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Physicochemical Characterization of the Four-Iron–Four-Sulphide Ferredoxin from *Bacillus stearothermophilus*

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1. A stable ferredoxin was prepared from *Bacillus stearothermophilus* and purified by chromatography on DEAE-cellulose and by electrophoresis. 2. The minimum molecular weight determined from the amino acid composition was about 7900 and this was in reasonable agreement with a value of 8500 determined by polyacrylamide-gel electrophoresis. The ferredoxin contained four iron atoms and four labile sulphide groups per molecule. 3. The optical absorption, optical-rotatory-dispersion and circular-dichroism spectra are typical of ferredoxins containing 4Fe–4S clusters. 4. Oxidation–reduction titrations, combined with electron-paramagnetic-resonance (e.p.r.) spectroscopy, showed that the protein has a mid-point potential, at pH 8, of -280 ± 10mV, and that only one electron-accepting paramagnetic species is present. 5. The e.p.r. spectrum of the reduced ferredoxin is more readily saturated with microwave power at low temperatures than those of the eight-iron ferredoxins, indicating that there is another mechanism of electron-spin relaxation in the latter. 6. Mössbauer spectra of both redox states were observed over a range of temperatures and in magnetic fields. At high temperatures (77°K and above) both redox states appear as quadrupole-split doublets; in the reduced state two resolved doublets are seen, suggesting appreciable localization of the additional reducing electron. 7. The average chemical shift indicates formal valences of two Fe^{3+} and two Fe^{2+} in the oxidized state and three Fe^{2+} and one Fe^{3+} in the reduced state. However, the spectra indicate that there are differing degrees of electron delocalization over the iron atoms. 8. At low temperatures (4.2°K) the oxidized form shows no hyperfine magnetic interaction, even in an applied magnetic field, evidence that the oxidized ferredoxin is in a non-magnetic state as a result of antiferromagnetic coupling between the iron atoms. 9. At 4.2°K the reduced form shows a broad asymmetric pattern resulting from magnetic hyperfine interaction. This contrasts with the reduced ferredoxin of *Clostridium pasteurianum*, which shows a doublet, suggesting that in the latter there may be interaction between the two 4Fe–4S centres. 10. In large applied magnetic fields, positive and negative hyperfine fields are seen in the Mössbauer spectra of the reduced ferredoxin, evidence for antiferromagnetic coupling between the iron atoms in the 4Fe–4S centre. The high-field spectra of the reduced ferredoxin of *B. stearothermophilus* are similar to those of the reduced ferredoxin of *C. pasteurianum*.

Thus far three different types of iron–sulphur proteins containing very similar 4Fe–4S centres have been isolated from bacteria. These types are represented by the eight-iron ferredoxins of *Clostridium*, the four-iron ferredoxins of *Bacillus* and *Desulfovibrio* and the four-iron high-potential iron–sulphur protein of *Chromatium*.

The first type of bacterial ferredoxins to be isolated have been found to contain eight iron atoms and eight labile sulphur atoms per molecule (mol.wt. about 6000). This was confirmed in the careful analysis of the ferredoxin of *Clostridium acidi urici* (Hong & Rabinowitz, 1970), and in the X-ray-crystallographic analysis of that of *Peptococcus aerogenes* (Jensen *et al.*, 1973). These iron atoms appear to occur in two nearly identical four-iron units.

Bacterial ferredoxins containing just one four-iron centre have been reported in *Desulfovibrio gigas* (LeGall & Dragoni, 1966), *Desulfovibrio desulfuricans* (Zubieta *et al.*, 1973), *Bacillus polymyxa* (Shethna *et al.*, 1971) and *Spirochaeta aurantia* (Johnson & Canale-Parola, 1973). The first half of the primary

structure of the ferredoxin of *D. gigas* bears a strong resemblance to that of each half of the *Clostridium* ferredoxins (Travis *et al.*, 1971).

A third related type of iron–sulphur protein is the high-potential iron–sulphur protein from purple photosynthetic bacteria, e.g. *Chromatium*. This contains a four-iron centre like the ferredoxins, but has a considerably higher redox potential (+350mV compared with −400mV for the other bacterial ferredoxins). It has an e.p.r.* signal in the oxidized state whereas with the ferredoxins a signal is only observed in the reduced state. These differences are rationalized by the 'three-state hypothesis' of Carter *et al.* (1972). This hypothesis proposes that oxidized eight-iron ferredoxin and reduced high-potential iron–sulphur protein represent an equivalent non-magnetic state of the four-iron centre, C; the ferredoxin can be reduced to give a paramagnetic state, C^-, whereas the high-potential iron–sulphur protein can be oxidized to give a different paramagnetic state, C^+. It may be possible to prepare a 'super-oxidized' ferredoxin in a state C^+ and a 'super-reduced' high-potential iron–sulphur protein in a state C^-, and there is evidence that these states exist (Sweeney *et al.*, 1974; Cammack, 1973). Formal valences for the iron atoms in these states based on chemical shifts have been suggested by Thompson *et al.* (1974); these agree with those for the analogue compounds $Fe_4S_4[RS]_4$ (Herskovitz *et al.*, 1972).

