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Mössbauer Effect in *Scenedesmus* and Spinach Ferredoxins

THE MECHANISM OF ELECTRON TRANSFER IN PLANT-TYPE IRON–SULPHUR PROTEINS

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1. The Mössbauer spectra of *Scenedesmus* ferredoxin enriched in ^{57}Fe were measured and found to be identical with those of two other plant-type ferredoxins (from spinach and *Euglena*) that had been previously measured. Better resolved Mössbauer spectra of spinach ferredoxin are also reported from protein enriched in ^{57}Fe. All these iron–sulphur proteins are known to contain two iron atoms in a molecule that takes up one electron on reduction. 2. The Mössbauer spectra at 195°K have electric hyperfine structure only and show that on reduction the electron goes to one of the iron atoms, the other appearing to remain unchanged. 3. In the oxidized state, both iron atoms are in a similar chemical state, which appears from the chemical shift and quadrupole splitting to be high-spin Fe^{3+}, but they are in slightly different environments. In the reduced state the iron atoms are different and the molecule appears to contain one high-spin Fe^{2+} and one high-spin Fe^{3+} atom. 4. At lower temperatures (77 and 4.2°K) the spectra of both iron atoms in the reduced proteins show magnetic hyperfine structure which suggests that the iron in the oxidized state also has unpaired electrons. This provides experimental evidence for earlier suggestions that in the oxidized state there is antiferromagnetic exchange coupling, which would result in a low value for the magnetic susceptibility. 5. In a small magnetic field the spectrum of the reduced ferredoxin shows a Zeeman splitting with hyperfine field (H_n) of 180kG at the nuclei. On application of a strong magnetic field H the spectrum splits into two spectra with effective fields $H_n \pm H$, thus confirming the presence of the two antiferromagnetically coupled iron atoms. 6. These results are in agreement with the model proposed by Gibson, Hall, Thornley & Whatley (1966); in the oxidized state there are two Fe^{3+} atoms (high spin) antiferromagnetically coupled and on reduction of the ferredoxin by one electron one of the ferric atoms becomes Fe^{2+} (high spin).

Plant ferredoxins are among the simpler iron–sulphur proteins, since their molecules contain only two iron atoms and on reduction they take on only one electron (see review by Hall & Evans, 1969). Mössbauer spectra of ^{57}Fe have been measured in ferredoxins from spinach (Johnson & Hall, 1968; Johnson, Bray, Cammack & Hall, 1969) and from *Euglena* enriched by growth on ^{57}Fe (Johnson *et al.* 1968). The spectra were similar to those obtained from the more complex metalloflavoprotein, xanthine oxidase (Johnson, Knowles & Bray, 1967; Johnson *et al.* 1969).

We present here results on another plant-type ferredoxin from the green alga *Scenedesmus*, together with improved results on spinach ferredoxin. The purified proteins were enriched in ^{57}Fe by exchange, by using a method derived from that of Lovenberg, Buchanan & Rabinowitz (1963). This procedure greatly increases the sensitivity of the Mössbauer method, since naturally occurring iron contains only 2.2% of ^{57}Fe. However, the method is capable in some circumstances of producing artifacts that have a similar absorption spectrum to the plant-type ferredoxins (see Yang & Huennekens, 1970); moreover the product may be contaminated with excess of ^{57}Fe, which may be bound very tightly to the ferredoxin apoprotein (Keresztes-Nagy & Margoliash, 1966). This excess of iron would also contribute to the Mössbauer spectrum, producing two lines in the centre of the spectrum. These lines are similar to the spectrum of the oxidized ferredoxin, and are thus detectable only in the reduced state. Some previously published Mössbauer spectra of reduced spinach ferredoxin (Moss, Bearden, Bartsch, Cusanovitch & San Pietro, 1968; Johnson & Hall, 1968) showed these two lines to a

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greater or lesser extent and as a result were incorrectly interpreted. Therefore in the present work the ^{57}Fe-reconstituted ferredoxins were carefully purified by chromatography on DEAE-cellulose under anaerobic conditions and the products were examined by microchemical analysis, by biological activity and by a number of spectral methods to check that they were chemically identical with the native proteins.

The results on ^{57}Fe-enriched spinach ferredoxin agreed very well as far as could be seen with those on the unenriched protein, though of course there was far more detail observable in the present results. At the same temperature and magnetic field the spectra were essentially identical for *Scendesmus*, spinach and *Euglena* ferredoxins.

Gibson, Hall, Thornley & Whatley (1966) proposed a model for the active centre of spinach ferredoxin mainly from e.p.r.* evidence. In this model the oxidized ferredoxin molecule contains two high-spin ferric atoms and the reduced molecule contains one high-spin ferric atom and one high-spin ferrous atom. The low magnetic susceptibilities that have been reported (Thornley, Gibson, Whatley & Hall, 1966; Moss, Petering & Palmer, 1969) are explained by antiferromagnetic exchange coupling between the iron atoms. The present work was undertaken to examine carefully the model by using improved Mössbauer techniques.

The substance of this paper was presented at the 4th International Conference on Magnetic Resonance in Biological Systems, Oxford, August 1970 (C. E. Johnson, K. K. Rao, R. Cammack, M. C. W. Evans & D. O. Hall).

