Semi-met Oxidation Level of Chalcogenide Derivatives of Methemerythrin

MOSSBAUER AND EPR STUDIES*

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Donald M. Kurtz, Jr., J. J. Timothy Sage, Michael Hendrich, Peter G. Debrunner, and Gudrun S. Lukat

From the Department of Chemistry, Iowa State University, Ames, Iowa 50011 and the Department of Physics, University of Illinois at Urbana-Champaign, Urbana, Illinois, 61801

Conclusive evidence is presented for an S = 1/2 spin-coupled pair of high spin ferric and ferrous ions in the 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. Mossbauer 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 Mossbauer 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 iron.

The non-heme iron oxygen-carrying protein, Hr, is known to contain an antiferromagnetically coupled pair of high spin ferric ions 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-sulfur 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 Mossbauer and EPR spectroscopies.

MATERIALS AND METHODS

Preparation of Protein Samples—OxyHr was isolated from the coelomic fluid of worms 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)63- 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 Na2Se (purchased from Alfa Products, 95% pure) prepared under Ar in 0.5 mM 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.

Mossbauer 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 57Fe Mossbauer 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. 1a. 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. 1a, inset). Our crystalline sample of aquometHr (high spin ferric) gave the quadrupole splittings Δ (isomer shifts δ in m/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), S1 = 5/2, and Fe(II), S1 = 2, respectively. A solution obtained by addition of 0.1 mM Na2S to crystals of metHr yielded Mossbauer spectra at 200 K very similar to those of Fig. 1, including the contribution of the minor doublet.

In contrast to all previously published Mossbauer spectra of Hr derivatives (9-12), those of sulfideHr recorded at 4.2 K show resolved magnetic hyperfine splittings. The zero field spectrum, Fig. 1b, 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 diamagnetic, spin-coupled pair of high spin ferric ions and thus show no magnetic splitting of the Mossbauer spectra. The minor com-
Fig. 1: Mössbauer spectra of crystalline sulfide Hr. 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, A, B, and C. The average width of the Lorentzian lines is 0.33 mm/s. The inset lists the quadrupole splittings, A, B, and C, of the three components A, B, and C at 60 K, 100 K, and 200 K. The much larger splitting in spectrum a 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.

**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, 9.3 GHz; power, 100 microwatt; modulation, 10 G at 100 kHz; time constant, 0.5 s; receiver gain, 2.5 × 10^6.

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...
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 sulfideHr but a distinctive EPR spectrum. Shown in Fig. 3 are EPR spectra resulting from addition of Na₂Se 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, 1Fe, receiver gain, 2 × 10⁶ (---); −1 Se⁻²:1Fe, receiver gain, 3.2 × 10⁶ (--); −2 Se⁻²:1Fe, receiver gain, 3.2 × 10⁶ (.....).

Fig. 3. Derivative EPR spectra resulting from addition of Na₂Se 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, 1Fe, receiver gain, 2 × 10⁶ (---); −1 Se⁻²:1Fe, receiver gain, 3.2 × 10⁶ (--); −2 Se⁻²:1Fe, receiver gain, 3.2 × 10⁶ (.....).

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