Mössbauer Effect in Rubredoxin

DETERMINATION OF THE HYPERFINE FIELD OF THE IRON IN A SIMPLE IRON-SULPHUR PROTEIN

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1. Rubredoxin isolated from the green photosynthetic bacterium *Chloropseudomonas ethylica* was similar in composition to those from anaerobic fermentative bacteria. Amino acid analysis indicated a minimum molecular weight of 6352 with one iron atom per molecule. 2. The circular-dichroism and electron-paramagnetic-resonance spectra of *Ch. ethylica* rubredoxin showed many similarities to those of *Clostridium pasteurianum*, but suggested that there may be subtle differences in the protein conformation about the iron atom. 3. Mössbauer-effect measurements on rubredoxin from *Cl. pasteurianum* and *Ch. ethylica* showed that in the oxidized state the iron (high-spin Fe^{3+}) has a hyperfine field of 370 ± 3 kG, whereas in the reduced state (high-spin Fe^{2+}) the hyperfine field tensor is anisotropic with a component perpendicular to the symmetry axis of the ion of about -200 kG. For the reduced protein the sign of the electric-field gradient is negative, i.e. the ground state of the Fe^{2+} is a d_{z^2} orbital. There is a large non-cubic ligand-field splitting ($\Delta/k = 900^\circ\text{K}$), and a small spin-orbit splitting ($D \sim +4.4 \text{ cm}^{-1}$) of the Fe^{2+} levels. 4. The contributions of core polarization to the hyperfine field in the Fe^{3+} and Fe^{2+} ions are estimated to be -370 and -300 kG respectively. 5. The significance of these results in interpretation of the Mössbauer spectra of other iron-sulphur proteins is discussed.

The Mössbauer effect is becoming increasingly useful in studying the nature of iron atoms in proteins (see Lang, 1970; Johnson, 1971). Mössbauer spectra may show a chemical shift (relative to a standard iron sample), a quadrupole splitting and a magnetic splitting, each of which is characteristic of the iron in the protein molecule. The chemical shift in principle allows the oxidation state of the iron to be determined, although in practice it is not generally very sensitive when the iron has strong covalent bonds. An exception to this is high-spin Fe^{2+} which has a large positive shift (of the order of 1 mm/s relative to metallic iron). The quadrupole splitting may also provide an indication of the oxidation state, but only indirectly, so that it is usually not possible to draw unambiguous conclusions from it. Most powerful and useful is the measurement of magnetic hyperfine splitting. This is different for each charge and spin state of iron, and may therefore be used to determine the valence state of the metal ion. It may also provide more detailed information such as the degree of covalent bonding (i.e. chemical coupling to the neighbours) and magnetic interaction (i.e. exchange coupling).

The present paper describes measurements on the non-haem iron protein rubredoxin by using Mössbauer spectroscopy. Rubredoxin was first isolated from *Clostridium pasteurianum* by Lovenberg & Sobel (1965). Subsequently rubredoxins have been isolated from many species (see Meyer *et al.*, 1971; Tsibris & Woody, 1970). The 'labile' sulphur which is found in other types of non-haem iron proteins such as ferredoxins has not been detected in rubredoxins. All rubredoxins, except that isolated from *Pseudomonas oleovorans*, have a molecular weight of about 6000, contain one iron atom per molecule, accept one electron on reduction and have an oxidation-reduction potential of about -0.06 V at pH 7. *P. oleovorans* rubredoxin, however, has a molecular weight of 19000, contains one or two iron atoms per molecule depending on the method of preparation and accepts one electron per iron atom on reduction (Lode & Coon, 1971). Rubredoxin from the aerobe *P. oleovorans* is part of a hydroxylase system involved in the oxidation of long-chain hydrocarbons and fatty acids (Boyer *et al.*, 1971; Peterson *et al.*, 1966). All the other rubredoxins are isolated from anaerobic bacteria and no function has yet been ascribed to them.

Rubredoxin from *Micrococcus aerogenes* was studied by Bachmayer *et al.* (1967), who suggested on the basis of the e.p.r.* spectrum, position of the cysteine residues in the amino acid sequence, and the reactivity of the protein towards thiol reagents, that the iron atom in the oxidized rubredoxin is high-spin Fe^{3+} , with rhombic symmetry, bonded to four cysteine residues of the protein. An X-ray crystallographic analysis of *Cl. pasteurianum* rubredoxin to 0.5 nm (5 Å) resolution (Watenpaugh *et al.*, 1971), confirmed that the iron atom of the protein is bound to four cysteine sulphur atoms to form an approximately tetrahedral complex.