EXPERIMENTAL

Materials

Scenedesmus cells similar to those used by Matsubara (1968) were kindly supplied by Dr H. K. Gee, Richmond Field Station, University of California, U.S.A. Spinach was obtained from Covent Garden Market, London W.C.2, U.K. Iron enriched with 87.8% of ^{57}Fe was obtained as the element from the Isotope Separation Group of the Atomic Energy Research Establishment, Harwell, Berks., U.K.; the sample contained 0.03% of copper and less than 0.015% of other heavy metals. It was dissolved in 3M-H_2SO_4 and neutralized with NaOH before use. DEAE-cellulose (Whatman DE23 and DE52) was obtained from W. and R. Balston Ltd., Maidstone, Kent, U.K. NADP was from the Boehringer Corp. (London) Ltd., London W.5, U.K. All other chemicals were the purest products of B.D.H. Chemicals Ltd., Poole, Dorset, U.K.

Methods

Mössbauer spectra were measured in the apparatus previously described (Cranshaw, 1964). A strong narrow line (0.21 mm/s) source of ^{57}Co in palladium foil (0.00025in thick) was obtained from the Radiochemical Centre, Amersham, Bucks., U.K. E.p.r. spectra were recorded on a Varian E4 spectrometer (Varian Associates Ltd., Walton-on-Thames, Surrey, U.K.) by using a liquid-N_2 insert Dewar. Circular-dichroism spectra were recorded in a Spectropol I spectropolarimeter (SoFICA, St Denis, France). Iron was determined, after digestion of the protein with 1M-HCl at 80°C for 15min, by using bathophenanthroline (4,7-diphenyl-1,10-phenanthroline) as described by Diehl & Smith (1965). Labile sulphur was determined by the method of Fogo & Popowsky (1949), as modified by Lovenberg *et al.* (1963). Protein was determined by Kjeldahl nitrogen determination, the nitrogen content being taken to be 16%.

Purification of spinach ferredoxin

The method used was developed from the methods of Keresztes-Nagy & Margoliash (1966) and Matsubara (1968), and was designed for the handling of large quantities of plant material. Ferredoxin was recovered from the plant homogenate by adding DEAE-cellulose and recovering it by centrifugation; this procedure proved to be much more rapid than passing the homogenate directly through a column of DEAE-cellulose as in previously used methods, and has been found to be applicable to the preparation of a wide variety of iron–sulphur proteins. A 20mM-potassium phosphate buffer, pH7.5, was used throughout the procedure instead of tris–HCl because of the affinity of tris for adventitious iron. Samples of ferredoxin prepared with phosphate did not show the e.p.r. signal at $g = 4.27$ in the oxidized state due to contaminant iron that was seen in some samples prepared in tris (Hall, Gibson & Whatley, 1966). To minimize denaturation of the protein, all steps were carried out at 0–4°C, and buffers for chromatography and dialysis were bubbled with N_2 to remove O_2.

Homogenization. Washed spinach leaves (20kg) were homogenized in a 1-gallon Waring Blendor in 800g batches, each with 1.5 litres of cold buffer, at maximum speed for 2min. The homogenate was allowed to settle in a large aspirator and the copious froth was removed. The liquid was run from the bottom and centrifuged at 2500rev./min in the 6×1-litre rotor of an MSE Mistral refrigerated centrifuge for 15min. The dark-green supernatant, about 40 litres in all, was poured through muslin.

Batchwise DEAE-cellulose treatment. Solid NaCl (6g/l) and Whatman DE23 DEAE-cellulose equilibrated with buffer (20 ml packed wet volume/l) were added with stirring and the suspension was centrifuged at 500*g* for 1 min. The supernatant was discarded and the precipitate washed three times by resuspension in 6 litres of buffer and centrifugation. Finally the DEAE-cellulose was transferred to a column (30cm× 8 8cm) and washed with 2 litres of 0.2M-NaCl in buffer. The ferredoxin was eluted with 0.5M-NaCl in about 600ml of dark-red solution.

Ammonium sulphate treatment. $(NH_4)_2SO_4$ (50%, w/v) was added to the solution with stirring. After 15min the solution was centrifuged at 20000*g* for 30min. The precipitate of greenish protein material was discarded and the

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

supernatant dialysed overnight against 30 vol. of buffer.

DEAE-cellulose chromatography. The ferredoxin was concentrated by passing the solution through a column (15 cm × 2.5 cm) of Whatman DE23 DEAE-cellulose and eluting with 0.8 M-NaCl in buffer. It was then diluted threefold and applied to a column (60 cm × 4.4 cm) of Whatman DE52 DEAE-cellulose equilibrated with 0.32 M-NaCl in buffer. More 0.32 M-NaCl was run through and the ferredoxin was eluted in about 1.5 column vol. Eluted fractions with an E_{420}/E_{275} ratio of more than 0.3 were collected.

Chromatography on hydroxyapatite. The ferredoxin was concentrated by collecting it on a column (5 cm × 2.2 cm) of Whatman DE23 DEAE-cellulose and eluting with 0.8 M-NaCl in buffer, and dialysed overnight against 2 mM-potassium phosphate buffer, pH 7.5. Chromatography on hydroxyapatite was then carried out by the method of Matsubara (1968). Fractions with an E_{420}/E_{275} ratio of more than 0.45 were collected. The final yield was about 2.2 μmol (25 mg)/kg of leaves, with E_{420}/E_{275} ratio 0.46.

