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# Mössbauer Effect in the Eight-Iron Ferredoxin from *Clostridium pasteurianum*

## EVIDENCE FOR THE STATE OF THE IRON ATOMS

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1. Mössbauer spectra of both redox states of the eight-iron ferredoxin from *Clostridium pasteurianum* were observed over a range of temperatures and in magnetic fields. 2. At high temperatures (77°K and above) the spectra of both states consist essentially of the superposition of two or more closely similar doublets. 3. The average chemical shift for the oxidized protein leads to the proposal that each of the two four-iron active centres consists formally of two $Fe^{3+}$ and two $Fe^{2+}$ atoms. 4. The average chemical shift and quadrupole splitting increase on reduction, consistent with there being one $Fe^{3+}$ and three $Fe^{2+}$ atoms per centre in the reduced molecule. 5. The spectral changes on reduction show that all the iron atoms are affected when one electron is added to each four-iron centre. 6. No separate $Fe^{3+}$ and $Fe^{2+}$ spectra were observed (as they were, for instance, in the reduced two-iron plant ferredoxins) suggesting that the d electrons are not localized on particular atoms, but are shared approximately equally by all four atoms in the four-iron centres. 7. At low temperatures (4°K and below) no magnetic hyperfine interaction was observed in the oxidized protein even in an applied magnetic field, confirming the non-magnetic nature of the molecule in the oxidized state, and suggesting that the four iron atoms in each centre are antiferromagnetically coupled together to give zero spin. 8. Magnetic hyperfine interaction was observed in the reduced protein at low temperatures, and showed that all the iron atoms were magnetic. This demonstrates that one electron goes to each centre on reduction. 9. On application of a large magnetic field to the reduced protein at low temperatures, both positive and negative hyperfine fields were shown to be present, thus directly showing that antiferromagnetic coupling exists between the iron atoms in the reduced state.

The ferredoxin from *Clostridium pasteurianum*, an iron–sulphur protein with a high negative redox potential, is an electron-transport component involved in a number of reactions of fermentation and nitrogen fixation (Buchanan & Arnon, 1970; Orme-Johnson, 1973; Hall *et al.*, 1974). It has a relative molecular mass of 6000 with eight iron atoms and eight labile sulphur atoms per molecule (Hong & Rabinowitz, 1970). Each molecule gains two electrons on reduction (Evans *et al.*, 1968), and it has been proposed that one may be assigned to each centre (Orme-Johnson & Beinert, 1969). X-ray-crystallographic determinations on the closely related ferredoxin from *Peptococcus aerogenes* (Sieker *et al.*, 1972; Adman *et al.*, 1973) show that it has two active centres each containing four iron atoms at four points of a cube of side 0.28 nm with the four labile sulphur atoms at the alternate points of a concentric cube with sides 25% larger. The iron atoms are co-ordinated along the diagonals of the cube to four sulphur atoms in the cysteine residues of the amino acid chain of the protein. Each iron atom has a tetrahedral environment of four sulphur atoms, similar to that proposed for the two-iron plant ferredoxins (Rao *et al.*, 1971). Evidence from redox-potential measurements (Eisenstein & Wang, 1969) and from e.p.r.† measurements during reductive titration (Orme-Johnson & Beinert, 1969) suggest that there are small differences between the two active centres in the ferredoxin from *C. pasteurianum*. It may be noted that the two four-iron active centres of the eight-iron bacterial ferredoxin are identical, within the resolution of the latest structural determinations, with the four-iron active centre of high-potential iron–sulphur protein from *Chromatium* (Carter *et al.*, 1972*a*).

