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Mössbauer Studies of Adrenodoxin

THE MECHANISM OF ELECTRON TRANSFER IN A HYDROXYLASE IRON–SULPHUR PROTEIN

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1. Mössbauer spectra were measured of adrenodoxin purified from porcine adrenal glands. They show similarities to the spectra of the plant ferredoxins. All of these proteins contain two atoms of iron and two of inorganic sulphide per molecule, and on reduction accept one electron. 2. As with the plant ferredoxins the adrenodoxin for these measurements was enriched with ^{57}Fe by reconstitution of the apoprotein, and subsequently was carefully purified and checked by a number of methods to ensure that it was in the same conformation as the native protein and contained no extraneous iron. 3. The Mössbauer spectra of oxidized adrenodoxin at temperatures from 4.2°K to 197°K show that the iron atoms are probably high-spin Fe^{3+}, and in similar environments, and experience little or no magnetic field from the electrons. 4. Mössbauer spectra of reduced adrenodoxin showed magnetic hyperfine structure at all temperatures from 1.7°K to 244°K, in contrast with the reduced plant ferredoxins, which showed it only at lower temperatures. This is a consequence of a longer electron-spin relaxation time in reduced adrenodoxin. 5. At 4.2°K in a small magnetic field the spectrum of reduced adrenodoxin shows a six-line Zeeman pattern due to Fe^{3+} superimposed upon a combined magnetic and quadrupole spectrum due to Fe^{2+}. 6. In a large magnetic field (30kG) each hyperfine pattern is further split into two. Analysis of these spectra at 4.2°K and 1.7°K shows that the effective fields at the Fe^{3+} and Fe^{2+} nuclei are in opposite directions. This agrees with the proposal, first made for the ferredoxins, that the iron atoms are antiferromagnetically coupled. 7. In accord with the model for the ferredoxins, it is proposed that the oxidized adrenodoxin contains two high-spin Fe^{3+} atoms which are antiferromagnetically coupled; on reduction one iron atom becomes high-spin Fe^{2+}.

Adrenodoxin is an iron–sulphur protein that can be isolated from mammalian adrenal glands. It is a component of the hydroxylase systems involved in hormone biosynthesis. Adrenodoxin is a relatively simple protein, with mol. wt. approx. 13000, containing two atoms each of iron and labile sulphur per molecule, and accepting one electron on reduction (Kimura, 1968).

The iron–sulphur proteins have been recognized in the last few years as components of a wide variety of electron-transport systems, including the mitochondrial respiratory chain, photosynthetic electron transport and nitrogen fixation (see Hall & Evans, 1969). Many of these iron–sulphur proteins are very complex, with high molecular weights and containing other electron-transferring components such as flavin and molybdenum. For this reason most of the studies of the structure and mechanism of action of the iron–sulphur group have been directed at the simpler proteins of this class, namely the ferredoxins and the hydroxylase iron–sulphur proteins, which are all small, soluble proteins. The hydroxylase iron–sulphur proteins and the plant ferredoxins contain two atoms each of iron and labile sulphur per molecule, and accept one electron. The bacterial ferredoxins are more complex; they contain six to eight atoms of iron and of labile sulphur per molecule, and accept two electrons.

A model was proposed for the active group of spinach ferredoxin by Gibson, Hall, Thornley & Whatley (1966) and Thornley, Gibson, Whatley & Hall (1966) on the basis of e.p.r.* experiments. According to this model, the oxidized ferredoxin contains two high-spin ferric atoms with their spins antiferromagnetically coupled together to give no

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

net spin. In the reduced form one iron atom is reduced to the high-spin ferrous state, and the net spin of the coupled atoms is then $S = \frac{1}{2}$. This model has been confirmed by Mössbauer spectroscopy of ^{57}Fe-enriched ferredoxins from spinach and *Scenedesmus* (Rao, Cammack, Hall & Johnson, 1971). At 195°K the Mössbauer spectrum of reduced ferredoxin showed two quadrupole split doublets corresponding to the two different iron atoms. At 4.2°K and 1.7°K the spectra were complicated by magnetic hyperfine interaction, but could be resolved by the technique of applying an external magnetic field to the sample (Johnson, 1967). In a small applied field (0.3kG) two overlapping spectra could be seen, corresponding to the two iron atoms. In a large applied field (30kG) the two patterns separated, indicating that the applied field was adding to the hyperfine field at the nucleus of the Fe^{2+} atom, and subtracting from the hyperfine field at the Fe^{3+} atom. This showed once more that the two atoms were different, and confirmed the antiferromagnetic coupling between them.