Preparation of Scenedesmus *ferredoxin*

The algal cells were broken by homogenization with glass beads by the method of Matsubara (1968). The supernatant, after removal of glass beads, was centrifuged and ferredoxin was extracted and purified in the same way as from the spinach homogenate. About 20 mg of ferredoxin with an E_{420}/E_{275} ratio of 0.65 was obtained/kg wet wt. of cells.

All ferredoxin samples after preparation were stored frozen under liquid N_2.

Reconstitution of ferredoxins with ^{57}Fe

The method was a modification of the methods of Lovenberg *et al.* (1963) and Bayer *et al.* (1967). Unless otherwise stated, operations were carried out under N_2 at 4°C. All reagents were flushed with N_2 before use.

Preparation of apoprotein. Ferredoxin (5 μmol) in 5 ml of phosphate buffer, pH 7.5, was treated with 1.7 ml of 20% (w/v) trichloroacetic acid and flushed with N_2 for 3 h to expel H_2S. The mixture was centrifuged at 10000*g* for 10 min. The precipitate was washed once with 5% (w/v) trichloroacetic acid and twice with water, then resuspended in 3 ml of 0.2 M-tris–HCl buffer, pH 8.5, and centrifuged as before. The precipitate was discarded and the protein in the supernatant was precipitated, washed and resuspended as before. The resulting solution of the apoprotein had no extinction peaks at 420 and 465 nm (Fig. 1). Analysis showed no detectable amounts of iron or labile sulphide.

Reconstitution. The apoferredoxin solution was incubated with 0.2 ml of 2-mercaptoethanol for 2 h at room temperature. Freshly prepared 0.1 M-Na_2S (0.2 ml) and 20 μmol of $^{57}FeSO_4$ were added and the mixture was warmed at 37°C for 15 min. The reconstituted ferredoxin was separated from excess of reagents by passage through a column (15 cm × 2.5 cm) of Sephadex G-25. It was then adsorbed on a small column of Whatman DE23 DEAE-cellulose, washed with phosphate buffer and eluted with 0.8 M-NaCl in buffer.

Chromatography. The ferredoxin at this stage contained contaminant ^{57}Fe, which was very tightly bound to apoferredoxin (see Keresztes-Nagy & Margoliash, 1966). The solution was diluted threefold with phosphate buffer and adsorbed on a column (35 cm × 2.5 cm) of Whatman DE52 DEAE-cellulose. Then 0.32 M-NaCl in phosphate buffer was passed slowly through the column. The apoferredoxin was eluted first, followed by a separate band of ferredoxin. Fractions with the highest E_{420}/E_{275} ratio were pooled (more than 0.42 for spinach ferredoxin, or 0.60 for *Scenedesmus* ferredoxin). Finally the ferredoxins were concentrated by adsorption on a column (0.5 cm × 1 cm) of Whatman DE23 DEAE-cellulose and elution with 1.5 M-NaCl–50 mM-phosphate buffer, pH 7.5. The yield of reconstituted ferredoxin was 40–60%.

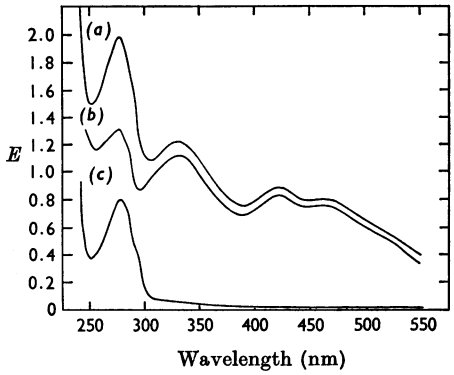


Fig. 1. Absorption spectra of: (*a*) ^{57}Fe-enriched reconstituted spinach ferredoxin, 0.092 mM; (*b*) ^{57}Fe-enriched reconstituted *Scenedesmus* ferredoxin, 0.083 mM; (*c*) spinach apoferredoxin, 0.062 mM.

Preparation of reduced ferredoxin samples

The Mössbauer cell, containing 0.7 ml of approx. 1 mM-ferredoxin, was placed in a glass phial fitted with a rubber cap, and flushed with argon. Then 10 μl of 0.14 M-sodium dithionite in 0.1 M-phosphate buffer, pH 7.5, was added with a microlitre syringe (final concentration, 2 mM). After 1 min the cell was frozen by immersing the phial in liquid N_2. The samples were stored in liquid N_2 at all times to prevent reoxidation of the ferredoxin.

RESULTS AND DISCUSSION

Characterization of the reconstituted ferredoxins. The ^{57}Fe-enriched reconstituted spinach and *Scenedesmus* ferredoxins had very similar absorption spectra to the native proteins (Fig. 1), and often showed a slightly higher E_{420}/E_{275} ratio. This indicates that they contained very little apoprotein.

Analysis of samples of ^{57}Fe-enriched reconstituted ferredoxin gave values of 2.1 g-atoms of iron and 2.0 of labile sulphide/mol for spinach, and 1.9 g-atoms of iron and 2.0 of labile sulphide/mol for *Scenedesmus*.