The Mössbauer effect has become increasingly useful in studying the nature of iron atoms in proteins (Lang, 1970; Johnson, 1971). Together with other measurements Mössbauer spectroscopy has contributed to the understanding of the nature of the

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

iron at the active centres of the one-iron (i.e., rubredoxins) and two-iron (i.e. plant-type ferredoxins) iron–sulphur proteins (see, e.g. Rao *et al.*, 1972; Cammack *et al.*, 1971; Dunham *et al.*, 1971). In the plant-type ferredoxins the two iron atoms have been shown to be coupled together antiferromagnetically (Gibson *et al.*, 1966; Thornley *et al.*, 1966) to give a resultant spin state which is non-magnetic ($S = 0$) when the protein is oxidized, and paramagnetic with $S = \frac{1}{2}$ and unusually low $g$ values averaging 1.96 when it is reduced.

Mössbauer spectroscopy of oxidized *C. pasteurianum* ferredoxin at 77°K by Blomstrom *et al.* (1964) indicated that it is non-magnetic. Proton-magnetic-resonance measurements at higher temperatures showed a low paramagnetic susceptibility with a temperature-dependence compatible with antiferromagnetic coupling (Poe *et al.*, 1971). Consistent with this the ferredoxin shows no e.p.r. signal in the oxidized state. In the reduced state, an e.p.r. signal with an average $g$ value of 1.96 is observed at temperatures below 40°K (Orme-Johnson & Beinert, 1969). The intensity of the signal corresponds to one unpaired electron ($S = \frac{1}{2}$) per four-iron centre.

The application of Mössbauer spectroscopy to the study of *C. pasteurianum* ferredoxin over a range of temperatures and applied magnetic fields should elucidate the nature of the iron atoms in the active centre and hence improve our understanding of the biologically important electron-transfer mechanism.

A preliminary report of this work has been presented (Rao *et al.*, 1973).

## Experimental

### *Materials*

*Clostridium pasteurianum* cells grown as described by Sargeant *et al.* (1968) were obtained from the Microbiological Research Establishment, Porton Down, Wilts, U.K. Iron enriched with 87.8% of $^{57}Fe$ was purchased as the element from the Atomic Energy Research Establishment, Harwell, Berks., U.K. It was dissolved in 3M-$H_2SO_4$ and the solution was neutralized with NaOH before use. DEAE-cellulose (Whatman DE23 and DE52) was obtained from Whatman Biochemicals, Maidstone, Kent, U.K. Sephadex G-25 was purchased from Pharmacia, London W.5., U.K. and Trizma pre-set pH crystals were obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. All other chemicals were the purest products of BDH Chemicals, Poole, Dorset, U.K.

### *Preparation of ferredoxin*

*C. pasteurianum* ferredoxin was prepared by a modification of the method of Mortenson *et al.* (1962). The operations were carried out in the cold (4°C) and all solutions were buffered with 20mM-Tris–HCl, pH8, and flushed with $N_2$. The cells, stored in liquid $N_2$, were thawed and suspended in about 4vol. of buffer and stirred well to a uniform suspension. The suspension was sonicated by using a Dawe Soniprobe (Dawe Instruments Ltd., London N.W.3, U.K.) at full power for about 10min, the container being immersed in an ice–NaCl mixture to maintain a temperature of 5–10°C. The sonicated material was centrifuged at approx. 23000$g$ for 2h. The residue was discarded and the supernatant was stirred with a suspension of DEAE-cellulose (approx. 10g of powder/litre of supernatant) for 5min and then the cellulose was allowed to settle. The DEAE-cellulose with the ferredoxin absorbed on it was transferred to a chromatographic column and washed with 0.1M-NaCl. The ferredoxin was then eluted with 0.8M-NaCl as a black solution. Solid $(NH_4)_2SO_4$ (0.6g/ml) was added to the eluate and the pH of the mixture was lowered to 5.8 by the addition of 1M-acetic acid. The mixture was centrifuged at 40000$g$ for 30min and the colourless supernatant was discarded. The sediment was dissolved in the minimum volume of buffer and the protein was desalted by passage through a long column of Sephadex G-25 and washing with buffer. The desalted material was adsorbed on top of a long column of DEAE-cellulose equilibrated with 0.2M-NaCl. The DEAE-cellulose was washed with 1 column volume of 0.25M-NaCl. A linear gradient of 0.28–0.5M-NaCl of 500ml or 1 litre total volume was then applied to the column. The early pink fractions contained rubredoxin and the later dark-brown fractions ferredoxin. The ferredoxin fractions were pooled, diluted with an equal volume of buffer and concentrated on a very small column of DEAE-cellulose. The whole process could be carried out in a day starting with 500g of wet cells. The yield was about 5mg of rubredoxin and 100mg of ferredoxin/kg of cells.

