Short Communications

Mössbauer Effect in the 'Super-Reduced' Form of the High-Potential Iron-Sulphur Protein from *Chromatium*

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Mössbauer-effect studies of the super-reduced form of *Chromatium* high-potential iron-sulphur protein indicate that the iron atoms are in a similar valency state to those in reduced ferredoxin from *Clostridium pasteurianum*, with possibly some inequivalence between the iron atoms within the four-iron centre. Mössbauer spectroscopy also shows magnetic differences between the four-iron centres in the two proteins.

The high-potential iron-sulphur protein from *Chromatium* has an active centre consisting of four iron atoms and four labile sulphur atoms. This 4Fe–4S centre is identical, within the limits of resolution of the latest X-ray crystallographic determinations, with each of the two such centres found in the eight-iron bacterial ferredoxin from *Peptococcus aerogenes* (Carter *et al.*, 1972). *Chromatium* high-potential iron-sulphur protein, which has a positive redox potential of +350 mV, gives an e.p.r.* signal with a $g_{av.}$ of 2.06 in the oxidized form, but is non-magnetic in the reduced form. The eight-iron bacterial ferredoxins, however, have a negative redox potential of about –400 mV, an e.p.r. signal with a $g_{av.}$ of about 1.96 in the reduced form and are non-magnetic when oxidized. To rationalize these differences between proteins with apparently identical active centres Carter *et al.* (1972) have proposed a 'three-state' hypothesis with the non-magnetic forms, reduced high-potential iron-sulphur protein and oxidized ferredoxin, representing an equivalent redox state, C, of the four-iron centre. In the high-potential iron-sulphur protein the four-iron centre can undergo oxidation to a magnetic state, C⁺, whereas in the ferredoxin the centre can be reduced to another magnetic state, C[–]. Under this scheme a 'super-oxidized' ferredoxin, with a centre in the C⁺ state, and a 'super-reduced' high-potential iron-sulphur protein, with a centre in the C[–] state, would be expected.

It was suggested that these chemical states are not normally observed, because the potentials of the two redox processes are modified by the protein environments of the iron-sulphur centres. If the high-potential iron-sulphur protein is treated

with aq. 80% (v/v) dimethyl sulphoxide, which alters the conformation of the protein, it can be further reduced with sodium dithionite to a super-reduced state equivalent to reduced ferredoxin (Cammack, 1973). As expected, this super-reduced state is paramagnetic, and gives an e.p.r. signal with $g_{||} = 2.04$, $g_{\perp} = 1.93$.

We here report measurements by Mössbauer spectroscopy of the super-reduced high-potential iron-sulphur protein. This technique can provide information about the chemical state of the iron atoms, and about their environment. Recent Mössbauer-effect measurements on *Clostridium pasteurianum* ferredoxin (Thompson *et al.*, 1974) and *Chromatium* high-potential iron-sulphur protein (Dickson *et al.*, 1974), in their normal oxidized and reduced states, suggest that in terms of formal valencies the C-state centre consists of two Fe³⁺ and two Fe²⁺ atoms, the C⁺ centre contains 3 Fe³⁺ + 1 Fe²⁺ and the C[–] centre contains 1 Fe³⁺ + 3 Fe²⁺, and that there is extensive electron delocalization between the iron atoms. These chemical states have also been observed in analogue compounds with a similar structure to the active site of these proteins (Herskovitz *et al.*, 1972; Frankel *et al.*, 1974).

Experimental

Preparation of samples. High-potential iron-sulphur protein was purified from *Chromatium* cells and enriched in ⁵⁷Fe by reconstitution (Dickson *et al.*, 1974). A sample of protein (1–2 μ mol) was adjusted to pH 9.5 and concentrated in a stream of dry N₂ to a volume of 0.17 ml in a final concentration of about 0.1 M-Tris-HCl buffer. Then 0.80 ml of O₂-free dimethyl sulphoxide was mixed with the solution at 0°C under Ar atmosphere and, after 3 min, 30 μ l of 0.2 M-sodium dithionite solution was added,

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

and the sample was frozen in liquid N₂ after a further 3 min.

