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Mössbauer Effect in the High-Potential Iron–Sulphur Protein from *Chromatium*

EVIDENCE FOR THE STATE OF THE IRON ATOMS

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1. The previous Mössbauer work on *Chromatium* high-potential iron–sulphur protein by Moss *et al.* (1968) and Evans *et al.* (1970) was extended to high applied magnetic fields. 2. Measurements of the reduced protein confirm that it is non-magnetic. 3. Spectra of the oxidized protein in applied magnetic fields clearly indicate that some iron atoms have a positive hyperfine field, which is evidence for antiferromagnetic coupling. 4. The spectra can be interpreted in terms of two types of iron atom with positive and negative hyperfine fields of 9 and 12T respectively. 5. A consideration of the chemical shifts and other evidence suggests formal valences of two Fe^{3+} and two Fe^{2+} atoms in the non-magnetic reduced state, and three Fe^{3+} atoms and one Fe^{2+} atom in the oxidized state. 6. However, no separate Fe^{3+} and Fe^{2+} spectra are seen, suggesting that the d electrons are not localized on particular iron atoms.

Chromatium high-potential iron–sulphur protein is an iron–sulphur protein with a high positive redox potential (Bartsch, 1963). This distinguishes it from the ferredoxins, which it resembles in many respects. *Chromatium* high-potential iron–sulphur protein has a relative molecular mass of 10000 with four iron atoms and four labile sulphur atoms per molecule (Dus *et al.*, 1967). As isolated from the organism the protein exists in the reduced state, and one electron per molecule is transferred in the oxidation–reduction process (Mayhew *et al.*, 1969). The reduced state is non-magnetic, but on oxidation the protein shows a paramagnetic susceptibility (Moss *et al.*, 1969), and e.p.r.* with $g_{\parallel} = 2.12$ and $g_{\perp} = 2.04$ (Palmer *et al.*, 1967).

X-ray-crystallographic determinations by Carter *et al.* (1971) show that high-potential iron–sulphur protein has an active centre identical, within the resolution of the present structual determinations, with the two four-iron centres in the eight-iron bacterial ferredoxin from *Peptococcus aerogenes* (Adman *et al.*, 1973). Carter *et al.* (1972) have proposed a 'three-state' hypothesis in which the non-magnetic forms of reduced high-potential iron–sulphur protein and oxidized eight-iron bacterial ferredoxin represent an equivalent state C of the four-iron centre. In the oxidized form of high-potential iron–sulphur protein this centre has undergone oxidation to a paramagnetic state C^+, whereas in the reduced form of the ferredoxin the cluster has undergone reduction to another paramagnetic state C^-. Confirmation of this hypothesis has come from the observation of a magnetic 'super-reduced' state of high-potential iron–sulphur protein (Cammack, 1973) in which the centre is presumably in the C^- paramagnetic state. Further evidence for this 'three-state' hypothesis comes from a consideration of the chemical shifts of high-potential iron–sulphur protein and *Clostridium pasteurianum* ferredoxin, which leads to the proposal that the state C centre contains two Fe^{3+} and two Fe^{2+} atoms coupled antiferromagnetically, with the C^+ state containing three Fe^{3+} and one Fe^{2+} atom, and the C^- state containing one Fe^{3+} and three Fe^{2+} atoms (Thompson *et al.*, 1974).

Previous Mössbauer spectra of high-potential iron–sulphur protein taken by Moss *et al.* (1968) and Evans *et al.* (1970) show no magnetic hyperfine interaction when the protein is in the reduced state. The spectrum of the oxidized protein at 1.3°K shows a broad flat band of absorption indicative of magnetic hyperfine interaction. The application of small magnetic fields helps to resolve the lines and leads to the proposal that the iron atoms are inequivalent in pairs with hyperfine fields of 12.1 and 9.0 T (Evans *et al.*, 1970). Proton-magnetic-resonance studies on high-potential iron–sulphur protein by Phillips *et al.* (1970) show contact shift patterns assigned to cysteine residues attached to the iron atoms. The temperature-dependence of these shifts indicates that the iron atoms are antiferromagnetically coupled in both redox states. In the oxidized state there is evidence for magnetic inequivalence between the iron atoms.

The purpose of the present work is to extend the previous measurements to high applied magnetic fields and to propose a model for the four-iron centre

* Abbreviation: e.p.r. electron paramagnetic resonance.

of high-potential iron–sulphur protein in the light of the work of Carter *et al.* (1972), Thompson *et al.* (1974) and Cammack (1973).