### *Preparation of $^{57}Fe$-enriched ferredoxin*

This was done essentially by published procedures (Hong & Rabinowitz, 1967; Rao *et al.*, 1971).

About 25mg (4$\mu$mol) of ferredoxin was desalted by passage through Sephadex G-25 and elution with water. To the desalted ferredoxin trichloroacetic acid was added to a final concentration of 8% (w/v). A stream of $N_2$ was passed over the mixture at 25°C to drive off $H_2S$ formed. The mixture was centrifuged at 5000$g$ for 10min. The sedimented protein was dissolved in 0.2M-Tris–HCl buffer, pH8.5, and reprecipitated with trichloroacetic acid and flushed with $N_2$. The precipitate was separated by centrifuging, washed with water and re-dissolved in Tris–HCl buffer. At this stage the solution showed no optical absorption at 390nm.

The solution of apoferredoxin was made 6M with respect to urea and then 0.5ml of 2-mercaptoethanol was added. The mixture was flushed with $N_2$ for 6h at room temperature. Then 100$\mu$mol of $Na_2S$ (as a fresh aqueous solution) and 100$\mu$mol of $^{57}FeSO_4$ were added and the mixture was incubated at 37°C for 10min. The mixture was then chromatographed on a Sephadex G-25 column and developed with 20mM-Tris–HCl buffer. The ferredoxin was eluted out of the column first and was further purified by chromatography on a long column of DE52 DEAE-cellulose developed with 0.32M-NaCl. The yield of pure $^{57}$Fe-substituted ferredoxin was about 50%, with a ratio $E_{390}/E_{278}$ of 0.80. The optical spectra of the reconstituted ferredoxin were indistinguishable from those of the native protein.

*Preparation of reduced ferredoxin samples*

During the early stages of this work it proved difficult to obtain highly concentrated ferredoxin samples in a state of complete reduction by using $Na_2S_2O_4$. These difficulties were attributed to the presence of denatured ferredoxin in the sample, which appears to have an inhibiting effect on reduction. This denatured ferredoxin sometimes gave a line at approx. 3mm/s in the Mössbauer spectra, as can be seen to a small extent in Fig. 2, spectrum (*b*). To avoid this it was necessary to make the Mössbauer samples from freshly prepared purified material. Of a number of procedures tried for reduction, the method giving the most consistent results was similar to that used by Poe *et al.* (1971). A solution of ferredoxin, containing 1–2$\mu$mol in 20mM-Tris–HCl buffer, pH8.0, was concentrated, by blowing a stream of dry $N_2$ over the surface, to a volume of 1.0ml. It was then transferred with a syringe, under $N_2$, to a polyethylene cell of 5mm thickness, containing 10mg of $Na_2S_2O_4$ and 10mg of Trizma pre-set pH crystals, pH8.5, and stirred for 3min at room temperature. The cell was then frozen by immersion in liquid $N_2$.

*Mössbauer spectra*

These were obtained with sources of $^{57}$Co in palladium and $^{57}$Co in rhodium, by using a velocity-drive system based on that of Cranshaw (1964). 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 $\gamma$-ray direction.