Mössbauer spectra of reduced eight-iron ferredoxins at high fields indicate the presence of antiferromagnetic coupling between the iron atoms in the four-iron centre. This is also supported by measurements of magnetic susceptibility and of contact-shifted proton resonances in the n.m.r. spectra (Poe *et al.*, 1970). Similar contact-shifted resonances have been observed in *B. polymyxa* four-iron ferredoxin in the oxidized and reduced states by Phillips *et al.* (1974).

It is important to compare the properties of the four-iron ferredoxins with those of the eight-iron ferredoxins in order to detect the extent to which the two centres interact in the latter. Poe *et al.* (1970) have shown that there is fast electron exchange (presumably intramolecular) between centres in the eight-iron ferredoxins which is not observed in the four-iron ferredoxins (Phillips *et al.*, 1974). In addition it has been proposed, on the basis of e.p.r. measurements, that the two centres in reduced eight-iron ferredoxins are spin-coupled (Mathews *et al.*, 1974). One may therefore expect the Mössbauer spectra to show a difference between the magnetic behaviour of the four-iron ferredoxins and that of the eight-iron ferredoxins.

* Abbreviations: e.p.r., electron paramagnetic resonance; o.r.d., optical rotatory dispersion; c.d., circular dichroism.

We have isolated a particularly stable four-iron ferredoxin from *B. stearothermophilus* and report here some physicochemical properties of the protein and a study by Mössbauer spectroscopy. These enable a comparison to be made between this four-iron ferredoxin and the eight-iron ferredoxins.

Experimental

Materials

Bacillus stearothermophilus cells were grown at 60°C in a medium based on that of Sargeant *et al.* (1971) and were obtained from the Microbiological Research Establishment, Porton Down, Wilts., U.K.

Analysis

Iron was determined by using bathophenanthroline as described by Evans *et al.* (1973); sulphide was determined as described by Lovenberg *et al.* (1963). After hydrolysis of samples in 6M-HCl for 24 and 48h, amino acids were determined on a Technicon AutoAnalyzer. Cysteine was determined after prior oxidation to cysteic acid as described by Hirs (1967). Protein concentration was determined by the microbiuret method (Itzhaki & Gill, 1964), by using *C. pasteurianum* ferredoxin as the standard. The concentration was confirmed by measuring the refractive increment of solutions of ferredoxin relative to their 48h diffusate. The refractive increment was measured with a Brice–Phoenix differential refractometer and the concentration determined by assuming a specific refractive increment of 1.83.

All operations were carried out at 4°C and all solutions were buffered with 20mM-Tris–HCl, pH8.5. The frozen cell paste (500g batches) was broken up and left overnight at 4°C with 1 litre of buffer containing 5mM-2-mercaptoethanol. The cell paste was filtered through muslin and the cells were smashed in a Manton–Gaulin homogenizer, run at a setting of 550kg·cm^{-2} (54MPa) pressure for 5min. The broken cells were centrifuged at 23000*g* for 1.5h. The supernatant was poured off and mixed with 50ml of a suspension of DEAE-cellulose (Whatman DE 23; Whatman Biochemicals, Maidstone, Kent, U.K.). The precipitated cells were mixed with 500ml of buffer and again centrifuged at 23000*g* for 1.5h. The supernatant was mixed with the initial supernatant and a further 25ml of a suspension of DEAE-cellulose was added. The suspension of DEAE-cellulose was stirred and left to settle for 30min, the supernatant was poured off and the DEAE-cellulose put in a column and washed with 0.1M-NaCl. The ferredoxin was eluted as a dark band with 0.8M-NaCl. The eluted impure ferredoxin was diluted fourfold with buffer and concentrated to about 40ml by ultrafiltration

(Diaflo PM 10 membrane; Amicon, High Wycombe, Bucks., U.K.). The solution was fed on to a column (30cm×2cm) of DE 23 cellulose and the column was washed with 150ml of 0.2M-NaCl. The column was further washed with 50ml of 0.25M-NaCl, and then eluted with a linear NaCl gradient (500ml of buffer containing 0.25M-NaCl and 500ml of buffer containing 0.5M-NaCl). Inspection of the eluted fractions showed a distinct brown colour from about 0.33M-NaCl onwards. The brown eluate was pooled, diluted with an equal volume of buffer and concentrated by pumping on to a small column (2cm×1cm) of DE 23 cellulose and eluting with buffer containing 0.8M-NaCl. The eluate was further concentrated by ultrafiltration on a collodion membrane (Sartorius, Göttingen, West Germany).