Gibson *et al.* (1966) suggested that their model might also be applicable to other types of iron–sulphur proteins, and recent results from physicochemical measurements have agreed with this. The iron–sulphur proteins mentioned so far have a number of properties in common: (1) they all contain equal numbers of atoms of iron and sulphur; (2) they all have very low redox potentials (around −400mV); (3) they all show an e.p.r. signal in the reduced form, centred around $g = 1.94$ (an exception to this is the high-potential iron protein from *Chromatium*; Mössbauer experiments indicate that its structure is very different from that of the plant ferredoxins; Evans, Hall & Johnson, 1970). It is possible that even the bacterial ferredoxins may contain groups similar to those found in the plant ferredoxins. Though they accept two electrons on reduction, measurements of redox potential (Eisenstein & Wang, 1969) and e.p.r. experiments (Orme-Johnson & Beinert, 1969*a*) suggest that these two electrons can be added separately, and therefore there may be two independent one-electron sites on the molecule. The presence of antiferromagnetically coupled iron atoms in *Clostridium* ferredoxin has been inferred from proton-magnetic-resonance studies (Poe, Phillips, McDonald & Lovenberg, 1970).

This paper describes studies using Mössbauer spectroscopy of adrenodoxin extracted from porcine adrenal glands. Though this protein is similar in many respects to the plant ferredoxins, there are differences. The amino acid sequences of the proteins are totally dissimilar (Tanaka, Hanui & Yasunobu, 1970). The e.p.r. signal of reduced adrenodoxin at $g = 1.94$ shows narrower line-widths than that of the plant ferredoxins, and indicates axial rather than rhombic symmetry. The physical properties of adrenodoxin are more closely similar to those of putidaredoxin, an iron–sulphur protein isolated from *Pseudomonas putida*, in which it functions as part of camphor hydroxylase (Cushman, Tsai & Gunsalus, 1967).

Cooke *et al.* (1968) have presented Mössbauer spectra of putidaredoxin. From these it was concluded that in the oxidized protein the iron atoms are equivalent, and therefore are probably coupled antiferromagnetically. No definite conclusions were reached about the state of the iron atoms in the reduced form.

For our studies on adrenodoxin, as with the plant ferredoxins, it was necessary to introduce enriched ^{57}Fe into the protein by reconstitution of the apoprotein, so as to increase the sensitivity of the Mössbauer spectroscopy. However, this method introduces the danger of contamination of the sample by excess of ^{57}Fe. Contaminant iron tends to appear as a doublet at the centre of the Mössbauer spectrum, and is particularly noticeable in the spectra of the reduced proteins (cf. Rao *et al.* 1971). Such doublets seem to be present in the previously published spectra of reduced putidaredoxin (Cooke *et al.* 1968) and suggest the presence of contaminant iron or unreduced protein. For the present studies, the ^{57}Fe-reconstituted adrenodoxin was purified by column chromatography and was checked by a number of physical techniques to ensure that it was in the same conformation as the native protein, and that contaminant iron was kept to a minimum.

EXPERIMENTAL

Materials. Pig adrenal glands were obtained through the courtesy of T. Wall and Sons (Meat and Handy Foods) Ltd., London, N.W.10, 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. Sephadex G-25 and G-75 were obtained from Pharmacia (G.B.) Ltd., London W.5, U.K. All other chemicals were the purest products of BDH Chemicals Ltd., Poole, Dorset, U.K.

Methods. Mössbauer spectra were measured in the apparatus described by Cranshaw (1964). A 100mCi source of ^{57}Co in palladium foil (0.00025in thick) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. The spectra were calibrated against the metal iron, which also gave the zero of the chemical shift. 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 spectro-

polarimeter (SoFICA, St Denis, France). Iron was determined, after digestion of the protein with 1M-HCl at 80°C for 15min, by using a Schweizerhall test kit obtained from Pye Unicam Ltd., Cambridge, U.K. This spectrophotometric test uses a bathophenanthroline sulphonate (4,7-diphenyl-1,10-phenanthroline sulphonate) reagent after reduction of the Fe^{3+} with ascorbate. Labile sulphur was determined by the method of Fogo & Popowsky (1949), as modified by Lovenberg, Buchanan & Rabinowitz (1963). The adrenodoxin concentration was determined by extinction by using a value for ϵ_{414} of 9800 (Kimura, 1968).