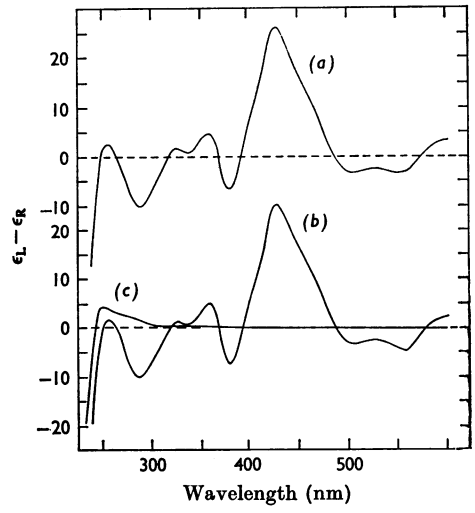


Fig. 2. Circular dichroism of: (*a*) native spinach ferredoxin; (*b*) ^{57}Fe-enriched spinach ferredoxin; (*c*) spinach apoferredoxin.

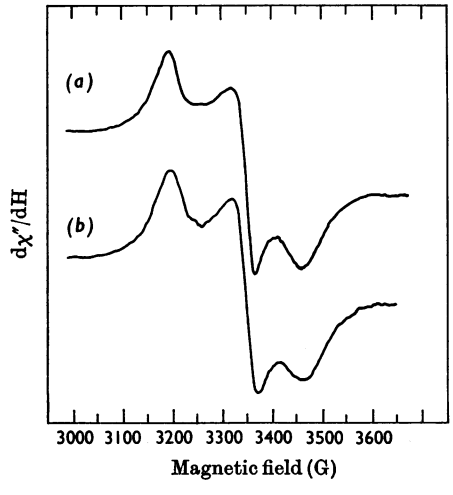


Fig. 3. E.p.r. signals from reduced spinach ferredoxin at 77°K: (*a*) native, 0.24mM; (*b*) reconstituted with ^{57}Fe, 0.24mM. Samples (0.1ml) in 3mm-bore tubes were reduced under argon with 2mM-sodium dithionite at 20°C for 1min before freezing. The settings for measurement were: microwave frequency 9.154GHz; power 10mW; modulation frequency 100KHz; modulation amplitude 4G.

No iron or labile sulphide could be detected in the apoproteins prepared from either ferredoxin.

Circular dichroism. Fig. 2 shows the circular-dichroism spectra of spinach ferredoxin, in the native, apo- and ^{57}Fe-enriched reconstituted forms. Very similar results were obtained with *Scenedesmus*. The spectrum of reconstituted ferredoxin is essentially identical with that of the native protein. The circular-dichroism spectrum is probably more sensitive than the absorption spectrum to changes in the overall conformation of ferredoxin, since it changes drastically on denaturation by reagents such as urea, whereas the absorption spectrum remains relatively unchanged (Garbett, Gillard, Knowles & Stangroom, 1967).

Table 1. *Observed g values of reduced ferredoxins*

	g_x	g_y	g_z
Scenedesmus	1.888	1.944	2.037
Spinach	1.884	1.949	2.041

The optical-rotatory-dispersion spectra of the reconstituted ferredoxins were also very similar to those of the native proteins.

E.p.r. spectra. In the reduced forms the native and reconstituted ferredoxins had similar e.p.r. signals centred around g 1.95 (Fig. 3 shows that for spinach ferredoxin). A small broadening was observed in the ^{57}Fe-enriched reconstituted ferredoxin spectrum, probably due to nuclear hyperfine interaction (Palmer, 1967). The observed g values are shown in Table 1. No e.p.r. signal was detected in the apoprotein on addition of sodium dithionite. The oxidized forms of the proteins did not show signals in the region of g 4.3, where contaminant ferric iron might be expected to absorb.

Biological activity. The biological activity of ^{57}Fe-enriched reconstituted spinach ferredoxin was measured by $NADP^+$ reduction by illuminated chloroplasts, by using the method of San Pietro (1963). These experiments were kindly performed for us by Dr M. C. W. Evans. The rate of $NADP^+$ reduction was proportional to ferredoxin concentration in the range 0–0.5μM. With 0.47μM-native spinach ferredoxin the rate was 48μmol/h per mg of chlorophyll, and with 0.45μM-^{57}Fe-enriched reconstituted ferredoxin the rate was 50μmol/h per mg of chlorophyll. No activity was detected with apoferredoxin.

Mössbauer spectra. The Mössbauer spectra of reduced *Scenedesmus* ferredoxin at several temperatures between 195 and 4.2°K, and with and without applied magnetic fields, are shown in Fig. 4. Spectra for reduced spinach ferredoxin are shown in Fig. 5. For the same physical conditions the spectra are essentially the same for the two species. The spectra of the oxidized proteins were also very similar for spinach and *Scenedesmus* (see Fig. 6, and Johnson *et al.* 1968, 1969). No Mössbauer absorption was detected in the apoproteins.