The concentrated sample was then subjected to electrophoresis on polyacrylamide gel. About 20 gel tubes of internal diameter 7mm were half-filled with polyacrylamide gel containing 10% (w/v) acrylamide and 0.35% methylenebisacrylamide. When the brown ferredoxin bands had travelled about 20mm, they were cut out and left overnight at 4°C with a suspension of DE 23 cellulose. The DE 23 cellulose suspension was put in a column, washed with buffer and the ferredoxin eluted with buffer containing 0.8M-NaCl. About 10mg of ferredoxin with an E_{390}/E_{280} ratio of 0.61 was prepared from 500g of cell paste.

^{57}Fe-enriched ferredoxin was prepared as described by Thompson *et al.* (1974). The yield of pure ^{57}Fe-substituted ferredoxin was about 80% with an E_{390}/E_{280} ratio of 0.67.

Molecular-weight estimation

The molecular weight was estimated from a plot of the logarithm of the mean residue molecular weight against the distance travelled during electrophoresis in polyacrylamide gel containing 15% (w/v) polyacrylamide, 0.4% methylenebisacrylamide, 0.1% sodium dodecyl sulphate and 3% urea. The ferredoxins of spinach, *Chromatium*, *C. pasteurianum* and pig adrenal were used for comparison.

Physical properties

O.r.d. and c.d. spectra were recorded on a Fica recording spectropolarimeter (Sofica, St. Denis, France). E.p.r. spectra were recorded on a Varian E4 spectrometer, with a flow of cold He gas to cool the samples.

Redox potential

The mid-point potential of the ferredoxin was determined by measuring the size of the e.p.r. signal of the reduced form as a function of the potential. A solution of ferredoxin (approx. 10μM) in 0.1M-Tris–HCl, pH8.0, was stirred under Ar at 25°C in an apparatus similar to that described by Dutton (1971). The redox potential was continuously monitored with platinum and calomel electrodes, and the pH with a glass electrode. Mediators present were 2-hydroxy-1,4-naphthoquinone, phenosafranine, Benzyl Viologen and Methyl Viologen (BDH Chemicals Ltd., Poole, Dorset, U.K.), all at a concentration of 0.2mM. The potential was adjusted with small additions of 25mM-$Na_2S_2O_4$ solution and after equilibration for 1min e.p.r. samples were taken under Ar and frozen.

Mössbauer spectra

These were obtained with a source of ^{57}Co in rhodium. Ferredoxin samples, at a concentration of 1.5mM, were held in nylon cells of approx. 5mm thickness. 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 small applied magnetic fields and the large fields were obtained with a superconducting magnet.

Results and Discussion

During chromatography and electrophoresis, only one form of ferredoxin was detected in *B. stearothermophilus*. No ferredoxin was detectable by e.p.r. spectroscopy of the fractions that came off the DEAE-cellulose column before the brown ferredoxin band, and only one band was observed on electrophoresis. This is in contrast with *B. polymyxa*, where two forms of four-iron ferredoxin have been reported (Yoch, 1973; Stombaugh *et al.*, 1973). The ferredoxin of *B. stearothermophilus* resembled the more highly charged ferredoxin of *B. polymyxa*, since it was eluted with a similar concentration of NaCl from DEAE-cellulose.

The amino acid composition (Table 1) showed a high proportion of acidic amino acids and an absence of histidine and arginine, as reported for several other four-iron ferredoxins (Zubieta *et al.*, 1973; Yoch, 1973). The minimum molecular weight was calculated from these results to be about 7900 (including four iron atoms and four sulphur atoms per molecule). This was in reasonable agreement with the value of 8500 obtained by polyacrylamide-gel electrophoresis (Fig. 1). The graph of distance migrated against logarithm of the molecular weight of ferredoxins was curved, as has been previously reported for proteins with molecular weights of less than about 15000 (Neville, 1971). The molecular weight is a little lower than that reported for *B. polymyxa* (Shethna *et al.*, 1971), but higher than the mol.wt. of approx. 6000 reported for the ferredoxins of *D. gigas* and *D. desulfuricans* (Zubieta *et al.*, 1973).

Table 1. *Amino acid composition of B. stearothermophilus ferredoxin*

Values are given as the nearest integer for the minimum molecular weight.