Preparation of adrenodoxin. The method of preparation was similar to that described for spinach ferredoxin (Rao *et al.* 1971). This general method was developed for the rapid preparation of iron–sulphur proteins from large quantities of starting material, and uses a batchwise DEAE-cellulose step for the initial recovery of the protein, followed by several stages of column chromatography. A somewhat similar process has been described by Orme-Johnson & Beinert (1969*b*), who used preparative electrophoresis for the final stage of purification. The buffer used was 20mM-potassium phosphate, pH7.5. All steps were carried out at 0°–4°C. Solutions for chromatography and dialysis were bubbled with N_2 to remove O_2.

Approx. 1.8kg of freshly extracted pig adrenal glands (including 20–25%, w/w, of associated fat) was homogenized in four batches. Each batch was homogenized in a 1-gallon Waring Blendor with 750ml of 50mM-potassium phosphate, pH7.5, at maximum speed for 2min. The homogenate was centrifuged at 2500rev./min in the 6×1-litre rotor of an MSE Mistral refrigerated centrifuge for 15min. The upper layer of fatty material was skimmed off and discarded. The precipitate was stirred up with the soluble material and the resulting suspension from each batch was transferred to a 1-litre Pyrex beaker surrounded by ice–water and subjected to ultrasonic oscillation by using a Dawe Soniprobe at full power (about 4.5A) for 15min. The suspension was centrifuged at 12000rev./min for 15min in the 6×250ml rotor of an MSE 18 refrigerated centrifuge. The supernatant was collected and the precipitate washed in buffer (400ml/batch) by resuspension and centrifugation. The pink, slightly cloudy supernatant and washings were pooled. Adrenodoxin was extracted by using DEAE-cellulose, and purified by chromatography on DEAE-cellulose and hydroxyapatite columns as described for ferredoxin (Rao *et al.* 1971). The final yield was about 100mg, with E_{415}/E_{276} ratio approx. 0.67. A small degree of further purification with decreased yield could be achieved by chromatography on Sephadex G-75, but this was not normally done. The adrenodoxin samples were stored frozen under liquid N_2.

Preparation of samples for Mössbauer spectroscopy. The adrenodoxin was reconstituted with ^{57}Fe in the same way as for the plant ferredoxins (Rao *et al.* 1971). The method involves precipitation with 5% (w/v) trichloroacetic acid to prepare the apoprotein, followed by incubation under N_2 with 2-mercaptoethanol, Na_2S and $^{57}FeSO_4$.

The reconstituted adrenodoxin was separated from excess of reagents and apoprotein by chromatography on columns of Sephadex G-25 (15cm×2.5cm) and Sephadex G-75 (35cm×2.5cm). Finally it was concentrated by adsorption on a column (0.5cm×1cm) of DEAE-cellulose and elution with 1.5M-NaCl–50mM-potassium phosphate, pH7.5. The overall yield of purified reconstituted adrenodoxin was 40–50%.

For measurements on reduced adrenodoxin, 0.7ml samples of 1–2mM-adrenodoxin were reduced with sodium dithionite soln. (final concn. 2mM) under argon at 20°C. The reduction of adrenodoxin by dithionite is much slower than reduction of spinach ferredoxin. However, the reduction was essentially complete under these conditions in about 10min. The samples were then frozen by immersion in liquid N_2.

RESULTS AND DISCUSSION

Characterization of the purified adrenodoxin. The purified pig adrenodoxin appeared to be very similar to the preparations of bovine adrenodoxin described by Palmer, Brintzinger & Estabrook (1967) and Kimura (1968), in its absorption, circular-dichroism, optical-rotatory-dispersion and e.p.r. spectra. The amino acid composition of a highly purified sample of pig adrenodoxin was kindly determined for us by Professor K. T. Yasunobu and Dr R. Chung, of the Department of Biochemistry and Biophysics, University of Hawaii. In Table 1, this is compared with the composition of bovine adrenodoxin and shows obvious similarities.

Table 1. *Comparison of amino acid composition of porcine and bovine adrenodoxin*

Values for bovine adrenodoxin are taken from Tanaka *et al.* (1970). The values for porcine adrenodoxin were determined by R. Chung and K. T. Yasunobu. n.d., Not determined.

	Composition (residues/molecule)	
Amino acid	Porcine adrenodoxin	Bovine adrenodoxin
Lysine	6	5
Histidine	2	3
Arginine	4	4
Aspartic acid+asparagine	18	19
Threonine	8	10
Serine	7	7
Glutamic acid+glutamine	12	11
Proline	2	1
Glycine	9	8
Alanine	8	7
Half-cysteine	5	5
Valine	4	7
Methionine	3	3
Isoleucine	7	8
Leucine	13	12
Tyrosine	1	1
Phenylalanine	4	4
Tryptophan	n.d.	0
Total	113	115

Characterization of the reconstituted adrenodoxin. The ^{57}Fe-enriched reconstituted adrenodoxin had a very similar absorption spectrum to the native protein (Fig. 1). The E_{415}/E_{276} ratio was often higher for the reconstituted protein, probably owing to removal of contaminant protein or nucleic acid during purification; a similar effect was observed with the plant ferredoxins (Rao *et al.* 1971).