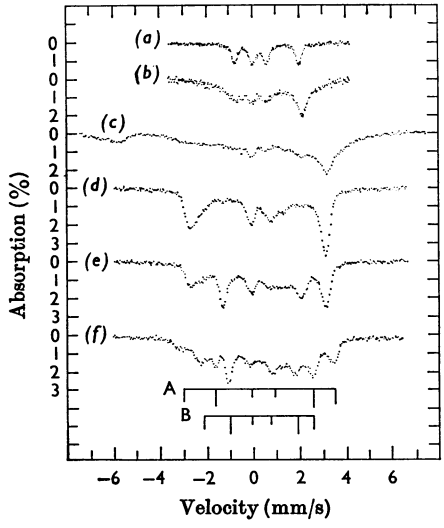


Fig. 4. Mössbauer spectra of reduced *Scenedesmus* ferredoxin, 1.9mM, enriched with ^{57}Fe: (*a*) at 195°K; (*b*) at 77°K; (*c*) at 4.2°K; (*d*) at 4.2°K, with a field of 0.1kG applied parallel to the γ-rays; (*e*) at 4.2°K, with a field of 0.3kG applied perpendicular to the γ-rays; (*f*) at 4.2°K, with a field of 30kG applied perpendicular to the γ-rays.

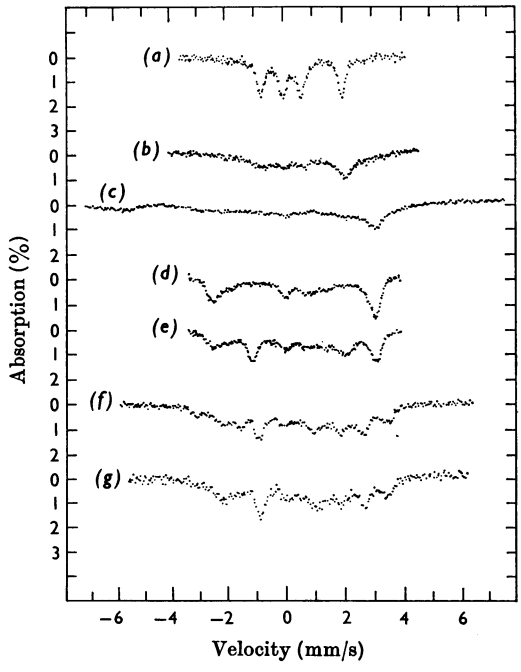


Fig. 5. Mössbauer spectra of reduced spinach ferredoxin, 1.0mM, enriched with ^{57}Fe: (*a*) at 195°K; (*b*) at 77°K; (*c*) at 4.2°K; (*d*) at 4.2°K, with a field of 0.1kG applied parallel to the γ-rays; (*e*) at 4.2°K, with a field of 0.3kG applied perpendicular to the γ-rays; (*f*) at 4.2°K, with a field of 30kG applied perpendicular to the γ-rays; (*g*) at 1.7°K, with a field of 30kG applied perpendicular to the γ-rays.

(1) High-temperature results (electric hyperfine structure). At high temperatures the magnetic hyperfine interaction averages to zero as the electron-spin-relaxation rates become very rapid compared with the nuclear-precession frequency. The Mössbauer spectrum then shows only electric hyperfine interactions: the chemical shift and quadrupole splitting (Figs. 4*a* and 5*a*).

Fig. 6 shows a comparison of the spectra of oxidized and reduced *Scenedesmus* ferredoxin measured at 195°K. These results, as remarked above, are identical with those reported for ferredoxins from spinach (Johnson *et al.* 1969) and *Euglena* (Johnson *et al.* 1968). The spectra of the oxidized ferredoxin (Fig. 6*a*) show a quadrupole splitting into two lines of almost equal width, with a chemical shift δ of +0.20mm/s relative to metallic iron and a quadrupole splitting ΔE_Q of +0.60mm/s. Close examination shows that the higher-energy line is always slightly broader than the lower-energy line and its depth slightly less; possibly this is due to a slight inequivalence of the two iron atoms even in the oxidized state.

The spectra of the reduced ferredoxins (Figs. 5*a* and 6*b*) show four lines of almost equal width (0.28mm/s, which is close to the natural width) and depth. These are a superposition of two quadrupole split doublets with the following parameters:

$$\delta_1 = +0.22\text{mm/s}, \quad (\Delta E_Q)_1 = 0.59\text{mm/s};$$
$$\delta_2 = +0.56\text{mm/s}, \quad (\Delta E_Q)_2 = 2.75\text{mm/s}$$

(errors are $\pm$0.005mm/s; shifts are relative to metallic iron). The first doublet is very similar to those of the oxidized protein, and it seems likely that it arises from iron atoms that are unchanged in state after the reduction. The second doublet has a larger chemical shift, which corresponds to a larger number of *d* electrons on those iron atoms, i.e. it strongly suggests that the electron transferred on reduction is entirely localized on one of the two iron atoms of the molecule, whereas the other remains unchanged.

