Hyperfine Structure of [^{57}Fe]Iron in the Mössbauer Spectrum of the High-Potential Iron Protein from *Chromatium*

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The magnetic hyperfine structure observed in the ^{57}Fe Mössbauer spectra of the high-potential iron protein from *Chromatium* shows that the iron atoms are inequivalent in pairs, with hyperfine fields of 121 and 90 kG.

Magnetic hyperfine interaction has been observed previously in the Mössbauer spectra of ^{57}Fe in a number of reduced iron–sulphur proteins with large negative redox potentials (Johnson, Bray, Cammack & Hall, 1969). In this paper we report similar observations for the oxidized state of the high-potential iron protein from *Chromatium*, which has a positive redox potential. Moss, Bearden, Bartsch, Cusanovich & San Pietro (1968) have previously shown that the Mössbauer spectrum of oxidized high-potential iron protein becomes broadened at low temperatures ('diffuse hyperfine splitting'), indicating that the electron-spin-relaxation times have become long enough for magnetic hyperfine interaction to be observed.

Chromatium high-potential iron protein has four iron atoms and four labile sulphur atoms per molecule with a molecular weight of 10000 (Dus, De Klerk, Sletten & Bartsch, 1967). It has a redox potential of +350 mV and one electron per molecule is transferred in the oxidation–reduction process (Bartsch, 1963; Mayhew, Petering, Palmer & Foust, 1969). X-ray studies with 4 Å resolution failed to distinguish the four atoms, so that they must form a very compact unit in the molecule (Strahs & Kraut, 1968). In the reduced state the iron is non-magnetic but on oxidation it shows a paramagnetic susceptibility (Moss, Petering & Palmer, 1969) and electron spin resonance with $g_{\parallel} = 2.12$ and $g_{\perp} = 2.04$ (Palmer, Brintzinger, Estabrook & Sands, 1967).

EXPERIMENTAL

Growth of bacteria. *Chromatium* Strain D was grown in a modified Pfennig's medium (Evans & Buchanan, 1965) supplemented with sodium malate (7.0 g/l). The iron used was enriched to 30% with ^{57}Fe . The cells were harvested with a continuous flow centrifuge and the cell paste was stored frozen until required.

Preparation of high-potential iron protein. The purification procedure was based on that of Bartsch (1963). The cell paste was suspended (1:2, w/v) in 0.02 M-potassium phosphate buffer, pH 7.3, and the cells were broken by sonic oscillation. The extract was fractionated with acetone and the 50% acetone supernatant was passed through a column (5 cm $\times$ 8 cm) of DEAE-cellulose to remove ferredoxin. The eluate was diluted with an equal volume of water and passed through a column (25 cm $\times$ 5 cm) of DEAE-cellulose to collect the high-potential iron protein. The protein was eluted with 0.05 M-potassium phosphate buffer, pH 7.3. The eluate was fractionated with $(\text{NH}_4)_2\text{SO}_4$. The fraction precipitated between 55 and 90% saturation with $(\text{NH}_4)_2\text{SO}_4$ was collected, and after dialysis the protein was finally purified by chromatography on a column (40 cm $\times$ 2.5 cm) of DEAE-cellulose; it was eluted with 0.02 M-potassium phosphate buffer, pH 7.3. The protein was isolated in the reduced form. It was spectroscopically identical with the protein described by Bartsch (1963) and Dus *et al.* (1967).

Oxidation of the protein. A dilute solution of the protein in 0.02 M-potassium phosphate buffer, pH 7.3, was mixed with a tenfold excess of potassium ferricyanide. The protein was then precipitated by the addition of $(\text{NH}_4)_2\text{SO}_4$; the precipitate was dissolved in 0.02 M-potassium phosphate buffer, pH 7.3, and then desalted on a column of Sephadex G-25.

The protein was finally concentrated by collection on a column (1 cm $\times$ 0.5 cm) of DEAE-cellulose (Whatman DE52) from which it was eluted with 0.1 M-potassium phosphate buffer, pH 7.3, directly into the Mössbauer cuvette. The concentration of solution used for spectroscopy varied between 60 and 100 mg of protein/ml as estimated by using the extinction coefficients given by Bartsch (1963).

Whatman DEAE-celluloses DE23 and DE52 were used. All chemicals used in the purification procedure were A.R. grade.

Mössbauer spectra. These were obtained with a source of ^{57}Co in palladium by using the velocity-drive system of Cranshaw (1964). Calibration was done with an absorber of pure iron, and the spectra were plotted with the centre of the iron spectrum as the zero of velocity.

RESULTS AND DISCUSSION

At 77°K the Mössbauer spectra both in the oxidized and reduced states consisted of a quadrupole-split doublet. The quadrupole splittings and chemical shifts are given in Table 1, and are close to the data of Moss *et al.* (1968) who observed that all four atoms in the molecule are equally affected in the oxidation-reduction process. It may be noted that the smaller chemical shift in the oxidized state corresponds to a smaller number of d-electrons on the iron ions, which is consistent with oxidation.

The spectrum of the reduced protein did not change on cooling from 77°K to 4.2°K. On application of an external magnetic field at low temperatures no hyperfine interaction was observed, confirming that the iron atoms do not have a magnetic moment (cf. Moss *et al.* 1968).