Analysis of a sample of ^{57}Fe-enriched reconstituted adrenodoxin gave values of 2.1g-atoms of iron and 1.8g-atoms of labile sulphur/mol. No iron or labile sulphur could be detected in the apoprotein.

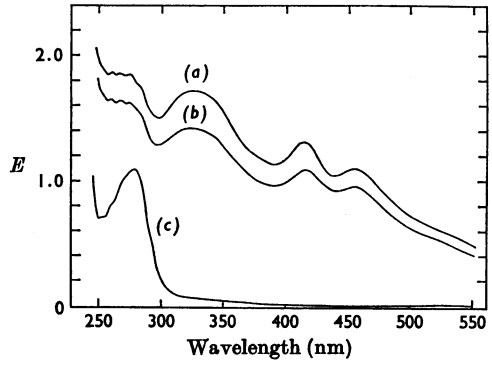


Fig. 1. Absorption spectra of (*a*) native adrenodoxin, 0.134mM; (*b*) ^{57}Fe-enriched reconstituted adrenodoxin, 0.113mM; (*c*) apo-adrenodoxin, 0.143mM.

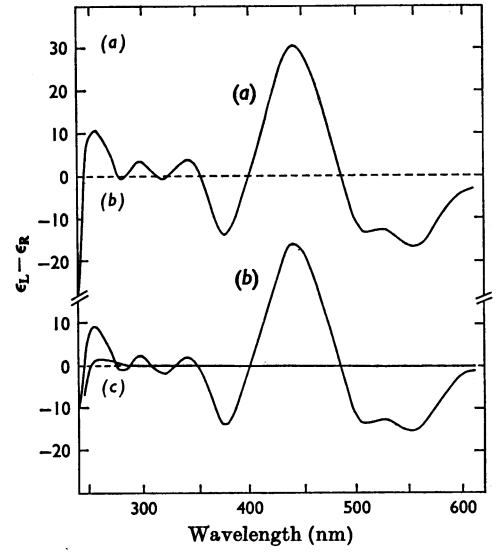


Fig. 2. Circular-dichroism spectra: (*a*) native adrenodoxin; (*b*) ^{57}Fe-enriched reconstituted adrenodoxin; (*c*) apo-adrenodoxin.

Circular-dichroism spectra. Fig. 2 shows the circular-dichroism spectra of adrenodoxin in the native, apo- and ^{57}Fe-enriched reconstituted forms. The spectrum of the reconstituted adrenodoxin is essentially identical with that of the native protein. The optical-rotatory-dispersion spectrum of the reconstituted adrenodoxin was also very similar to that of the native protein.

E.p.r. spectra. The e.p.r. spectra of the reduced native (^{56}Fe) and ^{57}Fe-enriched reconstituted adrenodoxin are shown in Fig. 3. The spectra show approximately axial symmetry ($g_x = g_y \neq g_z$) and are similar to those reported by other workers (e.g. Beinert & Orme-Johnson, 1969). The spectrum of the ^{57}Fe-enriched protein is split by nuclear hyperfine interaction into three lines with relative intensities 1:2:1. This can be seen most clearly in the $g_{\parallel}$ (g_z) peak. The significance of this effect is discussed elsewhere (see below, and Johnson, Cammack, Rao & Hall, 1971).

Mössbauer spectra. Measurements were made at a series of temperatures between 1.7°K and 244°K, and in magnetic fields between zero and 30kG. Very similar spectra were obtained from two samples of reconstituted adrenodoxin that had been prepared independently from two batches of adrenals.