On cooling of the reduced protein to 77°K (Figs. 4*b* and 5*b*) all the lines broaden as the magnetic hyperfine structure begins to appear; hence both the iron atoms are magnetic, i.e. they have unpaired *d* electrons. Since iron atoms of type 1 (small quadrupole splittings; δ_1) seem to be identical with those of oxidized ferredoxin, the latter must contain unpaired electrons also. The observations by e.p.r. (Palmer & Sands, 1966; Gibson *et al.* 1966), Mössbauer effect (Johnson *et al.* 1968) and magnetic susceptibility (Thornley *et al.* 1966; Moss *et al.* 1969) that the oxidized protein does not exhibit any bulk

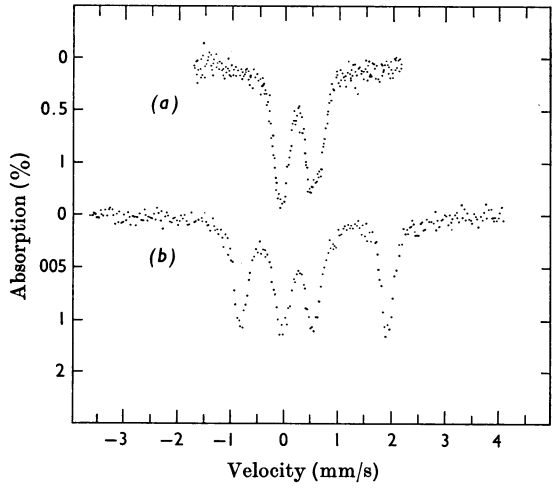


Fig. 6. Mössbauer spectra of *Scenedesmus* ferredoxin, enriched with ^{57}Fe, at 195°K: (*a*) oxidized, 1.75mM; (*b*) reduced, 1.9mM.

paramagnetic effects can only be reconciled with this conclusion if the magnetic moments of the two iron atoms in the molecule are antiferromagnetically coupled by exchange interactions to give a non-magnetic ground state. This has been suggested by Gibson *et al.* 1966 (see also Thornley *et al.* 1966) to account for the observed g values <2, and the Mössbauer spectra now provide direct evidence for spin coupling.

The larger quadrupole coupling (δ_2) for iron atoms of type 2 in the reduced state is entirely consistent with the asymmetrical charge distribution that could arise when a spherically symmetrical high-spin Fe^{3+} ion gains an electron to become a high-spin Fe^{2+} ion. Although it is always difficult to be sure of assignments of chemical states from Mössbauer results, especially when there may be strong covalent bonding present, the chemical shifts are consistent with a model where the oxidized protein contains two high-spin Fe^{3+} atoms and the reduced protein contains one high-spin Fe^{3+} and one high-spin Fe^{2+} atom, as previously suggested (Gibson *et al.* 1966).

(2) Low-temperature results (magnetic hyperfine structure). As the temperature of the reduced ferredoxin is lowered below 77°K the spin-lattice relaxation time increases and magnetic hyperfine splitting is observed in the Mössbauer spectrum. At 4.2°K the relaxation time is very much longer than the precession period of the nuclei in the hyperfine field, and the spectrum (Figs. 4*c* and 5*c*) does not change on cooling to lower temperatures.

This spectrum is very complicated and shows many very broad absorption lines as well as a few more prominent ones. The arrangement of the lines is asymmetrical and cannot be described in terms of an effective magnetic field at the iron nuclei, but has been interpreted (Johnson *et al.* 1968, 1969) by an approximately isotropic hyperfine coupling of the form $\mathscr{H} = A\boldsymbol{S}\cdot\boldsymbol{I}$. This would give a three-line spectrum in the zero field, which would become a symmetrical Zeeman pattern if a small magnetic field H, such that $g\beta H > A$, is applied to decouple the nuclear and electronic moments, and this is observed (Figs. 4*d*, 4*e*, 5*d* and 5*e*).

However, although the zero-field spectrum fits this picture in a rough qualitative way, in detail it is more complicated than this and contains many more than three lines. In a small field the spectrum seems to consist of a single Zeeman pattern only, i.e. it appears that the iron atoms are all equivalent, although the high-temperature spectrum (Figs. 4*a* and 5*a*) clearly showed that they are different. To pursue this further we compared the Mössbauer spectra of reduced spinach ferredoxin containing iron enriched in ^{57}Fe with that containing ordinary natural iron, which contains 2.2% of the ^{57}Fe, the isotope which is detected by the Mössbauer effect. E.p.r. results on two other isotopically enriched two-iron one-electron proteins (putidaredoxin and adrenodoxin, which may be different from plant-type ferredoxins) have been interpreted as providing evidence that the two iron nuclei are coupled to one unpaired electron (Tsibris *et al.* 1968; Orme-Johnson & Beinert, 1969*a*). However, e.p.r. results of ^{57}Fe-enriched spinach ferredoxin did not show similar behaviour (Palmer, 1967). If the two iron nuclei are equivalent and coupled to a single electron spin S, the zero-field spectrum of a sample enriched in ^{57}Fe will be described by the spin-hamiltonian:

$$\mathscr{H}_{\text{pair}} = A\boldsymbol{S}\cdot\boldsymbol{I}_1 + A\boldsymbol{S}\cdot\boldsymbol{I}_2 = A\boldsymbol{S}\cdot(\boldsymbol{I}_1+\boldsymbol{I}_2)$$

where I_1 and I_2 are the spins of the ^{57}Fe nuclei. The Mössbauer spectrum would then be different from that of an unenriched sample, where the majority of the molecules have only one ^{57}Fe nucleus, the other iron atom having an isotope with no nuclear-magnetic moment, each giving a Mössbauer spectrum arising from:

$$\mathscr{H} = A\boldsymbol{S}\cdot\boldsymbol{I}.$$

Fig. 7 compares the Mössbauer spectra measured at 4.2°K for reduced spinach ferredoxin (*a*) made from natural iron, and (*b*) made from iron enriched to 90% in ^{57}Fe. The unenriched sample had a concentration of 8mM and the enriched sample was 1mM. From the ratio of about 5:1 found for the intensities of the Mössbauer absorption in the two specimens, it can be seen that the enrichment of ^{57}Fe obtained in the ferredoxin is close to 90%,