	Content (residues/molecule)		
Asx	15	½ Cys	4
Thr	3	Met	2
Ser	1	Ile	7
Glx	6	Leu	2
Pro	5	Phe	2
Gly	5	Lys	4
Ala	6	Tyr	3
Val	4	His	Not present
		Arg	Not present

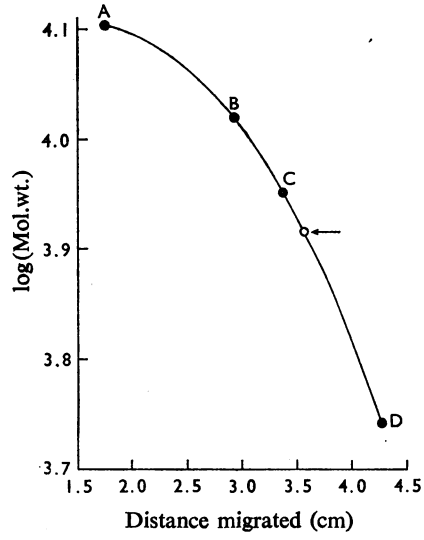

Fig. 1. *Logarithm of molecular weight of ferredoxins plotted against the distance moved during polyacrylamide-gel electrophoresis*

The standard ferredoxins were: A, pig adrenal; B, spinach; C, *Chromatium*; D, *C. pasteurianum*; the *B. stearothermophilus* ferredoxin is marked with an arrow.

Analysis of the iron content showed four iron atoms per molecule, but the sulphide content was lower than expected, being nearer three groups per molecule. Unexpectedly low values of sulphide have been obtained with other bacterial ferredoxins (Lovenberg, *et al.*, 1963).

Optical spectra

The optical absorption, o.r.d. and c.d. spectra of the ferredoxins and rubredoxins are characteristic

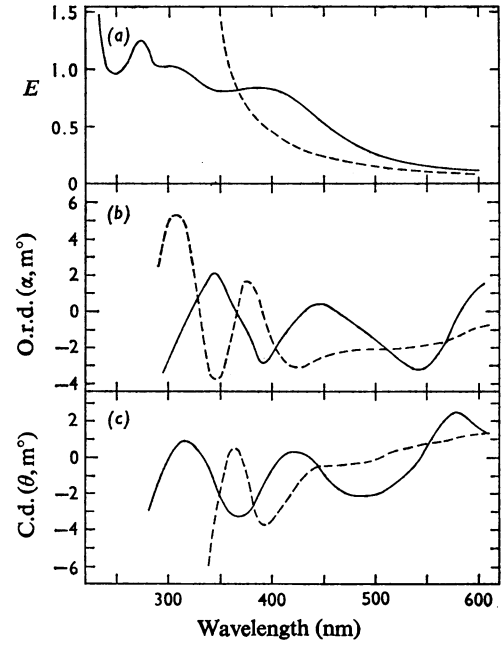

Fig. 2. (*a*) *Optical absorption,* (*b*) *o.r.d. and* (*c*) *c.d. spectra of a sample of B. stearothermophilus ferredoxin*

——, Oxidized form; – – – –, form reduced with excess of $Na_2S_2O_4$ solution. Ferredoxin concentration, approx. 81 μM.

of the type of iron–sulphur centres that they contain (see Hall *et al.*, 1974). These spectra for the oxidized and reduced states of *B. stearothermophilus* ferredoxin are shown in Fig. 2. The optical absorption spectra are similar to those of *B. polymyxa* ferredoxin (Yoch, 1973), with a broad maximum at 390nm and a peak at 278nm. The corresponding spectra are similar to those of the oxidized ferredoxin of *C. pasteurianum* or the reduced high-potential iron–sulphur protein of *Chromatium*, which are in an equivalent oxidation state. The absorption at 390nm decreases by about 40% on reduction by dithionite.

The o.r.d. and c.d. spectra in the region 300–600nm are weak in comparison with those of the plant-type two-iron ferredoxins (Garbett *et al.*, 1967). In their intensity and shape, the spectra of *B. stearothermophilus* ferredoxin resemble those of eight-iron ferredoxins of *C. pasteurianum* and *Chromatium*, which is strong evidence that the iron–sulphur centres in these proteins are similar.