Mössbauer spectra. These were obtained with a source of ⁵⁷Co 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 permanent magnet provided the magnetic fields.

Results and discussion

Although the conditions of sample preparation were chosen to give optimum yield of the super-reduced form, measurements of e.p.r. signal intensity indicate that some of the protein remains in the normal reduced form, probably because the redox potential required for super-reduction is very low (Cammack, 1973). This is borne out by Mössbauer spectroscopy, which is sensitive to all the iron atoms present and therefore enables us to see superimposed spectra for all forms of the protein present.

The spectrum at 77°K consists of two or more overlapping quadrupole-split doublets. A computer fit of this spectrum to two quadrupole-split doublets gives Mössbauer parameters of (0.46 ± 0.04) mm/s for the chemical shift and (1.09 ± 0.06) mm/s for the quadrupole splitting of one doublet and (0.59 ± 0.04) mm/s for the chemical shift and (1.28 ± 0.06) mm/s for the quadrupole splitting of the other doublet. The Mössbauer parameters of the former doublet are identical, within the errors, with

those of the reduced form of *Chromatium* high-potential iron-sulphur protein (Dickson *et al.*, 1974), and it may therefore be presumed to arise from the presence of that species. A denaturation product would be unlikely to give these parameters. The other doublet can therefore be associated with the super-reduced form and has parameters close to those found in the reduced form of *C. pasteurianum* ferredoxin (Thompson *et al.*, 1974), which under the 'three-state' scheme also has a centre in the C⁻ oxidation state. By comparison of the chemical shift at 77°K of the super-reduced form of the high-potential iron-sulphur protein with the chemical shifts given in Thompson *et al.* (1974) it may be seen that the shift is consistent with a centre of one Fe³⁺ and three Fe²⁺ atoms, as would be expected for the C⁻ state centre. The quality of the fit to two quadrupole-split doublets, and the line widths obtained, imply that the spectrum of the super-reduced form may itself consist of more than one component. This would suggest an inequivalence between the four iron atoms in the active centre, i.e. some degree of localization of the 3d electrons.

Fig. 1 shows the spectra taken at 4.2°K, and with small applied magnetic fields. The quadrupole-split doublets seen in the centre of these spectra may be attributed to the presence of approximately 20% of the protein in the reduced form, and the Mössbauer parameters of these doublets confirm this. The rest of these spectra, arising from the super-reduced form, appear to consist of broad and rather flat bands of absorption, arising from magnetic hyperfine interaction. These spectra are rather similar to the spectra of the oxidized form (which is also magnetic) but shifted to the right by the greater chemical shift. They are also similar to those reported for the reduced form of the analogue compound (Frankel *et al.*, 1974).

The comparable spectra of reduced *C. pasteurianum* ferredoxin (which under the 'three-state' scheme also has a C⁻ centre) are somewhat different, consisting essentially of quadrupole-split doublets with some broadening attributable to magnetic hyperfine interaction. This difference is magnetic in origin and must arise from the centre being in a different protein, possibly because of a difference in the spin-relaxation times; the e.p.r. spectrum of super-reduced high-potential iron-sulphur protein is more readily saturated than that of reduced ferredoxin, indicating that spin relaxation is more rapid in the latter. An alternative explanation is that in the ferredoxin there are two centres and there may be spin coupling between them (Gersonde *et al.*, 1974).

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Fig. 1. Mössbauer spectra of super-reduced *Chromatium* high-potential iron-sulphur protein at 4.2°K

(a) In zero field, (b) with a small magnetic field (approx. 0.05 T) applied parallel to the γ -ray direction, (c) with a small magnetic field (approx. 0.05 T) applied perpendicular to the γ -ray direction.

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