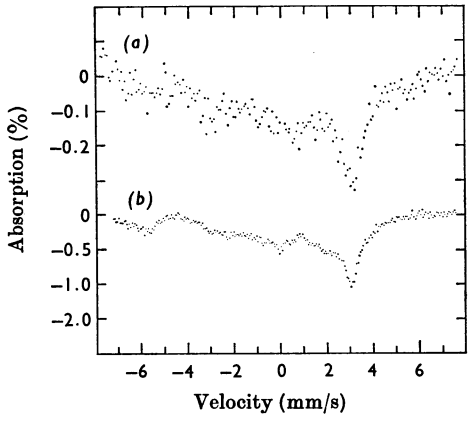


Fig. 7. Mössbauer spectra of reduced spinach ferredoxin at 77°K: (*a*) native ferredoxin, 8.0mM; (*b*) ^{57}Fe-enriched ferredoxin, 1.0mM.

i.e. approximately that of the isotopically enriched iron used for the exchange process. Although the ratio of the absorption to statistical error is much worse in the unenriched specimen, the results are consistent with the two spectra being the same. The hamiltonian $\mathscr{H}_{\text{pair}}$, therefore, does not describe the hyperfine interaction in the Mössbauer spectrum of spinach ferredoxin, and confirms e.p.r. results, which showed very little splitting of the spectrum by ^{57}Fe enrichment (Palmer, 1967).

Our results are, however, more compatible with a model of the reduced ferredoxin in which the two iron atoms are inequivalent, with different spins S_1 and S_2, and hyperfine couplings A_1 and A_2, which are not necessarily isotropic. To fit this with our conclusions from high-temperature spectra we should take $S_1 = 5/2$ in the Fe^{3+} atom and $S_2 = 2$ in the Fe^{2+} atom. The two iron nuclei are now coupled to a single electron spin and we would then expect two hyperfine spectra from energy levels given by the hamiltonians:

$$\mathscr{H}_1 = \boldsymbol{S}_1 \cdot \boldsymbol{A}_1 \cdot \boldsymbol{I}$$
$$\mathscr{H}_2 = \boldsymbol{S}_2 \cdot \boldsymbol{A}_2 \cdot \boldsymbol{I}$$

When a small magnetic field H is applied such that $g\beta H > \hbar/A_1$ and $\hbar/A_2$, the effective hyperfine couplings become:

$$\mathscr{H}_1 = A_{1z} S_{1z} I_z$$
$$\mathscr{H}_2 = A_{2z} S_{2z} I_z$$

where z is the direction about which the resultant electron spin $S = S_1 + S_2$ precesses, i.e., since S is approximately isotropic, z is the direction of the magnetic field. The effective magnetic fields at the nuclei at the two sites are:

$$H_1^n = A_{1z}\langle S_1 \rangle / g_n \beta_n$$

and

$$H_2^n = A_{2z}\langle S_2 \rangle / g_n \beta_n$$

where g_n and β_n are the nuclear g-value and magneton respectively.

When a small field is applied, only one Zeeman hyperfine pattern is observed (see Figs. 4*d*, 4*e*, 5*d* and 5*e*). The lines are broad because the hyperfine interaction is not isotropic; nor is the quadrupole splitting zero, and so the energy of the γ-rays depends on the angle the field makes with the axes of the magnetic hyperfine and electric field gradient tensors. The asymmetric line positions show that there is a quadrupole coupling whose component is negative along the direction of the effective hyperfine field (the value of this component is small probably because the effective hyperfine field lies at a large angle to the symmetry axes of the electric field gradient tensor). The asymmetry in the line shapes shows that the anisotropy of the hyperfine interaction is such that $|A_{x,y}| > |A_z|$. Thus, unless our conclusions up to this point are wrong, the components of the hyperfine interaction along the axis of S are by coincidence equal. This can be tested by applying a magnetic field large enough to produce a detectable direct shift in the Mössbauer lines.

When a large magnetic field H is applied the spectrum would be expected to split into two Zeeman patterns, with effective fields of $|H_1^n - H|$ and $|H_2^n + H|$. These correspond to the external field adding to the hyperfine (internal) field at the iron nucleus in the Fe^{2+} atom, and subtracting at the Fe^{3+} (the sign of the hyperfine field is negative, i.e. opposite to the spin of the atom, in each ion). This confirms that S_1 and S_2 are coupled antiparallel to each other, and that the hyperfine fields are almost equal.

On the model proposed by Gibson *et al.* (1966) and Thornley *et al.* (1966) $\langle S_1 \rangle = 7/6$ and $\langle S_2 \rangle = -2/3$. Since for the Fe^{3+} ion the hyperfine field is isotropic and arises from the contact interaction:

$$H_1^n = \left(\frac{7/6}{5/2}\right) H_0$$

where H_0 is the hyperfine field for an uncoupled high-spin Fe^{3+} ion in the same environment. The experimental result shows that $H_1^n = -180$kG and so $H_0 \simeq -380$kG; this is considerably less than that found in ionic ferric compounds, which have values typically around 500–600kG, but the decrease can be plausibly attributed to the effect of covalency producing electron delocalization on to the sulphur ligands.