Mössbauer-effect measurements on *C. pasteurianum* rubredoxin were reported by Phillips *et al.* (1970) in a study which also included proton magnetic resonance and magnetic susceptibility. In the oxidized form of rubredoxin a hyperfine interaction with an effective field at the nucleus of 375 ± 5 kG was observed. It was concluded that the iron atom in rubredoxin is high-spin Fe^{3+} in the oxidized form and high-spin Fe^{2+} in the reduced form. The e.p.r. spectrum of the oxidized protein (Lovenberg, 1966) has a signal at $g = 4.3$, which shows that the Fe^{3+} is in a strongly distorted non-axial ligand field which splits the ^6S ground state by several cm^{-1} .

The present paper describes more extensive Mössbauer studies on rubredoxin over a wider range of temperature and making use of applied magnetic fields to study the hyperfine splitting (Johnson, 1967a). One objective of the investigations was to determine the effect of covalent bonding to sulphur atoms on the hyperfine interaction of iron in a protein environment. In general it is known that covalency implies a strong overlap and admixing of *s*-electrons from the ligand atoms with the *d*-electrons of the iron (and vice versa), and this decreases the hyperfine interaction compared with that of a free ion. The values of the hyperfine fields in the Fe^{3+} and Fe^{2+} atoms in a sulphur environment would be valuable in obtaining a more quantitative interpretation of the Mössbauer results in the ferredoxins.

In this study we have used the rubredoxins from *Cl. pasteurianum* and the green photosynthetic bacterium *Chloropseudomonas ethylica*. The isolation of rubredoxin from the latter organism and from the related green photosynthetic bacterium *Chlorobium thiosulphatophilum* has recently been reported by Meyer *et al.* (1971), but the physicochemical properties of *Ch. ethylica* rubredoxin were not described. During our investigation, the chemical and physical properties of *Ch. ethylica* rubredoxin were investigated in some detail. Any difference between the rubredoxins of the anaerobic fermentative bacteria and the green photosynthetic bacteria would be of interest from the point of view of comparative bio-

chemistry. As in previous work with the ferredoxins, the iron in the native rubredoxins was replaced by ^{57}Fe isotope to increase the sensitivity of the Mössbauer measurements. The reconstituted proteins were freed from contaminating iron by gel filtration and chromatography on DEAE-cellulose. The purity and conformational intactness of the proteins were determined by measuring the optical absorption, electron-paramagnetic-resonance and circular-dichroism spectra.

Experimental

Cl. pasteurianum cells grown with NH_3 as nitrogen source were obtained from the Microbiological Research Establishment, Porton Down, Wilts., U.K. *Ch. ethylica* was grown in a modified Pfennig's medium with N_2 gas as nitrogen source as described previously (Evans & Smith, 1971). Protein was determined by the phenol method as described by Rabinowitz & Pricer (1962). Performic acid oxidation of aporubredoxin was done as described by Hirs (1967). Amino acid analysis of native and oxidized rubredoxin was carried out with the assistance of Mrs. R. Hynes by using a Beckman model B amino acid analyser.

Mössbauer spectra were observed by using a conventional velocity drive and a monochromatic source of ^{57}Co in palladium, and were accumulated on a multichannel analyser. Chemical shifts are expressed relative to metallic iron at room temperature.

Other materials used, and the methods of spectroscopic measurement and analysis were as described previously (Rao *et al.*, 1971).

Preparation of rubredoxins

The *Ch. ethylica* cells were processed in such a way as to isolate the nitrogenase, rubredoxin and the ferredoxin-type proteins. Unless otherwise specified the buffer used was 20 mM-tris-HCl, pH 8.5. All eluting solutions contained the buffer and were flushed with N_2 .