Experimental

Sample preparation

Chromatium strain D was grown as described by Evans *et al.* (1973) in a medium containing NH_4Cl (0.2 g/litre) as nitrogen source. High-potential iron–sulphur protein was prepared by a modification of the method of Bartsch (1963). The protein was enriched with ^{57}Fe as described by Rao *et al.* (1971). The yield of purified enriched protein was 70%, with a ratio E_{388}/E_{282} of 0.38 in the reduced form. Oxidized samples were prepared by the method of Evans *et al.* (1970). The circular-dichroism spectra of the enriched material were identical with those of the native protein in both the oxidized and reduced states.

Mössbauer spectra

These were obtained with sources of ^{57}Co in palladium and ^{57}Co in rhodium. An absorber of pure iron was used for calibration and the spectra were plotted with the centre of the iron spectrum as the zero of velocity. A superconducting solenoid was used to provide the high magnetic fields perpendicular to the γ-ray direction.

Results

Reduced high-potential iron–sulphur protein

The Mössbauer spectra of the reduced protein are shown in Fig. 1. The spectra at 195°K, 77°K and 4.2°K show quadrupole-split doublets. The chemical shifts and quadrupole splittings obtained from least-squares computer fits are given in Table 1 together with these parameters for the oxidized protein. Within the limits of experimental error these agree with the data of Evans *et al.* (1970). When the strong magnetic fields are applied to the reduced protein (Fig. 1, spectra *d* and *e*) the spectra show a splitting equal to that expected from the applied field, i.e. with no internal hyperfine field. The form of these spectra suggest a positive sign for the electric field gradient. The lack of an internal hyperfine field confirms the e.p.r. and susceptibility evidence that the iron atoms are coupled to give zero total spin.

Oxidized high-potential iron–sulphur protein

The Mössbauer spectra of the oxidized protein at 195° and 77°K show quadrupole-split doublets with smaller chemical shifts and quadrupole splittings than for the reduced protein, indicative of the decreased ferrous character of the oxidized molecule, which has one less electron. The spectra of the oxidized protein at 4.2°K and in applied magnetic fields are shown in Fig. 2. The spectra in zero applied magnetic field and in small magnetic fields applied parallel and perpendicular to the γ-ray direction (Fig. 2, spectra *a*, *b* and *c*) are essentially identical with those of high-potential iron–sulphur protein obtained from cells grown on a medium containing ^{57}Fe, taken at 1.3°K (Evans *et al.*, 1970). The high-field spectra (Fig. 2, spectra *d*, *e* and *f*) give useful additional information. The outermost lines (shown arrowed

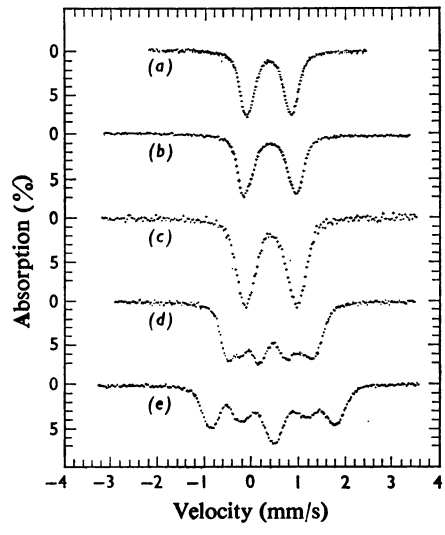

Fig. 1. *Mössbauer spectra of reduced Chromatium high-potential iron–sulphur protein*

Protein was used at 1 mM concentration. (*a*) At 195°K, (*b*) at 77°K; (*c*) at 4.2°K; (*d*) at 4.2°K in a magnetic field of 3.0T applied perpendicular to the γ-ray direction; (*e*) at 4.2°K in a magnetic field of 6.0T applied perpendicular to the γ-ray direction. For details see the text.

Table 1. *Mössbauer data on Chromatium high-potential iron–sulphur protein at* 195°, 77° *and* 4.2°K

The chemical shift δ (relative to pure iron) and the quadrupole splitting ΔE_Q were determined by computer fitting of the observed spectra to single quadrupole doublets. Errors are ±0.01 mm/s.