(1) Spectra of oxidized adrenodoxin. The spectra of oxidized adrenodoxin are shown in Fig. 4, and consist of two lines of equal intensity with widths of 0.35mm/s. As with the plant ferredoxins, this is presumably the superimposition of two quadrupole-

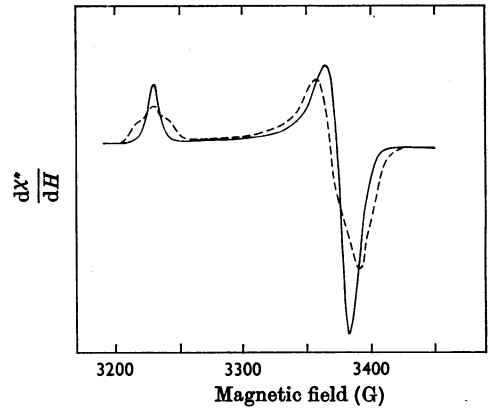


Fig. 3. E.p.r. signals from reduced adrenodoxin at 77°K; ——, native, 0.19mM; – – – –, ^{57}Fe-enriched, 0.21mM. Samples (0.1ml) in 3mm-bore tubes were reduced under argon with 2mM-sodium dithionite at 20°C for 10min before freezing. The settings for measurement were: microwave frequency 9.17GHz; power 20mW; modulation frequency 100kHz: modulation amplitude 1G.

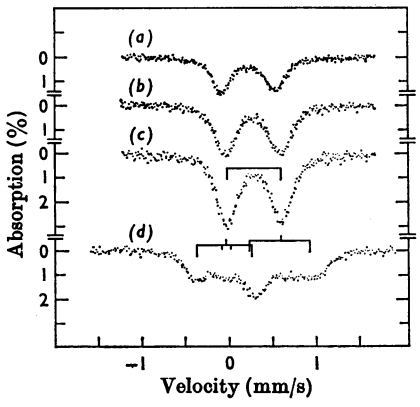


Fig. 4. Mössbauer spectra of oxidized adrenodoxin enriched with ^{57}Fe (0.42mM): (*a*) at 197°K; (*b*) at 77°K; (*c*) at 4.2°K; (*d*) at 4.2°K in a field of 30kG applied perpendicular to the γ-rays.

split doublets caused by the two types of iron atom. In the plant ferredoxins (Rao *et al.* 1971) the spectra of the two iron atoms were slightly dissimilar, but this is not readily detectable for adrenodoxin. The two iron atoms therefore must be in the same chemical state and in very similar environments. The values of the quadrupole splitting and chemical shift at three different temperatures were determined by computer fitting and are given in Table 2. The quadrupole splitting is fairly constant, whereas the shift increases significantly with decreased temperature, owing to the second-order Doppler shift.

On application of a magnetic field of 30kG (Fig. 4*d*) the lower-energy line splits into three components and the higher-energy line splits into two broad lines. This splitting is due to the direct effects of the applied magnetic field on the iron nuclei, and indicates that there is little or no magnetic field at the nuclei due to free electrons. This is consistent with the low magnetic susceptibility of oxidized adrenodoxin (Kimura, Tasaki & Watari, 1970; Moleski, Moss, Orme-Johnson & Tsibris, 1970). The sign of the electric field gradient ($q = \partial^2 V/\partial x^2$, where V is the electric potential at the iron atoms) is therefore positive, i.e. $\frac{1}{2}e^2Qq > 0$ (where Q is the quadrupole moment). The same result has also been found for ferredoxin from *Euglena* (Johnson *et al.* 1968).

(2) Spectra of reduced adrenodoxin. The Mössbauer spectra of reduced adrenodoxin are shown in Fig. 5. The spectra are qualitatively similar to those found for the plant ferredoxins, e.g. from spinach (Johnson, Bray, Cammack & Hall, 1969), *Euglena* (Johnson *et al.* 1968) and *Scenedesmus* (Rao *et al.* 1971), but the temperatures at which

Table 2. *Parameters of the Mössbauer spectrum of oxidized adrenodoxin at various temperatures*

The chemical shift δ (relative to the metal iron) and quadrupole splitting ΔE_Q were determined by computer fitting of the observed spectra. Errors are ±0.005mm/s.

Temp. (°K)	δ (mm/s)	ΔE_Q (mm/s)
197	+0.222	0.614
77	+0.263	0.632
4.2	+0.285	0.622

similar spectra are found are in general higher for adrenodoxin. For example the magnetic hyperfine splitting in the plant ferredoxins is not fully resolved at 77°K and is completely absent (i.e. it averages to zero) at 195°K, whereas in adrenodoxin it is still observable (Fig. 5*a*) at the highest temperatures at which measurement were made [244°K, in Freon (difluorodichloromethane)]. These differences are probably consequences of a longer spin-lattice relaxation time T_1 of iron in adrenodoxin, and are consistent with the observations that the e.p.r. signal has narrower line-widths than for spinach ferredoxin, and is detectable at considerably higher temperatures (Palmer *et al.* 1967; Kimura, 1968). This might be expected from the observed g values, which are nearer to 2 for adrenodoxin than for ferredoxin; this implies a smaller orbital angular momentum, which would result in a longer spin-lattice relaxation time.