Frozen cells (stored in liquid N_2) were broken into small pieces and suspended in four times their volume of buffer containing 50 mM- MgCl_2 . The suspension was passed through a Manton-Gaulin homogenizer (A.P.V. Co. Ltd., Crawley, Sussex, U.K.) at a setting of 55.2 MN/m² (8000 lbf/in²) and homogenized with recycling. The homogenate was centrifuged for 20 min at 12000 rev./min in the 6 × 250 ml rotor of an MSE 18 refrigerated centrifuge. The supernatant was treated with polyethylene glycol (mol.wt. 6000; 10%, w/v) at pH 7.1 to remove the photosynthetic lamellae and then with protamine sulphate (20% by weight on a protein basis) to precipitate the nitrogenase. The supernatant contained ferredoxin, rubredoxin, and cytochromes. It was

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

diluted twofold with buffer and stirred with a suspension of DEAE-cellulose (Whatman DE 23; W. and R. Balston Ltd., Maidstone, Kent, U.K.). The DEAE-cellulose was transferred to a column (30cm×8.8cm), washed with 0.2M-NaCl and eluted with 0.8M-NaCl. The eluate was diluted fivefold with buffer and adsorbed on top of a column (45cm×2.5cm) of DE 23 cellulose equilibrated with 0.1M-NaCl. The proteins were eluted from the column by chromatography by using successively 0.25M- and 0.4M-NaCl. Eluates containing rubredoxin were monitored by measuring E_{490} . The rubredoxin was further purified by chromatography on a column (35cm×2.5cm) of Sephadex G-50 followed by a column (35cm×2.5cm) of DE 23 cellulose in 0.25M-NaCl. The yield of rubredoxin was about 10mg/kg wet wt. of cells.

Cl. pasteurianum cells were broken in the Manton-Gaulin homogenizer; the extract was centrifuged and the supernatant was used for isolation of rubredoxin by DE 23 cellulose chromatography as described above.

Preparation of ^{57}Fe -substituted rubredoxins

Rubredoxin (approx. 20mg) in 5ml of buffer was treated with 3ml of 20% (w/v) trichloroacetic acid for 15min at 35°C. The mixture was centrifuged at 10000g for 10min. The precipitate was washed with 5% trichloroacetic acid and with water and then dissolved in 3ml of 0.2M-tris-HCl buffer, pH8.5. The protein was reprecipitated with trichloroacetic acid, centrifuged and washed as before. The precipitate was dissolved in 3ml of 0.2M-tris-HCl buffer as before. This solution of aporubredoxin showed no optical absorption or circular dichroism in the visible region. The apoprotein solution was incubated with 0.2ml of 2-mercaptoethanol for 2h at room temperature in N_2 . Then 20 μmol of $^{57}\text{FeSO}_4$ was added and the mixture was incubated at 37°C for 10min. The reconstituted rubredoxin was separated from excess of reagents by passage through a column of Sephadex G-25. It was further purified by chromatography on DE 23 cellulose with 0.30M-NaCl. The protein was finally concentrated by adsorption on a small column of DE 23 cellulose, washing with buffer, and elution with 0.8M-NaCl containing 50mM-tris-HCl buffer, pH8.5. The yield of purified, reconstituted rubredoxin was 60–70%.

Samples for measurement of Mössbauer spectra were contained in polyethylene cells of 0.5cm path-length, and were stored in liquid N_2 before use.

Results and Discussion

Composition of *Ch. ethylica* rubredoxin

The amino acid composition of *Ch. ethylica* rubredoxin was Lys₅, Trp₁, Asx₈, Thr₂, Ser₁, Glx₆,

Pro₆, Gly₅, Ala₂, Cys₄, Val₅, Met₁, Ile₁, Leu₂, Tyr₄, Phe₃. This analysis gives a value for the minimum molecular weight of 6352 including one iron atom per molecule. The composition is typical of those found in other rubredoxins (see Bachmayer *et al.*, 1968).

The extinction coefficient of *Ch. ethylica* rubredoxin was measured by using the Folin-Lowry protein-estimation method (Rabinowitz & Price, 1962), and the value obtained was $E_{490} = 6800 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$. Lovenberg & Williams (1969) reported that this method gave the same results with *Cl. pasteurianum* rubredoxin as those obtained from quantitative amino acid analysis. By using this value the iron content of the rubredoxin was calculated to be 1.14g-atoms/mol in the native protein, and 1.12g-atoms/mol in the ^{57}Fe -reconstituted protein, assuming a molecular weight of 6352.