	Temp. (°K)	δ (mm/s)	ΔE_Q(mm/s)
Oxidized	195	0.28	0.77
	77	0.31	0.80
Reduced	195	0.38	1.01
	77	0.42	1.13
	4.2	0.44	1.16

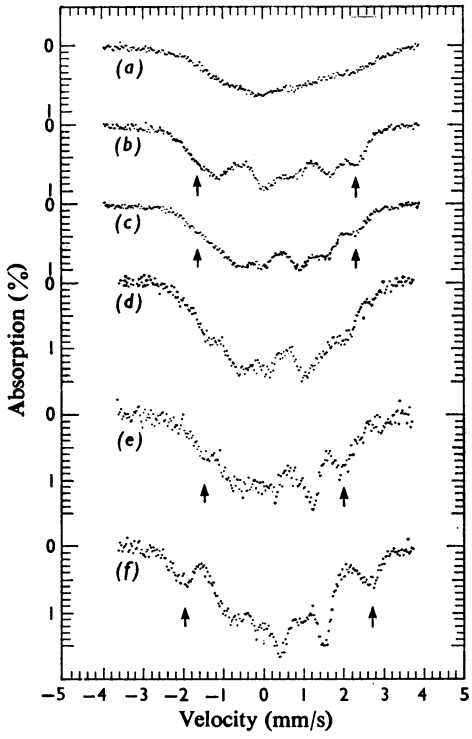

Fig. 2. *Mössbauer spectra of oxidized Chromatium high-potential iron–sulphur protein*

Protein was used at 0.35mM concentration at 4.2°K. (*a*) In zero applied magnetic field; (*b*) with a field of 0.05T applied parallel to the γ-ray direction; (*c*) with a field of 0.5T applied perpendicular to the γ-ray direction; (*d*) with a perpendicular field of 1.5T; (*e*) with a perpendicular field of 3.0T; (*f*) with a perpendicular field of 6.0T. For details see the text.

in Fig. 2, spectra *e* and *f*), which move out when the field increases from 3 to 6T, are indicative of iron atoms with a positive (i.e. parallel to the applied field) hyperfine field of 9.0T. Lines that move out on application of a large external magnetic field are strong evidence for the existence of antiferromagnetic coupling (Cammack *et al.*, 1971). No changes were seen in the high-field spectra when the temperature was lowered to 2.0°K. The outermost lines of the low-field spectra (shown arrowed in Fig. 2, spectra *b* and *c*) that move in as the applied field is increased indicate iron atoms with a negative hyperfine field of approx. 12T. These give rise to the lines in the central regions of the 3 and 6T spectra. The area of the spectrum due to the iron atoms with the positive hyperfine field (defined by the area of the outermost lines) is approximately half the total spectral intensity suggesting that the iron atoms are equally divided between those with positive and negative hyperfine fields.

Discussion

It is now possible to propose a model for the iron–sulphur centre of the protein in terms of formal valences that is consistent with the Mössbauer data and other evidence. This model is of an oxidized state with a centre of three Fe^{3+} atoms and one Fe^{2+} atom coupled to give a total spin $S = \frac{1}{2}$ and the observed magnetic behaviour. On reduction this centre becomes one of two Fe^{3+} atoms and two Fe^{2+} atoms coupled antiferromagnetically to yield the non-magnetic reduced state. Mössbauer evidence for this assertion comes from a consideration of the observed chemical shifts in high-potential iron–sulphur protein and in *C. pasteurianum* ferredoxin compared with the shifts for Fe^{3+} and Fe^{2+} atoms in other iron–sulphur proteins (Thompson *et al.*, 1974). Chemical shifts are used in preference to quadrupole splittings, as the latter are highly sensitive to deformation of the molecule. The chemical shifts show a consistent trend from Fe^{3+} to Fe^{2+}, and the proposed centres are consistent with the 'three-state' hypothesis of Carter *et al.* (1972). Of additional relevance is the model compound $(Et_4N)_2Fe_4S_4(SCH_2Ph)_4$ prepared by Herskovitz *et al.* (1972), which contains an Fe_4S_4 centre like that of high-potential iron–sulphur protein, and has an overall charge on the molecule corresponding to formal valences of two Fe^{3+} and two Fe^{2+} atoms. The model compound shows no e.p.r. signal, and it is proposed that it is an analogue of the non-magnetic reduced high-potential iron–sulphur protein.

The high-field spectra of the oxidized protein suggest that the iron atoms are in two pairs, one with spins parallel to the applied field and one with spins anti-parallel to the applied field, the two iron atoms in each pair being equivalent. This can be reconciled with the above assignments if there is some degree of electron delocalization. A noteworthy feature of the Mössbauer spectra of high-potential iron–sulphur protein at 77°K and above is that neither redox state shows separate Fe^{3+} and Fe^{2+} spectra. This also implies that the electron transferred in the oxidation–reduction process cannot be localized on one iron atom within the lifetime of the ^{57}Fe-excited state. By comparison the separate Fe^{3+} and Fe^{2+} spectra seen in the plant ferredoxins (Rao *et al.*, 1971) do show that the extra reducing electron is localized on one particular iron atom.

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