There are also small differences in the line positions of plant ferredoxins and of adrenodoxin. These can be seen clearly at 4.2°K on application of a small magnetic field (about 100G) sufficient to decouple the electronic and nuclear magnetic moments. Each type of iron nucleus will then show a spectrum characteristic of an effective field at the nucleus. The Fe^{3+} ion has a small quadrupole splitting relative to the magnetic field, so that its spectrum is a six-line Zeeman pattern. If the applied field is perpendicular to the direction of the γ-rays (Fig. 5*h*) the intensities of the six lines should be in the proportions 3:4:1:1:4:3; if it is parallel to the γ-rays (Fig. 5*g*) the intensities should be in the proportions 6:0:2:2:0:6 (i.e. effectively a four-line spectrum). The width of these Zeeman patterns is proportional to the hyperfine field at the nucleus caused by the electron (see Johnson *et al.* 1968). The Fe^{2+} ion has a large quadrupole splitting, comparable in magnitude with the magnetic splitting, and shows an asymmetrical spectrum with rather broad lines. The resulting spectra for reduced ferredoxin from spinach or *Scenedesmus* (Rao *et al.* 1971, Figs. 4*e* and 5*e*) showed only six lines, because the principal lines in the individual spectra were (by coincidence) overlapping. However, in the spectra for adrenodoxin (Figs. 5*g* and

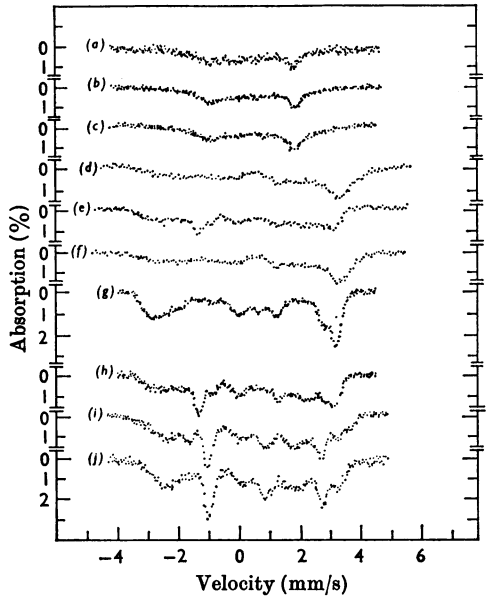


Fig. 5. Mössbauer spectra of reduced adrenodoxin, enriched with ^{57}Fe (1.71 mM): (*a*) at 244°K; (*b*) at 233°K; (*c*) at 198°K; (*d*) at 77°K; (*e*) at 77°K in a field of 0.5 kG applied perpendicular to the γ-rays; (*f*) at 4.2°K; (*g*) at 4.2°K in a field of 0.1 kG applied parallel to the γ-rays; (*h*) at 4.2°K in a field of 0.3 kG applied perpendicular to the γ-rays; (*i*) at 4.2°K in a field of 30 kG applied perpendicular to the γ-rays; (*j*) at 1.7°K in a field of 30 kG applied perpendicular to the γ-rays.

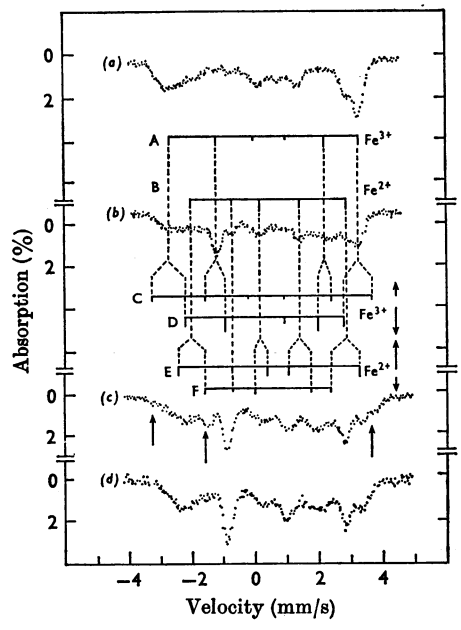


Fig. 6. Proposed assignments of hyperfine splitting patterns to the Mössbauer spectra of reduced adrenodoxin: (*a*) at 4.2°K in a field of 0.1 kG applied parallel to the γ-rays; (*b*) at 4.2°K in a field of 0.3 kG applied perpendicular to the γ-rays; (*c*) at 4.2°K in a field of 30 kG applied perpendicular to the γ-rays; (*d*) at 1.7°K in a field of 30 kG applied perpendicular to the γ-rays. The arrows under (*c*) indicate lines visible at 4.2°K and not visible at 1.7°K.