Optical spectra of the rubredoxin

The absorption spectra of the native and ^{57}Fe -reconstituted rubredoxins from *Ch. ethylica* were very similar to each other, with peaks at 276, 372, 492 and 575nm in the oxidized form, and 275, 313 and 340nm in the reduced form (Fig. 1). This spectrum is similar to those of other rubredoxins (e.g. Atherton *et al.*, 1966). There is a small difference between the spectra of the oxidized forms of *Ch. ethylica* and *Cl. pasteurianum* rubredoxins, in the region 300–400nm, where *Cl. pasteurianum* shows the presence of at least two absorption bands (Lovenberg & Sobel, 1965). The circular-dichroism spectra of the two rubredoxins showed more pronounced differences. Figs. 2(a) and 2(b) show the circular-dichroism spectra of oxidized

Fig. 1. Optical absorption spectra of *Ch. ethylica* rubredoxin

For experimental details see the text. —, Oxidized and ----, reduced rubredoxin.

Ch. ethylica and *Cl. pasteurianum* rubredoxins respectively. The spectra shown are of the ^{57}Fe -reconstituted rubredoxins, which were virtually identical to the spectra of the corresponding native proteins. Though the spectra of the rubredoxins from the two bacteria were similar to each other in general features, some of the bands appear to be changed in intensity and shifted. In particular, a shoulder at 410nm in *Cl. pasteurianum* rubredoxin is replaced by a band at 390nm in *Ch. ethylica*: this apparent shift may be associated with the difference in absorption spectrum. The optical absorption and circular dichroism in the wavelength region 300–600nm most probably arise from optically active charge-transfer transitions in the iron–sulphur chromophore (see Tsibris & Woody, 1970), and are probably very sensitive to changes in conformation.

E.p.r. spectra

The oxidized rubredoxins showed e.p.r. signals at $g = 9.4$ and $g = 4.3$ (Fig. 3). Peisach *et al.* (1971) have pointed out that these two signals arise from the lowest and middle Kramer's doublets respectively of the high-spin Fe^{3+} atom. At temperatures between 4.2° and 77°K the signal at $g = 4.3$ is much more prominent than that at $g = 9.4$. The e.p.r. spectrum of *Ch. ethylica* rubredoxin (Fig. 3a) was significantly different from those reported in other rubredoxins

(Lovenberg, 1966; Atherton *et al.*, 1966; Bachmayer *et al.*, 1967; Newman & Postgate, 1968; Peterson & Coon, 1968), in which the signal at $g = 4.3$ is associated with broader absorptions at around $g = 4.6$ and $g = 4.1$ (Fig. 3b). These latter absorptions were attributed by Peisach *et al.* (1971) to absorptions from the other two principal directions, although it is not clear why these should be so broad when the central absorption is very sharp. In the e.p.r. spectrum of *Ch. ethylica* rubredoxin these associated absorptions were never observed, and the signal at $g = 4.3$ was consequently much more intense. Fig. 3 shows a comparison of the e.p.r. spectra of oxidized rubredoxins from *Ch. ethylica* and *Cl. pasteurianum*.

These chemical and spectroscopic analyses indicate that the rubredoxin of *Ch. ethylica* is very similar to those of the anaerobic fermentative bacteria such as *Cl. pasteurianum*, which have molecular weights of about 6000 and contain one iron atom per molecule. Some minor differences in the circular-dichroism and e.p.r. spectra of the proteins may be a consequence of small differences in the chelate structure of the chromophore, which may in turn reflect differences in amino acid sequence. Reconstitution with ^{57}Fe did not appear to alter the structure of the molecule.

Fig. 2. Circular-dichroism spectra of oxidized rubredoxins from (a) *Cl. pasteurianum* ($E_{490} = 0.88$) and (b) *Ch. ethylica* ($E_{490} = 0.68$).

The rubredoxins were enriched with ^{57}Fe isotope; the spectra were indistinguishable from those of the native proteins.

Fig. 3. E.p.r. spectra of oxidized rubredoxins from (a) *Ch. ethylica* and (b) *Cl. pasteurianum*.

The gain setting for the signals centred at $g = 9.4$ (left-hand side) was 100 times that for the signal at $g = 4.3$ (right-hand side). Other settings were: microwave frequency, 9.17GHz; power, 0.5mW; temperature, 23°K.