The oxidized sample showed magnetic hyperfine splitting in the Mössbauer spectra at 4.2°K and 1.7°K. In the absence of an applied magnetic field the spectrum did not show any clearly resolved lines, but has a broad flat band of absorption between velocities of about -2 and $+2$ mm/s (Fig. 1a). The advantages of applying a magnetic field in removing ambiguities in the interpretation of the Mössbauer spectra of biological compounds have been stressed by Johnson (1967) and Johnson & Hall (1968). On applying a small magnetic field (50 G) the spectrum sharpened up and definite absorption lines were observed (Figs. 1b and 1c). These lines were broad (full width at half height = 1 mm/s) presumably because we are dealing with a sample with anisotropic ions whose molecular axes are randomly oriented relative to the external field. Although there appear at first sight to be six strong lines in the spectra, they do not correspond to a simple Zeeman spectrum for a single type of iron atom. The actual situation is more complicated. This may be seen by comparing the spectrum in a small longitudinal field (i.e. parallel to the γ -ray beam) (Fig. 1b) with the spectrum in a small transverse field (i.e. perpendicular to the γ -ray beam) (Fig. 1c). If a magnetic field H is applied an iron atom with hyperfine coupling of the form $\mathcal{H} = S.A.I$, where S and I are the electronic and nuclear spins, the Mössbauer spectrum will become a simple Zeeman splitting if $g\beta H \gg A$ with an effective field at the nuclei $H_{\text{eff}} \approx AS/g\beta$ (Johnson *et al.* 1968). This condition is realized for a field of 50 G. In a longitudinal field a Zeeman splitting would give rise to four lines in the Mössbauer spectrum, since the two $\Delta m = 0$ transitions do not emit along the direction of the field. However, with oxidized *Chromatium* high-potential iron protein more than four lines are observed under these conditions (Fig. 1b). The extra two lines at about -1.2 and $+1.7$ mm/s (marked a and f in Fig. 1) cannot be

Table 1. Mössbauer data on *Chromatium* high-potential iron protein at 77°K

The data were computer-fitted by a least-squares programme. At 4.2°K, $\Delta E_Q = 1.18 \pm 0.02$ mm/s and $\delta = 0.44 \pm 0.02$ mm/s for the reduced protein.

	Quadrupole splitting ΔE_Q (mm/s)	Chemical shift δ (mm/s) relative to pure iron
Reduced	1.12 ± 0.01	0.42 ± 0.01
Oxidized	0.79 ± 0.01	0.32 ± 0.01

Fig. 1. Mössbauer spectra of ^{57}Fe in oxidized *Chromatium* high-potential iron protein at 1.3°K; concentration of protein was 100 mg/ml. (a) In zero external magnetic field; (b) in 50 G field applied longitudinal to the γ -ray beam; (c) in 50 G-field applied perpendicular to the γ -ray beam.

associated with a Zeeman spectrum with extreme lines at -1.6 and $+2.4$ mm/s (marked A and F) for two reasons. First, the positions of these lines are not consistent with a Zeeman splitting for ^{57}Fe , which would require lines at B and E. Even when the small quadrupole splitting is taken into account, agreement cannot be obtained. Secondly, when the field is rotated into the transverse direction the $\Delta m = 0$ transitions will give rise to strong lines. (Johnson *et al.* 1968). These are indeed observed (Fig. 1c) and they occur at positions B and E as expected for a Zeeman spectrum with outer lines

at positions A and F, and not at the positions of the extra lines at a and f. These extra lines a and f are, therefore, the outer lines of another Zeeman spectrum with a smaller effective field at the nucleus. The spectrum thus appears to be a superposition of two six-line spectra with effective fields of 121 and 90 kG, which are shown in Fig. 1 as A-F and a-f respectively.

The spectra are the same for a given direction of field or fields between 20 and 5000 G, and do not vary with temperature from 4.2°K down to 1.3°K, indicating that there are no low-lying states of the iron complex close to the ground state. At a magnetic field of 30 kG the position of the lines changes, probably mainly due to the direct effect of the applied field on the nucleus.

Detailed interpretation of the hyperfine spectra is not possible unambiguously at present, partly because of the complexity of the iron environment. The four iron atoms are close together (Strahs & Kraut, 1968) and the unpaired electron in the oxidized state is shared between them. In zero field the Mössbauer spectrum will be strongly influenced by the interaction of the electron with the other three neighbouring ^{57}Fe nuclei and any other nearby nuclei with finite magnetic moments (see Lang, 1967). Prediction of the spectrum is possible in principle but requires making assumptions about the form of the hyperfine interaction tensor, A . In an applied field the electronic and nuclear spins are decoupled from each other and the neighbouring nuclei effects are not important.

The implications of the observation of two hyperfine spectra at low temperatures in small fields, whereas only one quadrupole splitting is observed at high temperatures, again are not unambiguous in view of the possible complexity of a four-iron unit and the scarcity of data to go on. Although the obvious conclusion that the iron atoms are inequivalent in pairs seems likely, it may not be the only possibility. It could be that the combined anisotropies of the hyperfine interaction and the magnetic susceptibility conspire to make the

effective field in this system of randomly oriented molecules have a distribution of values with two peaks. However, it seems quite likely that there are two pairs of iron atoms that are slightly different structurally, and that the magnetic hyperfine interaction is more sensitive to this than the quadrupole splitting or chemical shift.

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