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Electrochemical and spectroscopic characterization of the conversion of the 7Fe into the 8Fe form of ferredoxin III from *Desulfovibrio africanus*

Identification of a [4Fe-4S] cluster with one non-cysteine ligand

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Desulfovibrio africanus ferredoxin III is a protein (M_r 6585) containing one $[3Fe\text{-}4S]^{1+,0}$ and one $[4Fe\text{-}4S]^{2+,1+}$ core cluster when aerobically isolated. The amino acid sequence contains only seven cysteine residues, the minimum required to ligand these two clusters. Cyclic voltammetry by means of direct electrochemistry at a pyrolytic-graphite-'edge' electrode promoted by neomycin shows that, when reduced, the $[3Fe\text{-}4S]^0$ centre reacts rapidly with Fe(II) ion to form a $[4Fe\text{-}4S]^{2+}$ cluster. The latter, which can be reduced at a redox potential similar to that of the other [4Fe-4S] cluster, must include non-thiolate ligation. We propose that the carboxylate side chain of aspartic acid-14 is the most likely candidate, since this amino acid occupies the position of a cysteine residue in the sequence typical of an 8Fe ferredoxin. The magnetic properties at liquid-He temperature of this novel cluster, studied by low-temperature magnetic-c.d. and e.p.r. spectroscopy, are diamagnetic in the oxidized state and $S = \frac{3}{2}$ in the one-electron-reduced state. This cluster provides a plausible model for the ligation states of the $[4Fe\text{-}4S]^{1+}$ core in the $S = \frac{3}{2}$ cluster of the iron protein of nitrogenase and in *Bacillus subtilis* glutamine:phosphoribosyl pyrophosphate amidotransferase.

INTRODUCTION

An intriguing aspect of biological iron–sulphur cluster chemistry stems from the ability of some proteins to bind either the $[4Fe\text{-}4S]^{2+,1+}$ or the $[3Fe\text{-}4S]^{1+,0}$ core. In certain cases these are known to interconvert by loss or uptake of an Fe subsite [1]. Ease of cluster interconversion, however, varies substantially with the protein. In some systems, for example, conversion of [4Fe-4S] to [3Fe-4S] occurs during protein isolation [2,3] or on exposure to air [4], whereas in others an oxidant such as $K_3Fe(CN)_6$ is required [5,6]. In addition, there are a number of proteins containing 4Fe and 3Fe clusters that apparently do not exhibit this chemistry. These observations have raised a number of questions, many of which are largely unresolved. For example: might some [3Fe-4S] clusters be artifacts generated by cluster degradation during purification? Which particular protein structures and amino acid sequences either facilitate cluster interconversion or stabilize one form over another? What is the mechanism of cluster interconversion, and does this chemistry have a physiological role?

In order to provide an improved experimental basis for understanding these systems, we have developed a combined direct electrochemical and spectroscopic approach for their study. The use of direct electrochemistry enables dynamic monitoring of cluster interconversion as well as providing control over this chemistry. Furthermore, it has been necessary to identify relatively simple proteins for which interconversion occurs readily and apparently without gross rearrangement of tertiary or quaternary structure. In the present paper we describe the results of our studies on one such protein, namely ferredoxin (Fd) III from the sulphate-reducing bacterium *Desulfovibrio africanus*.

In the preceding paper [7], the combination of direct electrochemistry with e.p.r. and low-temperature m.c.d. spectroscopies was used to show that, when aerobically isolated, *D. africanus* Fd III contains one $[3Fe\text{-}4S]^{1+,0}$ and one $[4Fe\text{-}4S]^{2+,1+}$ cluster within a polypeptide of M_r of 6585. This protein is of special interest as its amino acid sequence contains only seven cysteine residues [8], the minimum required to co-ordinate the two iron–sulphur clusters. As a result of our analyses, and in accordance with the detailed structural analysis of low-M_r Fds by Fukuyama *et al.* [9], we assigned the distribution of cysteine ligands to the iron–sulphur centres as indicated in Fig. 7 of the preceding paper [7]. The 4Fe cluster is co-ordinated by short sequences with the pattern Cys-Xaa-Xaa-Cys-Xaa-Xaa-Cys and Cys-Pro. These provide what may be termed a 'classic' domain known to co-ordinate $[4Fe\text{-}4S]^{2+,1+}$ centres in numerous Fds [9]. The 3Fe cluster is co-ordinated by a similar arrangement of amino acid residues, but, however, two aspects of this binding domain are unusual. The proline residue that would be expected to be adjacent to the remote cysteine residue has been replaced by glutamic acid (position 52), and, more interestingly, the cysteine residue in the centre of the main sequence has been replaced by aspartic acid (position 14). Although aspartic acid is not known to be