Mössbauer spectra

The spectra of rubredoxins from *Ch. ethylica* and *Cl. pasteurianum* were not significantly different, indicating that the iron atoms in the two proteins are in very similar electronic states. Most of the spectra shown in this paper are for *Ch. ethylica* rubredoxin.

Oxidized rubredoxin. The Mössbauer spectra of oxidized rubredoxin measured at various temperatures are shown for *Ch. ethylica* in Fig. 4, and for *Cl. pasteurianum* in Fig. 5.

At the lower temperature (1.3°K and 4.2°K) magnetic hyperfine splitting was observed, with an effective field at the iron nucleus of 370 ± 3 kG, in good agreement with the results of Phillips *et al.* (1970). The hyperfine field was not discernibly altered by application of external magnetic fields of up to 30 kG.

At high temperatures (77°K and 195°K) the electron-spin-relaxation rates have become rapid and the hyperfine splitting collapses (Fig. 4a). The spectrum in fact was extremely difficult to observe, which could be due either (a) to the relaxation rate being close to the precession frequency of the nucleus in the hyperfine field or (b) to a very small Mössbauer effect at these temperatures arising from a weak binding of the iron atom in the molecule. The former is the most likely explanation. Our spectra of oxidized *Ch. ethylica* and *Cl. pasteurianum* rubredoxins did not show the two strong lines in the centre of the spectra at 4.2°K and 77°K observed by Phillips *et al.* (1970).

Fig. 4. Mössbauer spectra of oxidized *Ch. ethylica* rubredoxin (1.2 mM) enriched with ^{57}Fe isotope

Temperatures were: (a) 77°K; (b) 4.2°K; (c) 1.4°K; (d) 4.2°K and a field of 30 kG was applied perpendicular to the γ -rays. For details see the text.

It may be that these two lines were due to the presence of an impurity containing iron, or to a different relaxation rate associated with the physical state of the rubredoxin (freeze-dried powder compared with the frozen solutions used in the present work).

Reduced rubredoxin. The reduced protein contains iron in the Fe^{2+} state and the Mössbauer spectrum shows the characteristic two-line quadrupole pattern at all temperatures. This is shown for *Ch. ethylica* rubredoxin (Fig. 6) and *Cl. pasteurianum* (Fig. 7). At 77°K the shift, δ , was 0.65 mm/s relative to iron at room temperature. The variation of quadrupole splitting ΔE_Q with temperature (Fig. 6) was small showing that the ligand field acting at the iron atom is very strong. The value is 3.10 mm/s at 198°K, and it increases to 3.16 mm/s at 77°K, then remains constant down to 1.4°K. It is concluded that the ground-state orbital is pure, and lies lower by a large energy ($\Delta/k \approx 900^\circ\text{K}$) than the first-excited orbital state. At low temperatures magnetic hyperfine splitting was observed by applying magnetic fields of up to 60 kG to the samples (Fig. 8). The splitting of the spectrum was much larger than that caused by the external field alone, showing that the effective field at the nuclei was mainly caused by the internal field arising from the unpaired electrons of the Fe^{2+} ion.

Fig. 5. Mössbauer spectra of oxidized *Cl. pasteurianum* rubredoxin (0.6 mM) enriched with ^{57}Fe isotope

Temperatures were: (a) 77°K; (b) 4.2°K and a small field was applied perpendicular to the γ -rays. (c) was at 1.3°K. For details see the text.

Fig. 6. Mössbauer spectra of reduced *Ch. ethylica* rubredoxin (1.2 mm) enriched with ^{57}Fe isotope

Temperatures were: (a) 198°K; (b) 77°K; (c) 4.2°K; (d) 1.4°K. For details see the text.

Fig. 7. Mössbauer spectrum of reduced *Cl. pasteurianum* rubredoxin (0.8 mm) enriched with ^{57}Fe isotope at 77°K

For details see the text.

From the behaviour of the two quadrupole lines in the field, the lower-energy line is seen to arise from the $I_z = \pm 3/2$ excited state of ^{57}Fe , i.e. the electric-field gradient, q has a negative sign. This identifies the ground-state orbital as d_{z^2} .