5*h*) the patterns for the two iron atoms are not identical. For example, consider the highest-energy line at about 3 mm/s in Fig. 5(*g*). This is split into two, although this is not the case in the plant ferredoxins. This could be due to the presence of two different proteins, but the fact that the same spectra were reproduced almost exactly with two different preparations renders this unlikely. Moreover the behaviour in a large magnetic field (see below) indicates that there is only one protein and the two iron atoms give different hyperfine splittings. The lower-energy component of the absorption at 3 mm/s corresponds to a smaller hyperfine field, and is broader and therefore less deep since it arises from the Fe^{2+}, which is magnetically anisotropic. Our assignments of the two hyperfine splitting patterns (Fe^{3+} and Fe^{2+}) to the spectra in a small magnetic field are shown as A and B in Fig. 6. The detailed interpretation of the spectrum is complicated as the magnetic hyperfine interactions are anisotropic, and a complete fit of the data has not been attempted. For the Fe^{3+} ion the hyperfine interaction is not too far from isotropic and the perpendicular component of the effective field at the nuclei is about 183 kG, the parallel component being slightly smaller.

The existence of two overlapping hyperfine spectra is confirmed by measurements in a magnetic field of 30 kG applied perpendicular to the γ-rays (Figs. 5*i*, 5*j*, 6*c* and 6*d*). When a large external magnetic field H is applied, the hyperfine pattern from each type of iron nucleus will split into two, with effective fields of $|H_n+H|$ ('spin up') and $|H_n-H|$ ('spin down'). These represent different energy levels and the two patterns for each iron atom will therefore have different intensities corresponding to the relative populations N_1 and N_2 in the two levels, according to the Boltzmann distribution:

$$\frac{N_1}{N_2} = \exp\left(\frac{-g\beta H}{kT}\right)$$

For an applied magnetic field of 30 kG at 4.2°K the expected populations would be 72% in the lower energy level and 28% in the upper level. Our assignments of the four-component spectra are shown above Fig. 6(*c*). Spectrum A (Fe^{3+}) is shown as being split by the field into spectra C and D, of

which D, corresponding to a decreased effective field, is the more intense. Spectrum B (Fe^{2+}) is shown as being split into spectra E and F, of which E, corresponding to an increased effective field, is the more intense. Once again spectrum D, due to Fe^{3+}, is sharper and deeper than E, due to Fe^{2+}. These assignments were confirmed by lowering the temperature to 1.7°K (by using pumped liquid helium), when the population of the upper energy levels should be only approx. 9% of the total. Some of the smaller lines visible at 4.2°K (indicated by arrows in Fig. 6c) are no longer visible at 1.7°K (Fig. 6*d*); these are lines corresponding to the weaker Zeeman patterns C and F, and thus correspond to energy levels which become depopulated at 1.7°K.

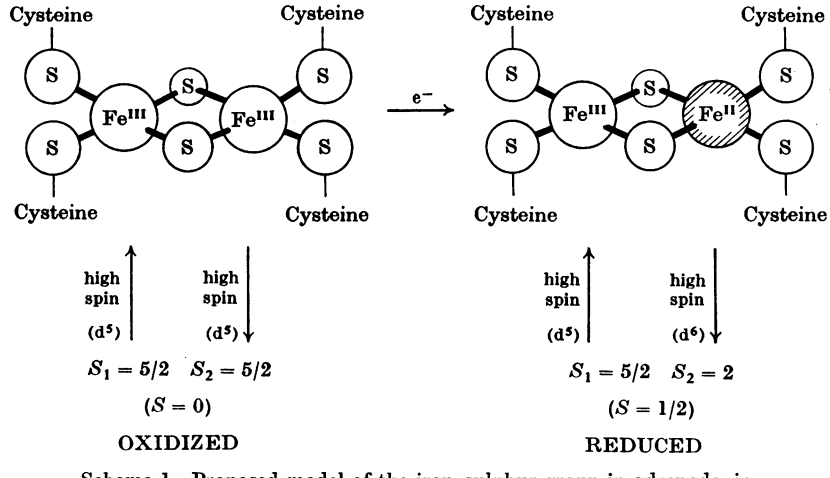
Thus the iron atoms with the smaller effective field at the nuclei (spectrum B, Fe^{2+}) tend to show an increase in effective field when a large external field is applied (spectrum E). This shows (since the effective field is negative at the iron nuclei) that these atoms have their magnetic moments aligned antiparallel to the total magnetic moment of the two iron centres.