## Results

*Oxidized ferredoxin*

The Mössbauer spectra for the oxidized ferredoxin are shown in Fig. 1. In the absence of an applied field the spectra consist of quadrupole-split doublets. The slight broadening and asymmetry of the lines can be attributed to the overlapping of at least two slightly differing spectra, indicating that the iron atoms in the four-iron centre may not be equivalent. In addition it should be remembered that each ferredoxin molecule contains two centres, which, though very similar, may not be exactly equivalent. The average chemical shifts and quadrupole splitting are given in Table 1 together with these parameters for the reduced ferredoxin.

The present results agree with those of Blomstrom *et al.* (1964) and confirm that the oxidized state is non-magnetic, but they give no definite indication of the state of the iron atoms. By comparison with plant ferredoxins (Rao *et al.*, 1971) it is possible that all the iron is in the high-spin $Fe^{3+}$ state antiferromagnetically coupled to produce zero total spin. The electric-field gradient necessary for quadrupole splitting would then come from the sulphur electrons covalently bonded into the $Fe^{3+}$ orbitals, as well as the effect of the neighbouring sulphur ligands. However, the quadrupole splittings and chemical shifts are significantly greater than those of the $Fe^{3+}$

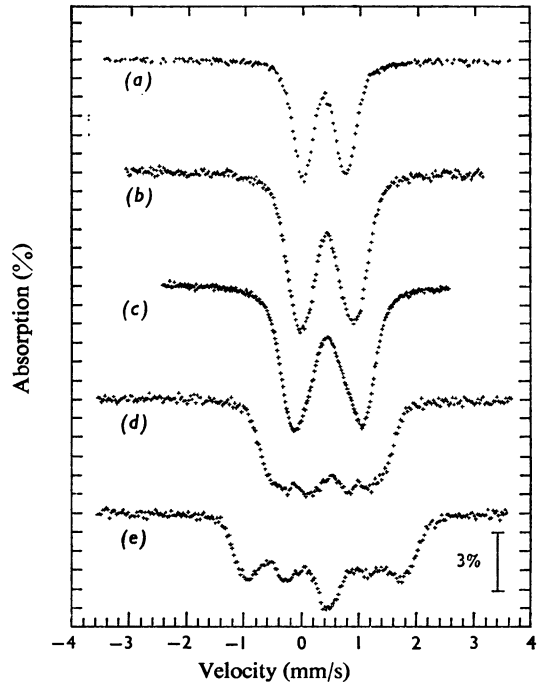


Fig. 1. *Mössbauer spectra of oxidized C. pasteurianum ferredoxin enriched with $^{57}$Fe*

(*a*) At 195°K, (*b*) at 77°K, (*c*) at 4.2°K, (*d*) at 4.2°K in a magnetic field of 3T applied perpendicular to the $\gamma$-ray direction, (*e*) at 4.2°K in a magnetic field of 6T applied perpendicular to the $\gamma$-ray direction. For details see the text.

Table 1. *Mössbauer data on C. pasteurianum ferredoxin at 198°, 77° and 4.2°K*

The chemical shift δ (relative to pure iron) and the quadrupole splitting $\Delta E_Q$ were determined by computer fitting of the observed spectra. For the oxidized ferredoxin the data for the average of the component doublets were taken. Errors are ±0.01 mm/s.

| | Temp. (°K) | δ (mm/s) | $\Delta E_Q$ (mm/s) |
|---|---|---|---|
| Oxidized | 195 | 0.39 | 0.75 |
| | 77 | 0.43 | 0.91 |
| | 4.2 | 0.44 | 1.08 |
| Reduced | 195 | 0.52 | 1.07 |
| | 77 | 0.57 | 1.25 |
| | 4.2 | 0.58 | 1.54 |

