portion of sulfideHr at the met oxidation level. Our current data do not allow us to distinguish bridging from nonbridging chalcogenide. Freier *et al.* (6) have proposed on the basis of resonance Raman spectra that added sulfide replaces the putative μ-oxo bridge between the irons (15). Our results provide an explanation for failure of their observed frequency to correlate with those of known iron-sulfide complexes. We are unaware of any Raman data on a synthetic analogue having the equivalent of the semi-met oxidation level of sulfideHr.

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