Abbreviations used: Fd, ferredoxin; m.c.d., magnetic circular dichroism; PGE, pyrolytic graphite edge.

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a ligand to biological iron–sulphur clusters, it does have the capability to bind Fe in other proteins, as seen in, for example, hemerythrin [10]. The iron-uptake properties of the [3Fe-4S] cluster at this site are therefore of considerable interest.

In the present paper we describe investigations into the reaction of *D. africanus* Fd III with Fe(II) ions. We again have employed a combination of direct electrochemistry with e.p.r. and low-temperature m.c.d. spectroscopies. We show that, when reduced, the [3Fe-4S] cluster in the 7Fe form of the protein reacts rapidly with a stoichiometric quantity of Fe(II) ions to produce an oxidized $[4Fe\text{-}4S]^{2+,1+}$ centre in an 8Fe form of the Fd. We further show that, when the 8Fe product Fd is reduced, one of the 4Fe clusters has the unusual spin state of $S = \frac{3}{2}$.

METHODS

The preparation of samples of *D. africanus* Fd III together with spectroscopic and electrochemical methods have been described in the preceding paper [7]. The work described in the present paper employed strictly anaerobic conditions, either with Ar-flushed apparatus or with an anaerobic glovebox ($O_2 < 1$ p.p.m.) (Faircrest). Protein samples were, in general, handled at temperatures not exceeding 4 °C. Care was taken to use non-metal apparatus in order to avoid accidental leaching of iron into solution. Electrochemical samples often contained 0.1 mM-EDTA or -EGTA to ensure the chelation of adventitous iron. All electrochemical experiments were performed at 2–3 °C with the standard calomel electrode reference maintained at 25 °C. Unless otherwise stated, all electrode and redox potentials are quoted versus the standard hydrogen electrode. When electrochemical reduction was not employed, protein solutions were reduced with microlitre quantities of 100 mM-$Na_2S_2O_4$. Where necessary, an appropriate amount of an organic redox dye was used to buffer the redox potential. Anaerobic stock solutions of Fe(II) ions were made up immediately before use from $Fe(NH_4)_2(SO_4)_2,6H_2O$ (AnalaR; BDH Chemicals) or from $FeSO_4$ three times anaerobically recrystallized.

RESULTS

Electrochemistry

As described in the preceding paper, *D. africanus* Fd III exhibits excellent cyclic voltammetry at a pyrolytic-graphite-'edge' (PGE) electrode. In the presence of small concentrations of the aminoglycoside neomycin, which promotes formation of an active protein/electrode interface, the faradaic responses due to reduction and oxidation of clusters are essentially reversible and diffusion-controlled at low scan rates. The reaction between *D. africanus* Fd III and Fe(II) ions can thus be studied electrochemically. This is illustrated in Fig. 1. Experimental details are given in the legend. Fig. 1(*a*) shows the cyclic voltammetry of the 7Fe Fd. Starting at +200 mV, low-potential cycles yield voltammetric waves due to the $[3Fe\text{-}4S]^{1+,0}$ couple (waves A) at −140 mV and the $[4Fe\text{-}4S]^{2+,1+}$ couple (waves B) at −410 mV. Also apparent at the very reducing potential of −726 mV are waves C. The nature of the species causing waves C is not discussed further in the present study.