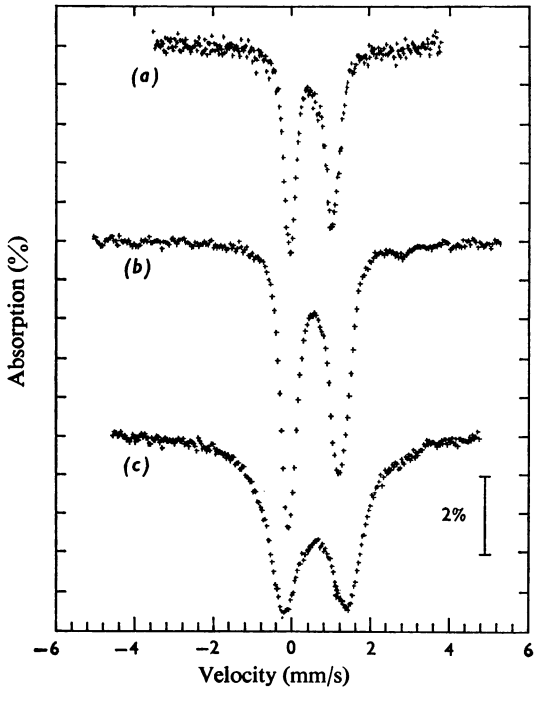


Fig. 2. *Mössbauer spectra of reduced C. pasteurianum ferredoxin*

(*a*) At 195°K, (*b*) at 77°K, (*c*) at 4.2°K. For details see the text.

ion in plant ferredoxins, suggesting that some of the iron atoms may be $Fe^{2+}$, although there is no evidence of separate $Fe^{3+}$ and $Fe^{2+}$ spectra as seen in the high-temperature spectra of reduced plant-type ferredoxins (see, e.g., Rao *et al.*, 1971).

When strong magnetic fields were applied to the oxidized ferredoxin (Fig. 1, spectra *d* and *e*) the spectra showed a splitting equal to that expected from the applied field, i.e. with no internal hyperfine field. The form of these spectra suggests a positive sign for the electric-field gradient, although with a large asymmetry parameter. The spectra gave good fits to theoretical spectra for quadrupole splittings in an applied magnetic field. This again confirms that the iron atoms are coupled to give zero total spin. The broadening of the lines with no applied field cannot therefore be due to magnetic effects, but must show that all eight iron atoms are not exactly equivalent.

*Reduced ferredoxin*

Fig. 2 shows the spectra from the reduced ferredoxin at 195°, 77° and 4.2°K. At 195° and 77°K the spectra are asymmetric quadrupole-split doublets with a larger chemical shift and quadrupole splitting than in the oxidized protein. The lines are again broad, with the much greater broadening in the 4.2°K spectrum being indicative of the onset of magnetic effects.

It is thought that on reduction each iron–sulphur centre of the ferredoxin gains one electron (Orme-Johnson & Beinert, 1969). If this electron were to reduce one $Fe^{3+}$ atom to $Fe^{2+}$, we would expect the spectra to contain a quadrupole-split doublet with a splitting of around 3 mm/s as for the $Fe^{2+}$ site in reduced ferredoxin from plants. Instead the spectra still show essentially only one doublet, but with a larger quadrupole splitting and chemical shift, indicative of the increased ferrous character produced by the extra reducing electron.

Useful information can be obtained from the line broadening on cooling and the effect of small magnetic fields parallel and perpendicular to the direction of the γ-ray beam, as shown in the spectra in Fig. 3. The line-width (full width at half maximum) at 4.2°K is 1.07 mm/s, more than twice that at room temperature. This does not increase on further cooling to 1.3°K. Line broadening on cooling is well known and arises from the hyperfine interaction between the electronic and nuclear spins. This has no effect at high temperature when the electron spin averages to zero, because of the short spin-lattice relaxation time. At lower temperatures the relaxation time becomes of the order of the measuring time (precession time of the $^{57}Fe$ nuclei) and the broadening occurs. Below 4.2°K temperature-independent spin–spin relaxation predominates and there is no further broadening. Thus the broadening on cooling is evidence that the iron sites are magnetic.