Fig. 8. Mössbauer spectra of reduced *Ch. ethylica* rubredoxin at 4.2°K, showing the effect of large magnetic fields applied perpendicular to the γ -rays

The fields were: (a) 5 kG; (b) 10 kG; (c) 15 kG; (d) 30 kG; (e) 60 kG. For spectra (a), (b), (c) and (e) the sample concentration was 0.7 mm; for spectrum (d) it was 1.2 mm. For further details see the text.

It is seen that the lines of the spectrum in magnetic fields are quite sharply defined. The fact that sharp lines are observed in a sample containing anisotropic ions, which are randomly oriented, is partly a consequence of the anisotropic susceptibility of the iron ions. There is a tendency in a randomly oriented sample for the effective field to be directed perpendicular to the axis of symmetry of the ion, since the probability $P(\theta)$ that the external field H will be oriented at an angle θ to the axis, is given by $P(\theta)d\theta = \sin\theta d\theta$ so that $P(\theta)$ is largest for $\theta = 90^\circ$. In addition if $\chi_{\perp} > \chi_{\parallel}$, this tendency will be enhanced, as the internal field H_n at the nucleus will then take up an angle ϕ with the axis where $\tan\phi = \chi_{\perp}/\chi_{\parallel}$, $\tan\theta$ (Johnson, 1967b). $\chi_{\parallel}$ and $\chi_{\perp}$ are the susceptibilities per mol measured parallel and perpendicular respectively to the symmetry axis and the ligand field. The effective field, H_{eff} , at the nuclei will then be:

$$H_{\text{eff.}} = H + H_n = H + \frac{\langle M \rangle}{M} H_{n\perp}(0)$$

For small-field magnetization $M = \chi_{\perp} H$ and

$$H_{\text{eff.}} = H + \frac{\chi_{\perp} H}{N\mu} H_{n\perp}(0)$$

where $H_{n\perp}(0)$ is the hyperfine field, i.e. the internal field at the nucleus which is observed when the magnetization is completely saturated, i.e. when $\langle M \rangle = M = N\mu$; μ is the magnetic moment of the Fe^{2+} ion, N is the number of ions per mol and $M (=N\mu)$ is the saturated magnetic moment per mol.

The spectra in magnetic fields do not depend upon the temperature between 4.2°K and 1.3°K, i.e. $\chi_{\perp}$ is independent of temperature. The spectrum for $H = 30$ kG is practically the same as that for 60 kG and has an effective field $|H_{\text{eff.}}|$ of 133 kG. This means that the internal field H_n is negative (as expected), and when $H = 30$ kG, H_n is -163 kG, and when $H = 60$ kG, H_n is -193 kG. The saturation value of the internal field $H_{n\perp}(0)$ is estimated to be about -200 kG.

The behaviour of the magnetization (as measured by the internal field H_n) at low temperatures shows that the splitting D of the ground-state orbital by the ligand field is not large, and is estimated to be about $+4.4\text{ cm}^{-1}$. Theoretically it would be expected to be of the order of λ^2/Δ where λ , the spin-orbit coupling parameter, is typically 100 cm^{-1} for a free Fe^{2+} ion, but would be decreased by covalency. This is consistent with a large ligand-field splitting as suggested by the small temperature variation of the quadrupole splitting and would lead to a value for $g_{\perp}$ of 2.04 or less, i.e. close to the free spin value of 2.0. The anisotropy of the susceptibility ($\chi_{\perp} > \chi_{\parallel}$) confirms the assignment of the negative sign of the quadrupole splitting, that the ground-state orbital is d_{z^2} . This would be produced if the effective distortion of the four sulphur ligands from tetrahedral symmetry were equivalent to a squashing about a tetragonal direction.

The interpretation of the hyperfine interaction tensor may be facilitated by comparing the data with those obtained (Johnson, 1967b) on Fe^{2+} in the salt ferrous fluosilicate, $\text{FeSiF}_6 \cdot 6\text{H}_2\text{O}$. This is the simplest and best understood ferrous salt and measurements on single crystals have enabled the different contributions to the field at the nucleus to be analysed. In ferrous fluosilicate the Fe^{2+} ion is octahedrally co-ordinated to six water molecules, and the ligand field has ligand symmetry leaving the ground state as a d_{z^2} orbital. From Mössbauer spectra of this salt in a magnetic field the various contributions to the hyperfine field have been analysed and a similar interpretation can be made for Fe^{2+} in reduced rubredoxin, which confirms the correctness of the assignments made.