E.p.r. spectra

Fig. 3 shows the spectrum of *B. stearothermophilus* ferredoxin in the reduced state. This spectrum is very similar to those observed in *B. polymyxa* ferredoxin

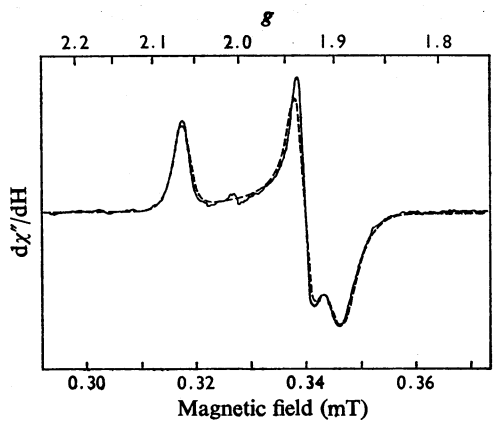

Fig. 3. *E.p.r. spectra of reduced B. stearothermophilus ferredoxin*

——, Native (^{56}Fe); – – – –, enriched with ^{57}Fe. Ferredoxin concentration, approx. 0.5mM. Instrument settings; modulation amplitude, 0.4mT (4G); modulation frequency, 100kHz; microwave power, 1mW; microwave frequency, 9.2GHz; temperature, 12°K.

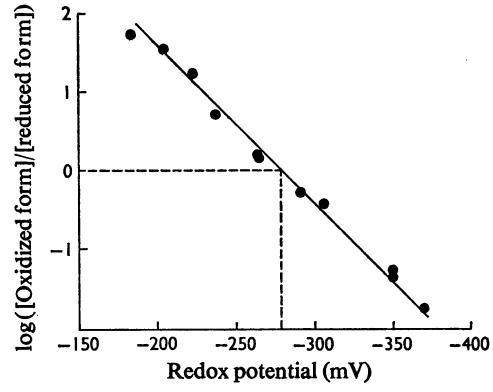

Fig. 4. *Redox-potential titration of B. stearothermophilus ferredoxin at pH* 8.0

Conditions of titrations were as described in the Experimental section. The proportions of oxidized and reduced forms were determined from the peak-to-peak height of the e.p.r. signal. The slope of the line is that expected for a one-electron reduction process.

(Shethna *et al.*, 1971) and that of *D. desulphuricans* (Zubieta *et al.*, 1973). In the oxidized state there was a signal, variable in intensity but always small, at $g = 2.02$, similar to that observed in oxidized *C. pasteurianum* ferredoxin (Orme-Johnson & Beinert, 1969). This latter signal might be due to a contaminant or to a higher oxidation state of the 4Fe–4S centre (Sweeney *et al.*, 1974).

Comparison of the e.p.r. spectra of native and ^{57}Fe-reconstituted ferredoxin (Fig. 3) indicates a broadening owing to hyperfine interaction with the ^{57}Fe nuclei. The large line-width of the spectrum and the small splitting [of the order of 1mT (10G)] prevents any detailed analysis of the hyperfine splitting.

The temperature-dependence of the e.p.r. spectrum is intermediate between that of the 2Fe–2S and that of 8Fe–8S ferredoxins. The signal is extremely broad at 77°K and is clearly seen at temperatures below 35°K, as in the eight-iron ferredoxin. However, at about 10°K, the signal is considerably saturated at a microwave power of 1mW, whereas the 8Fe–8S ferredoxins are not easily saturated at temperatures above 6°K. The saturation behaviour of *B. stearothermophilus* ferredoxin is similar to that of the 4Fe–4S high-potential iron–sulphur protein from *Chromatium* in its oxidized and 'super-reduced' forms. This suggests that, assuming that the 4Fe–4S clusters are the same in all three proteins, the clostridial-type ferredoxins have a mechanism of electron-spin relaxation at low temperatures which is not present in the four-iron proteins. Such a mechanism might be provided by spin–spin relaxation between the two reduced 4Fe–4S clusters.

In concentrated solutions of reduced eight-iron ferredoxins, a very weak e.p.r. signal at $g = 3.88$ is observed (Mathews *et al.*, 1974), and is assigned to a $\Delta M_s = 2$ transition as a result of spin-coupling between the four-iron centres. Examination of this region of the spectrum of *B. stearothermophilus* ferredoxin showed no detectable signal, which agrees with the above assignment.

Redox potential

E.p.r. samples taken at different redox potentials (see the Experimental section) showed an increase in signal intensity between −180mV and −389mV. The line-shape of the signal did not change during reduction, in contrast with the reduction of the eight-iron ferredoxins (Orme-Johnson & Beinert, 1969), which indicates the presence of only one paramagnetic species in reduced *B. stearothermophilus* ferredoxin.

Fig. 4 shows the data for e.p.r. signal intensity plotted according to the Nernst equation as a function of redox potential. The line has the theoretical slope for a one-electron-accepting species, and indicates a mid-point potential of -280 ± 10mV. This value is somewhat less reducing than the value of −330mV reported for the four-iron ferredoxin from *D. desulfuricans* (Zubieta *et al.*, 1973).