The contact part of the hyperfine field of the Fe^{2+} ions $[(H_2^n)_c]$ may be found by using this value of H_0, since the covalency for the two iron atoms is presumably very similar. Thus:

$$(H_2^n)_c = \left(\frac{-2/3}{2}\right)H_0$$

which is 130kG. The value found is 180kG. Relative to the spin S_2 on the Fe^{2+} ion these values are −130kG and −180kG respectively, and the difference of −50kG must be due to non-contact hyperfine fields. Since the g values are close to 2 the orbital field is presumably small, so we ascribe the −50kG to a dipolar hyperfine field H_d. The negative sign seems to be in accord with the negative value of the electric field gradient (q) observed in the hyperfine spectra (Figs. 4*e* and 5*e*), since for Fe^{2+} ions:

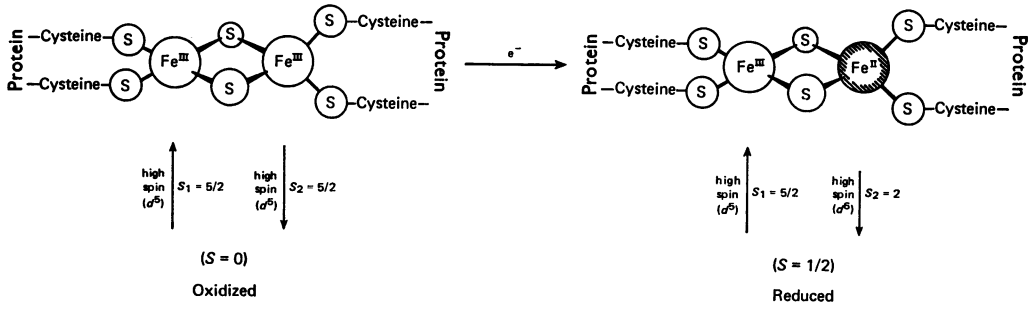
$$H_d = -\beta q$$

(Marshall & Johnson, 1962). The absence of information on the relative orientations of the electric field gradient and hyperfine tensors limits the amount of structural information that can be derived from the present results.

Proposed model. Scheme 1 illustrates the model we envisage for the iron–sulphur chromophore of plant-type ferredoxins. In the oxidized state the two high-spin ferric iron atoms have slightly different Mössbauer spectra and therefore have different environments (note that only one of these iron atoms accepts an electron on reduction). This difference is shown schematically as a difference in ligand bond angle to the cysteine sulphur atoms. The spins of the iron atoms are coupled in such a way that there is no net spin in the ground state ($S = 0$). When the ferredoxin is reduced one electron is transferred to the specific iron atom, which then becomes high-spin ferrous; thus in the reduced state there is one high-spin ferrous and one high-spin ferric iron atom. The spins on the iron atoms are still coupled, but there is a net spin $S = \frac{1}{2}$.

The rest of the model is based on recent results from other laboratories. The involvement of labile sulphide atoms in the active site was demonstrated by J. A. Fee & G. Palmer (unpublished work, presented at 4th International Congress on Magnetic Resonance in Biological Systems, Oxford, U.K., 1970), who found that the e.p.r. signal showed a small splitting if the sulphide was replaced by the selenium isotopes ^{77}Se and ^{80}Se; in other words, the electron in the reduced form was partly associated with the sulphur atoms. The other ligands to the iron are four cysteine residues of the protein, as shown by proton-magnetic-resonance contact shifts (W. D. Phillips, unpublished work presented at the 4th International Congress on Magnetic Resonance in Biological Systems, Oxford, U.K., 1970). These cysteine residues are presumably the invariant cysteines at positions 39, 44, 47, 77 of the amino acid sequence of the ferredoxin; the cysteine residue at position 19, which is present in spinach, *Scenedesmus*, alfalfa, *Lucaena glauca* and taro ferredoxins (see Rao & Matsubara, 1970) is absent from the ferredoxin from *Equisetum* (S. J. Aggarwal, K. K. Rao & H. Matsubara, unpublished work).

The photosynthetic system in which ferredoxin is involved requires that it should accept one electron at a very low redox potential (near to that of hydrogen at pH 7, −420mV). It is known that the plant ferredoxin molecule contains two iron atoms, although it accepts and donates only one electron. The model which we propose (Scheme 1) is consistent with this. At present it is not possible to predict accurately the redox potential of the proposed structure. However, it is noteworthy that the bacterial protein rubredoxin, which contains one high-spin ferric atom tetrahedrally co-ordinated to four sulphur ligands (from cysteine) (Herriot, Sieker, Jensen & Lovenberg, 1970) has a markedly higher redox potential, −60mV. In our model for ferredoxin, the high-spin ferric atom which undergoes reduction is also co-ordinated tetrahedrally to four sulphur ligands (two from cysteine and two from labile sulphide); its lower redox potential may



Scheme 1. Proposed model of the iron–sulphur group in plant ferredoxins.

be due to antiferromagnetic coupling to the second iron atom.

The improved Mössbauer results in this paper allow us to conclude that the oxidized ferredoxin molecule contains two high-spin ferric atoms which are not exactly equivalent; the reduced ferredoxin molecule contains one high-spin ferric atom and one high-spin ferrous atom.

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