In small fields perpendicular and parallel to the γ-ray direction (Fig. 3, spectra *b* and *c*) extra lines appear in the wings of the broadened doublet, suggesting the presence of at least two kinds of magnetic site. The outermost lines are more intense

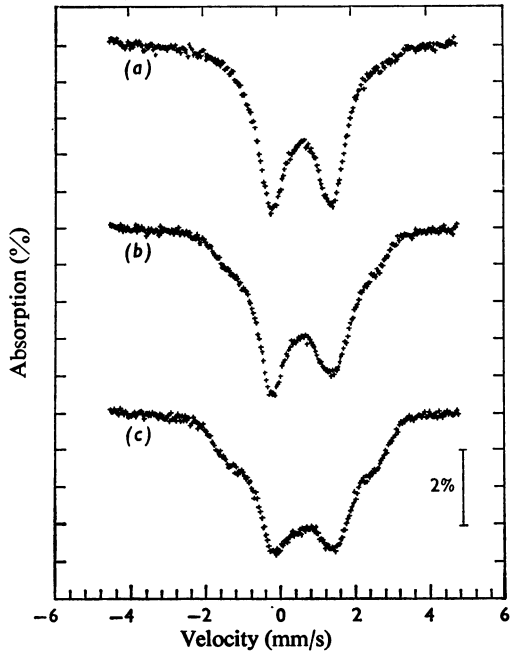


Fig. 3. *Mössbauer spectra of reduced C. pasteurianum ferredoxin at 4.2°K showing the effect of small magnetic fields*

(*a*) In zero field, (*b*) with a small field (approx. 0.05T) applied perpendicular to the $\gamma$-ray direction, (*c*) with a small field applied parallel to the $\gamma$-ray direction. For details see the text.

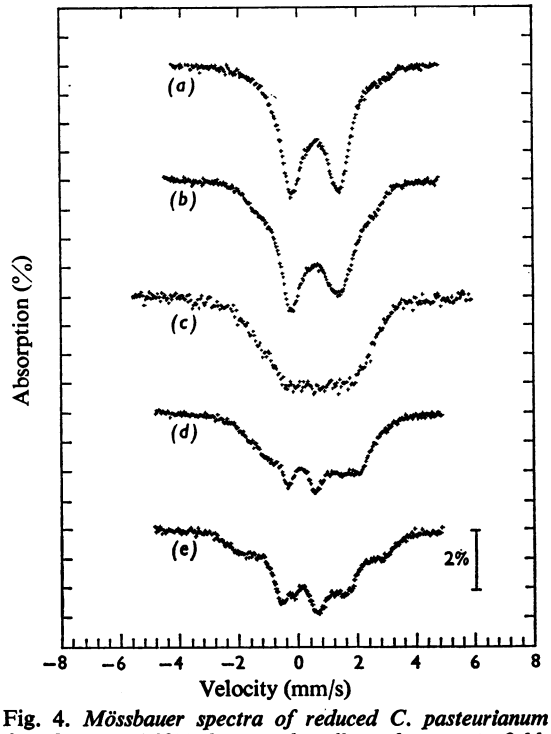


Fig. 4. *Mössbauer spectra of reduced C. pasteurianum ferredoxin at 4.2°K showing the effect of magnetic fields applied perpendicular to the $\gamma$-ray direction*

The fields were (*a*) zero, (*b*) approx. 0.05T, (*c*) 1.5T, (*d*) 3T, (*e*) 6T. For details see the text.

in a parallel applied field than in a perpendicular field. This effect is similar to the case of a hyperfine interaction at an isotropic magnetic site, which would produce a six-line spectrum with intensities 3:4:1:1:4:3 in a perpendicular field, and 6:0:2:2:0:6 in a parallel field. However, the presence of a quadrupole interaction means that the situation will be more complex than this.