Mössbauer spectroscopic studies

Oxidized ferredoxin. The Mössbauer spectra of the oxidized ferredoxin are shown in Fig. 5. In the absence

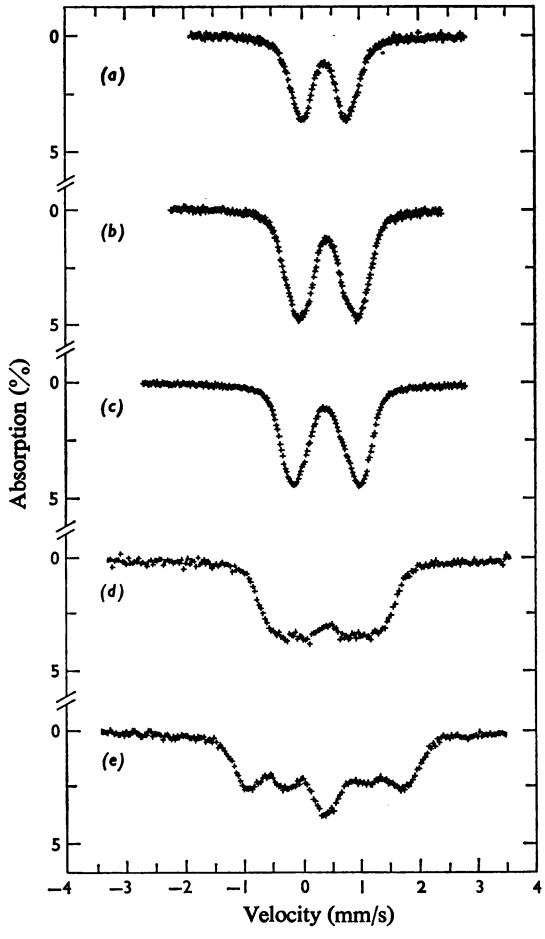

Fig. 5. *Mössbauer spectra of oxidized B. stearothermophilus ferredoxin*

(*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.

of an applied magnetic field, the spectra consist of quadrupole-split doublets. The 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 are not exactly equivalent. These spectra have been computer-fitted to two quadrupole-split doublet components and the chemical shifts and quadrupole splittings are given in Table 2. Because of the unreliability of the fitting procedure when the two doublets are unresolved, only average values of the Mössbauer parameters are quoted.

When strong magnetic fields are applied to the oxidized ferredoxin (Fig. 5, spectra *d* and *e*), the

Table 2. *Mössbauer data on B. stearothermophilus ferredoxin*

The chemical shifts δ (relative to pure iron) and the quadrupole splittings ΔE_Q were determined by least-squares computer fitting and are given in mm/s; for details see the text. Errors are ±0.01 mm/s for the chemical shifts and ±0.02 mm/s for the quadrupole splittings.

(*a*) Oxidized ferredoxin	T	$\delta_{av.}$	$\Delta E_{Q(av.)}$		
	195°K	0.37	0.77		
	77°K	0.42	0.98		
	4.2°K	0.42	1.08		
(*b*) Reduced ferredoxin	T	δ_1	ΔE_{Q1}	δ_2	ΔE_{Q2}
	195°K	0.55	1.53	0.44	1.15
	77°K	0.60	1.82	0.50	1.18
	35°K	0.62	1.83	—	—

spectra show a splitting that can be attributed to the applied field with no internal hyperfine field. The form of these spectra indicates a positive sign for the electric field gradient, although with a large asymmetry parameter. The lack of an internal hyperfine field confirms the e.p.r. evidence that the iron atoms are coupled to give a non-magnetic state with zero total spin.

Reduced ferredoxin. The Mössbauer spectra of the reduced ferredoxin at various temperatures and in applied magnetic fields are shown in Figs. 6 and 7. At 195°K and 77°K the spectra consist of two significantly different quadrupole-split doublets, which may themselves contain two slightly differing components. At 77°K the right-hand lines of these doublets are well resolved. These spectra were computer-fitted to two quadrupole-split doublets and the resulting parameters are given in Table 2. The two component doublets have almost equal areas, which implies that the four-iron centre contains two pairs of nearly equivalent iron atoms. In the 77°K spectrum the doublet with the larger quadrupole splitting and chemical shift arises from two atoms of ferrous character as confirmed by the temperature-dependence of the quadrupole splitting. The difference in resolution of the spectra between 77°K and 195°K might then be attributable to a slightly different temperature variation of the quadrupole interaction for these iron atoms.