This result is consistent with antiferromagnetic coupling between the two iron atoms in the reduced adrenodoxin molecule, and agrees with the model proposed by Gibson *et al.* (1966).

The e.p.r. spectrum of reduced, ^{57}Fe-enriched adrenodoxin (Fig. 3) shows splitting into three lines with relative intensities of 1:2:1. This indicates that the unpaired electron is somehow coupled magnetically to both ^{57}Fe nuclei (nuclear spin $\frac{1}{2}$). Orme-Johnson & Beinert (1969*b*) concluded from their isotopic substitution experiments that 'both iron atoms and both acid-labile sulphur atoms share the unpaired electron'. This would be inconsistent with our interpretation of the Mössbauer spectra and the model of Gibson *et al.* (1966), which require that only one iron atom is reduced. However, the e.p.r. spectrum can be reconciled with the Mössbauer results if the effect of the antiferromagnetic coupling between the iron atoms is taken into consideration (Johnson *et al.* 1971). As a result of this coupling, the unpaired electron associated with one iron atom would be expected to experience hyperfine interactions with both iron nuclei.

Proposed model. The structure that we envisage for the iron–sulphur group in adrenodoxin is illustrated in Scheme 1. It is similar in most respects to that proposed for the plant ferredoxins (Rao *et al.* 1971). In the oxidized state both iron atoms are very similar and, in accord with the model of Gibson *et al.* (1966), are probably high-spin ferric atoms ($S = \frac{5}{2}$), antiferromagnetically coupled so as to give no net spin. The Mössbauer spectra (Fig. 5) show no magnetic hyperfine interaction at temperatures up to 197°K. Measurements of magnetic susceptibility (Kimura *et al.* 1970; Moleski *et al.* 1970) indicate no temperature-dependent paramagnetism in oxidized adrenodoxin at temperatures up to 300°K. This indicates that the antiferromagnetic coupling must be very strong. In the reduced state, one iron atom is reduced to high-spin ferrous ($S = 2$) so that the net spin of the coupled system is $S = \frac{1}{2}$. This agrees with the measured magnetic susceptibility (Kimura *et al.* 1970; Moleski *et al.* 1970).

In the original model of Gibson *et al.* (1966) each iron atom was bound tetrahedrally to four sulphur atoms (though other possible structures were not ruled out). Subsequent results on adrenodoxin, putidaredoxin and the plant ferredoxins indicate



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

that the sulphur ligands to the two iron atoms are probably two atoms of labile sulphide and four sulphur atoms from cysteine residues in the protein, as shown in Scheme 1. The involvement of labile sulphide in the active site was demonstrated indirectly by Orme-Johnson *et al.* (1968). They replaced the labile sulphide in adrenodoxin and putidaredoxin with selenide; the products were biologically active. The e.p.r. spectrum of reduced adrenodoxin containing ^{77}Se (nuclear spin $= \frac{1}{2}$) showed hyperfine splitting corresponding to the interaction of the unpaired electron with both selenium atoms. The involvement of cysteine residues in iron binding in ferredoxins from spinach and parsley has been demonstrated by Poe, Phillips, Glickson, McDonald & San Pietro (1971), who found contact-shifted resonances in the proton-magnetic-resonance spectra caused by the β-CH_2 protons of cysteine. These experiments also confirmed the presence of antiferromagnetically coupled iron atoms in both the oxidized and reduced forms of the proteins. Similar contact-shifted resonances have been observed in oxidized adrenodoxin, although they were not detected in reduced adrenodoxin, probably because of the longer electron-spin relaxation time (W. D. Phillips, personal communication). Thus, as with the plant ferredoxins, it has been demonstrated that the ligands to iron in adrenodoxin are most probably four cysteine sulphur atoms. It may be noted that both types of adrenodoxin that have been isolated so far contain an adequate number of cysteine residues, in fact five in each case (Table 1).

In conclusion, experiments from Mössbauer spectroscopy indicate a common mechanism of action for two low-molecular-weight iron–sulphur proteins from higher organisms, namely plant ferredoxin and mammalian adrenodoxin. It is of great interest to determine whether a similar mechanism holds in the more complex iron–sulphur proteins of these organisms. At least for one of these, milk xanthine oxidase (a high-molecular-weight protein containing molybdenum and flavin as well as iron–sulphur groups), it has been shown that the Mössbauer spectra of the iron–sulphur group show a marked similarity to those of spinach ferredoxin (Johnson *et al.* 1969). It therefore appears that the structure of the simple iron–sulphur proteins is of relevance to the mechanism of action of the more complex biological electron-transport systems.

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