Fig. 4 shows the spectra of the reduced ferredoxin in high magnetic fields. The central regions of the 3 and 6T spectra show a spectrum from iron sites with a negative (i.e. antiparallel to the applied field) hyperfine field and large quadrupole splitting; the same sites that give the lines in the wings of the low-field spectra (Fig. 3, spectra *b* and *c*). The lines in the wings of the high-field spectra (Fig. 4, spectra *d* and *e*) come from the sites that give the broadened doublet in the centre of the low-field spectra, and are presumably resolved because the relaxation rate is slowed down in the field. These lines are seen to move out when the external field is increased from 3 to 6T, and this indicates a magnetic site with a positive (i.e. parallel to the applied field) hyperfine field of approx. 9T. A positive hyperfine field that

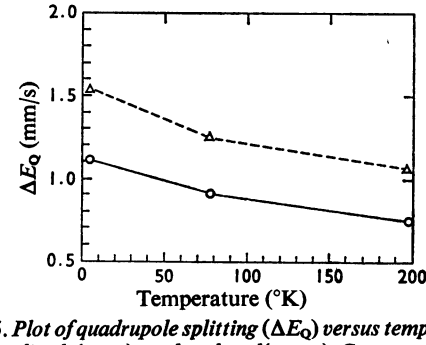


Fig. 5. *Plot of quadrupole splitting ($\Delta E_Q$) versus temperature for oxidized (——) and reduced(– – – –) C. pasteurianum ferredoxin*

For details see the text.

adds to the applied field is evidence of antiferromagnetic coupling. The fact that there is no non-magnetic behaviour in the spectra of the reduced ferredoxin confirms that each of the four-iron centres gains one electron on reduction.

Fig. 5 shows the quadrupole splitting for both redox states of the ferredoxin plotted against temperature. The temperature-dependence is greater than in

plant-type ferredoxins and also continues to low temperatures. The large temperature-dependence below 77°K is unusual and might be explained by a deformation of the molecule on cooling. The similar behaviour of both redox states suggests that the whole molecule contributes to this effect.

## Discussion

A striking feature of the high-temperature Mössbauer spectra of reduced *C. pasteurianum* ferredoxin is the absence of separate $Fe^{3+}$ and $Fe^{2+}$ spectra. This implies that the extra reducing electron 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 reducing electron is localized on one particular iron atom.

It is desirable to make some model for the iron–sulphur centre of the protein on the basis of the Mössbauer data and other evidence cited above, and although this cannot be done unambiguously at present, two possibilities are considered, of which the authors consider the second to be considerably more likely.

The first model is of an oxidized state having a centre of four $Fe^{3+}$ atoms coupled antiferromagnetically to yield a non-magnetic state. The addition of one electron to the centre on reduction would then give three $Fe^{3+}$ atoms and one $Fe^{2+}$ atom, coupled together to give a total spin $S=\frac{1}{2}$. This model is not entirely inconsistent with the present data; however, the quadrupole splittings and chemical shifts seen in the oxidized ferredoxin are significantly larger than one would expect if all the iron atoms are $Fe^{3+}$.

The second model is of a centre of two $Fe^{3+}$ atoms and two $Fe^{2+}$ atoms coupled antiferromagnetically to yield the non-magnetic oxidized state. On reduction this centre would become a centre of one $Fe^{3+}$ atom and three $Fe^{2+}$ atoms coupled to give a total spin $S=\frac{1}{2}$.

Mössbauer evidence for this assertion comes from a consideration of the observed chemical shifts in *C. pasteurianum* ferredoxin and in high-potential iron–sulphur protein compared with the shifts for $Fe^{3+}$ and $Fe^{2+}$ atoms in other iron–sulphur proteins. Chemical shifts are used here in preference to quadrupole splittings, as the latter are highly sensitive to deformation of the molecule. The chemical shifts, given in Table 2, show a consistent trend from $Fe^{3+}$ to $Fe^{2+}$, and the proposed centres are shown in the final column.