At 35°K (Fig. 6, spectrum *c*) the spectrum consists of a single quadrupole-split doublet with broad lines, superimposed on a wide band of absorption characteristic of a magnetic spectrum with intermediate spin relaxation. The Mössbauer parameters of this doublet correspond to those of the ferrous component (i.e. the component with the larger chemical shift and quadrupole splitting) of the higher-temperature spectra. Thus the broad magnetic component in the 35°K spectrum must arise from the iron atoms

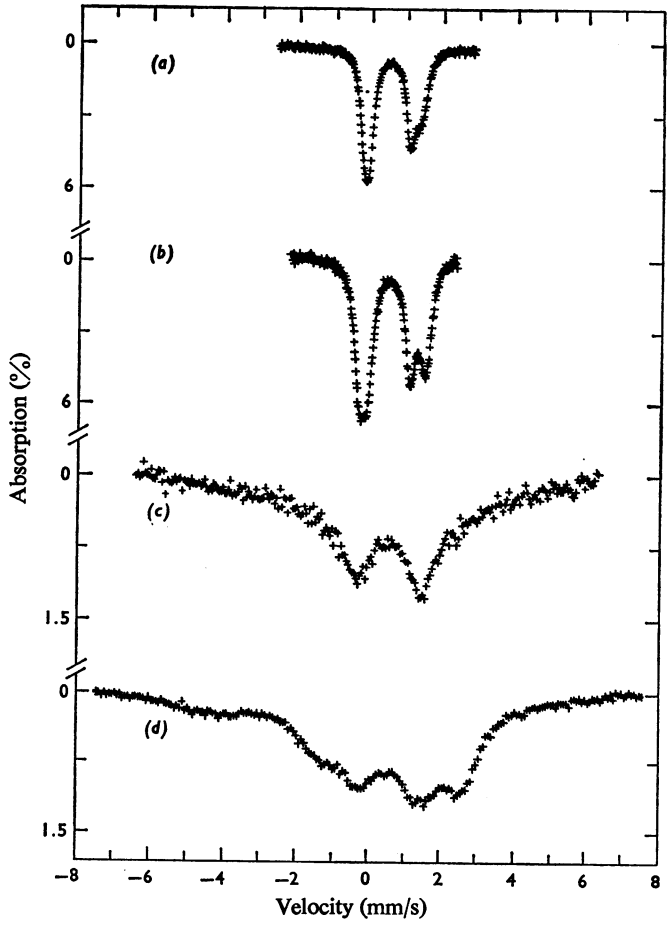

Fig. 6. *Mössbauer spectra of reduced B. stearothermophilus ferredoxin* (*a*) at 195°K, (*b*) at 77°K, (*c*) at 35°K, (*d*) at 4.2°K.

giving the more ferric component at the higher temperatures.

At 4.2°K the spectrum shows a broad asymmetric pattern of absorption resulting from magnetic hyperfine interaction and similar to that observed in reduced two-iron ferredoxins (Rao *et al.*, 1971) in 'super-reduced' *Chromatium* high-potential iron–sulphur protein (Dickson & Cammack, 1974), and in the reduced state of analogue compounds with 4Fe–4S centres (Frankel *et al.*, 1974). The effect of small magnetic fields (Fig. 7, spectra *b* and *c*) is to produce spectra resembling the familiar magnetic six-line pattern with broad lines, the intensity of the various lines being dependent on the applied field orientation. The 4.2°K, zero and low-field spectra of *B. stearothermophilus* ferredoxin are significantly different from those of *C. pasteurianum* ferredoxin (Thompson *et al.*, 1974), which at 4.2°K has a Mössbauer spectrum consisting of a broadened doublet.

The spectra of *B. stearothermophilus* ferredoxin in large applied magnetic fields are very closely similar to the equivalent spectra of *C. pasteurianum* ferredoxin (Thompson *et al.*, 1974). In the wings of the highest-applied-field spectra, lines (shown arrowed in Fig. 7, spectra *f* and *g*) can be distinguished which have moved out as the field increases, indicating iron atoms with a positive (i.e. parallel to the applied field) hyperfine field, which adds to the applied field to give the effective field at the nucleus. The lines in the wings of the low-field spectra (shown arrowed in Fig. 7, spectra *b* and *c*) which move in as the applied field is increased indicate iron atoms with a negative hyperfine field from which the applied field is subtracted to give the effective field at the nucleus. The presence of