The hyperfine field in Fe^{2+} salts may be considered to be the sum of the contributions:

$$H_n = H_s + H_L + H_d$$

where H_s arises from the s -electrons, H_L from the orbital current and H_d from the spin dipolar moment of the d -electrons. H_L is related to the g value by:

$$H_L = 2\mu_B \langle r^{-3} \rangle (g-2) S$$

where S is the electron spin, and r is the position of the electron relative to the nucleus. H_d is proportional to the quadrupole splitting:

$$H_d = \mu_B q$$

(Marshall & Johnson, 1962) where μ_B is the Bohr magneton. Table 1 gives a comparison of the data for $\text{FeSiF}_6 \cdot 6\text{H}_2\text{O}$ and reduced rubredoxin. For the rubredoxin the $|H_s|$ of 300 kG is estimated as four-fifths of the value for Fe^{3+} in oxidized rubredoxin which is presumably in a similar environment. The decreased value compared with that (421 kG) for $\text{FeSiF}_6 \cdot 6\text{H}_2\text{O}$ is attributable to the effects of covalency. The dipolar field H_d has the same sign in the two materials, being slightly smaller (as the quadrupole coupling is smaller) for rubredoxin. The orbital field is rather uncertain, but is much smaller for rubredoxin since its ground state is almost a pure d -orbital with quenched orbital angular momentum. Thus the total value for H_n of about -210 kG which is found is a reasonable one to be expected from the data available with randomly oriented molecules.

It should be noted that the analysis of the different contributions to the hyperfine interaction depends upon the symmetry of the ligands and not on their number. The fact that approximately similar values for H_n are found in rubredoxin and $\text{FeSiF}_6 \cdot 6\text{H}_2\text{O}$ arises from the smaller negative value for H_s in the protein almost cancelling the effect of smaller positive value for H_L .

Hence values of -370 kG and -300 kG seem reasonable values to take for the core polarized hyperfine field H_s for Fe^{3+} and Fe^{2+} ions when co-ordinated to four sulphur ligands in a protein environment.

Table 1. Comparison of magnetic and hyperfine-field data for Fe^{2+} in $\text{FeSiF}_6 \cdot 6\text{H}_2\text{O}$ and reduced rubredoxin

	$\text{FeSiF}_6 \cdot 6\text{H}_2\text{O}$	Rubredoxin
ΔE_a (mm/s)	3.8*	3.1
$g_{\perp}$	2.12	2.04
H_s (kG)	-421	-300
H_d (kG)	+64	+54
H_L (kG)	+109	+36
H_n (kG)	-248	-210

* Corrected for spin-orbit effects.

These values may assist in interpreting the structure of more complex iron-sulphur proteins such as ferredoxins. Several proteins of this important class have been shown to contain iron atoms co-ordinated to sulphur ligands (either inorganic sulphide or cysteine sulphur from the protein). The simplest of these are the two-iron, two-sulphur (plant-type) ferredoxins; although X-ray crystallography has not yet been applied successfully to these proteins, evidence from Mössbauer spectroscopy, electron-nuclear double resonance and proton magnetic resonance (see Rao *et al.*, 1971; Cammack *et al.*, 1971; Dunham *et al.*, 1971; Münck *et al.*, 1972; Poe *et al.*, 1971) confirms the model of Gibson *et al.* (1966) for these proteins, in which the two iron atoms are co-ordinated to sulphur ligands, probably with tetrahedral symmetry (Eaton *et al.*, 1971). The iron atoms, which are antiferromagnetically coupled together, are both high-spin Fe^{3+} in the oxidized ferredoxin, and one of them is reduced to high-spin Fe^{2+} in the reduced state. X-ray crystallography has been applied to the four-iron, four-sulphur high-potential iron protein from *Chromatium* (Carter *et al.*, 1971) and the eight-iron, eight-sulphur ferredoxin from *Micrococcus aerogenes* (Sieker *et al.*, 1972), and in both cases the iron atoms appear to be tetrahedrally co-ordinated to sulphur ligands. Therefore the iron atom in rubredoxin can be considered as a model for those in the more complex iron-sulphur proteins.

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