These results are in agreement with earlier work, reviewed by Orme-Johnson (1973), showing that on denaturation of oxidized ferredoxin, the iron atoms released are half ferric and half ferrous; on reduction, two more iron atoms per molecule become ferrous.

Table 2. *Chemical shifts (mm/s) at 77°K relative to pure iron*

Values are taken from the present work, from Evans *et al.* (1970), from Johnson *et al.* (1971), from Cammack *et al.* (1971) and from Rao *et al.* (1972).

| | δ | Proposed centre |
|---|---|---|
| $Fe^{3+}$ in rubredoxin | 0.25 | |
| $Fe^{3+}$ in adrenodoxin | 0.26 | |
| $Fe^{3+}$ in spinach ferredoxin | 0.22 | |
| Oxidized *Chromatium* high-potential iron–sulphur protein | 0.32 | $C^+$ $3Fe^{3+}+1Fe^{2+}$ |
| Reduced *Chromatium* high-potential iron–sulphur protein | 0.42 | C $2Fe^{3+}+2Fe^{2+}$ |
| Oxidized *C. pasteurianum* ferredoxin | 0.43 | |
| Reduced *C. pasteurianum* ferredoxin | 0.57 | $C^-$ $1Fe^{3+}+3Fe^{2+}$ |
| $Fe^{2+}$ in rubredoxin | 0.65 | |
| $Fe^{2+}$ in spinach ferredoxin | 0.56 | |

The absence of two types of Mössbauer spectra from two types of iron atoms in the intact protein indicates that these are formal valences, and the d electrons are not localized on particular iron atoms.

The results are also consistent with a hypothesis that has been suggested by Carter *et al.* (1972*b*) to explain why a bacterial eight-iron ferredoxin and a high-potential iron–sulphur protein from *Chromatium* have identical iron–sulphur centres (to within the limits of resolution of the latest X-ray-crystallographic determinations) yet very different redox properties. This 'three-state hypothesis' proposes that the oxidized ferredoxin and reduced high-potential iron–sulphur protein represent an equivalent diamagnetic state, C. The ferredoxin can be reduced to a state $C^-$, whereas high-potential iron–sulphur protein can be oxidized to a different paramagnetic state $C^+$. On this hypothesis one might expect to be able to produce a 'super-oxidized' ferredoxin in a state $C^+$, and a 'super-reduced' high-potential iron–sulphur protein in a state $C^-$. The latter state has recently been observed; it is magnetic and shows an e.p.r. signal with an average $g$ value of 1.96 (Cammack, 1973).

In terms of formal valences our proposals are of a state C with an active centre of two $Fe^{3+}$ and two $Fe^{2+}$ atoms coupled antiferromagnetically to give the observed diamagnetic behaviour, with the state $C^+$ having a centre of three $Fe^{3+}$ atoms and one $Fe^{2+}$ atom, and the state $C^-$ having a centre of one $Fe^{3+}$ atom and three $Fe^{2+}$ atoms. Of additional relevance to this problem is the model compound $(Et_4N)_2Fe_4S_4(SCH_2Ph)_4$ prepared by Herskovitz *et al.* (1972), which appears to contain a close analogue of the four-iron clusters in the eight-iron ferredoxins and the high-potential iron–sulphur protein. The structure of the $Fe_4S_4$ centre of the compound is like that of the

*P. aerogenes* ferredoxin (or high-potential iron–sulphur protein) with phenylmercaptan groups replacing the cysteines. The overall charge on the molecules corresponds to formal valences of two $Fe^{3+}$ atoms and two $Fe^{2+}$ atoms, although Mössbauer and other data, indicating that the iron atoms are actually equivalent, suggest a high degree of electron delocalization. As prepared the model compound shows no e.p.r. signal and it is proposed that it is an analogue of the non-magnetic oxidized ferredoxin or reduced high-potential iron–sulphur protein.

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