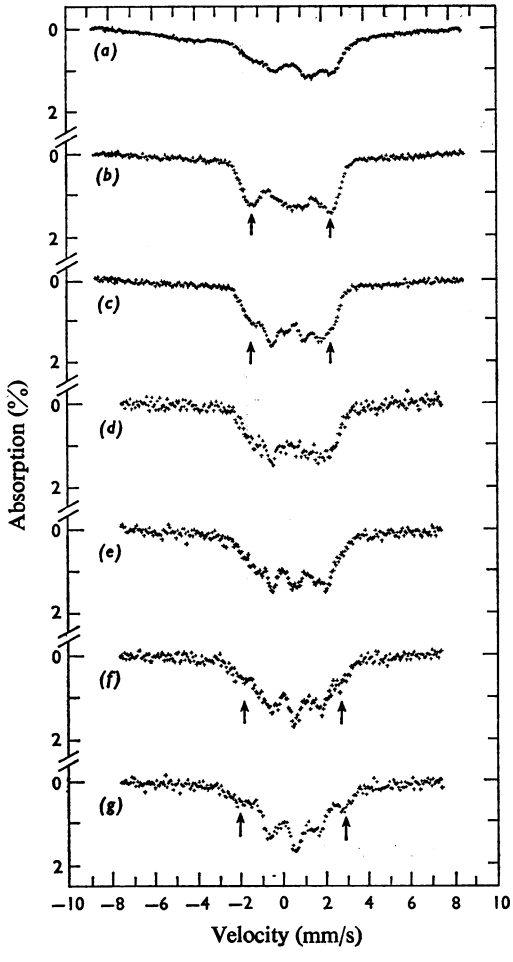

Fig. 7. *Mössbauer spectra of reduced B. stearothermophilus ferredoxin at 4.2°K showing the effect of applied magnetic fields*

The fields were (*a*) zero, (*b*) approx. 0.05T applied parallel to the γ-ray direction, (*c*) approx. 0.05T applied perpendicular to the γ-ray direction, (*d*) 1.5T perpendicular, (*e*) 3.0T perpendicular, (*f*) 4.5T perpendicular, (*g*) 6.0T perpendicular. For details of arrowed spectra see the text.

iron atoms with both positive and negative hyperfine fields implies the existence of antiparallel magnetic moments and hence antiferromagnetic coupling between the iron atoms.

Conclusions from Mössbauer-effect data. It is useful to consider the Mössbauer spectra of *B. stearothermophilus* ferredoxin in relation to the Mössbauer-effect data already obtained on the other iron–sulphur proteins with 4Fe–4S active centres.

It has been discussed how the chemical shift can be used to obtain information on the valence state of the iron in these proteins (Dickson *et al.*, 1974). The average values of the chemical shifts at 77°K, 0.42mm/s in the oxidized state and 0.55mm/s in the reduced state, are consistent with the oxidized form of the ferredoxin being a C state with formal valences of $2Fe^{3+}+2Fe^{2+}$ and the reduced form being a C^- state with formal valences of $1Fe^{3+}+3Fe^{2+}$.

The strong similarity between the Mössbauer spectra of the oxidized ferredoxin seen in Fig. 5, and the equivalent spectra of the oxidized ferredoxin of *C. pasteurianum* (Thompson *et al.*, 1974) and the reduced high-potential iron–sulphur protein of *Chromatium* (Dickson *et al.*, 1974), is confirmatory evidence that these proteins contain very closely related iron–sulphur centres, which in the case of the oxidized ferredoxins and reduced high-potential iron–sulphur protein are also in the C state of the 'three-state' scheme.

In the spectrum of reduced *B. stearothermophilus* ferredoxin at 77°K, although all iron atoms are affected on reduction, there is clear evidence of an appreciable localization of the extra reducing electron, as witnessed by the resolution of the quadrupole-split doublets from the different iron atoms. However, the Mössbauer parameters are not totally consistent with the presence of iron atoms with purely ferric and ferrous valences, as seen in the two-iron plant-type ferredoxins (Rao *et al.*, 1971). Some evidence for localization of the 3*d* electrons on particular iron atoms has also been found in the 'super-reduced' form of *Chromatium* high-potential iron–sulphur protein (Dickson & Cammack, 1974). The 77°K spectrum of the reduced ferredoxin of *C. pasteurianum* (Thompson *et al.*, 1974), however, is significantly different in that it indicates extensive electron delocalization, as shown by the lack of resolution of the component quadrupole-split doublets. This delocalization of the 3*d* electrons and equivalence of the iron atoms is also seen in the Mössbauer spectra of analogue compounds with 4Fe–4S centres (Frankel *et al.*, 1974).

The magnetic behaviour seen in the 4.2°K spectrum of *B. stearothermophilus* ferredoxin is similar to that observed in other systems with single 4Fe–4S centres, but contrasts with the broadened doublet seen in the 4.2°K spectrum of *C. pasteurianum* ferredoxin. The anomalous behaviour of the *C. pasteurianum* ferredoxin at 4.2°K may be due to rapid spin–spin relaxation arising from spin-coupling between its two centres, which has been previously suggested (Mathews *et al.*, 1974; Gersonde *et al.*, 1974). In large applied magnetic fields the spectra of *B. stearothermophilus* and *C. pasteurianum* ferredoxin are very similar, which implies that large applied magnetic fields destroy the interaction between the centres in the eight-iron ferredoxin and give a behaviour characteristic of the four-iron centres.

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