While the potential was held at +200 mV and the solution was stirred by 'microflea' a portion of Fe(II) ions was added sufficient to co-ordinate the EGTA (0.1 mM) and provide a stoichiometric equivalent to Fd III. Stirring was stopped and cyclic-voltammetry scans were commenced, first restricting the electrochemical perturbation to between −260 mV (cathodic limit) and +100 mV (return anodic limit), the region in which the $[3Fe\text{-}4S]^{1+,0}$ couple is active (Fig. 1*b*). On the first scan, reduction of the $[3Fe\text{-}4S]^{1+}$ centre (cathodic wave A) was observed as expected, but the anodic wave, corresponding to oxidation of the $[3Fe\text{-}4S]^{0}$ cluster, was much diminished in intensity. Subsequent restricted-range scans showed that both the cathodic and the anodic waves rapidly disappear. Extending the cyclic voltammetry to the full potential range +100 mV to −850 mV showed that waves C had also disappeared whereas waves B had increased in amplitude by 1.5–2-fold.

The product of the reaction exhibits a single pair of voltammetric waves with $E^{0\prime} = -400 \pm 5$ mV (Fig. 1*c*). Plots of peak current against (scan rate)$^{\frac{1}{2}}$ are linear at least to 160 mV·s^{-1}. There is no change in $E^{0\prime}$ or in the

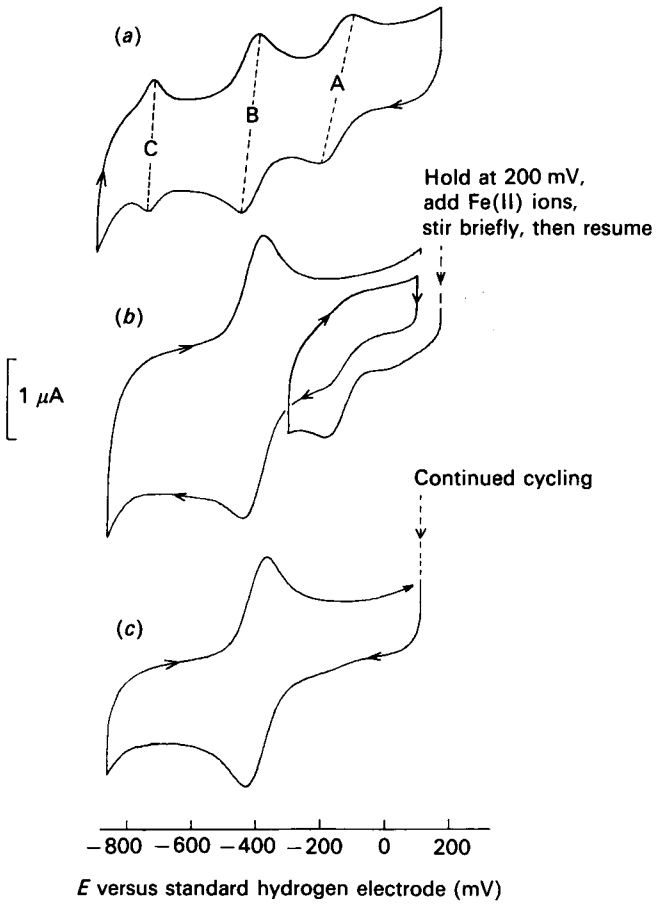


Fig. 1. Cyclic voltammograms of *D. africanus* Fd III showing the effect of adding Fe(II) ions

The scan rate was 16 mV·s^{-1} and the temperature was 2 °C. Protein concentration was 0.11 mM in electrolyte medium of 20 mM-Hepes buffer, pH 7.4, containing 0.10 M-$NaClO_4$, 0.10 mM-EGTA and 1.1 mM-neomycin. (*a*) Before addition of Fe(II) ions; (*b*) after hold at +200 mV for addition of Fe(II) ions (to total of 0.21 mM) and brief stirring by 'microflea'; (*c*) continued scan from (*b*) with no re-stirring, shows that waves associated with [3Fe-4S] have disappeared completely.

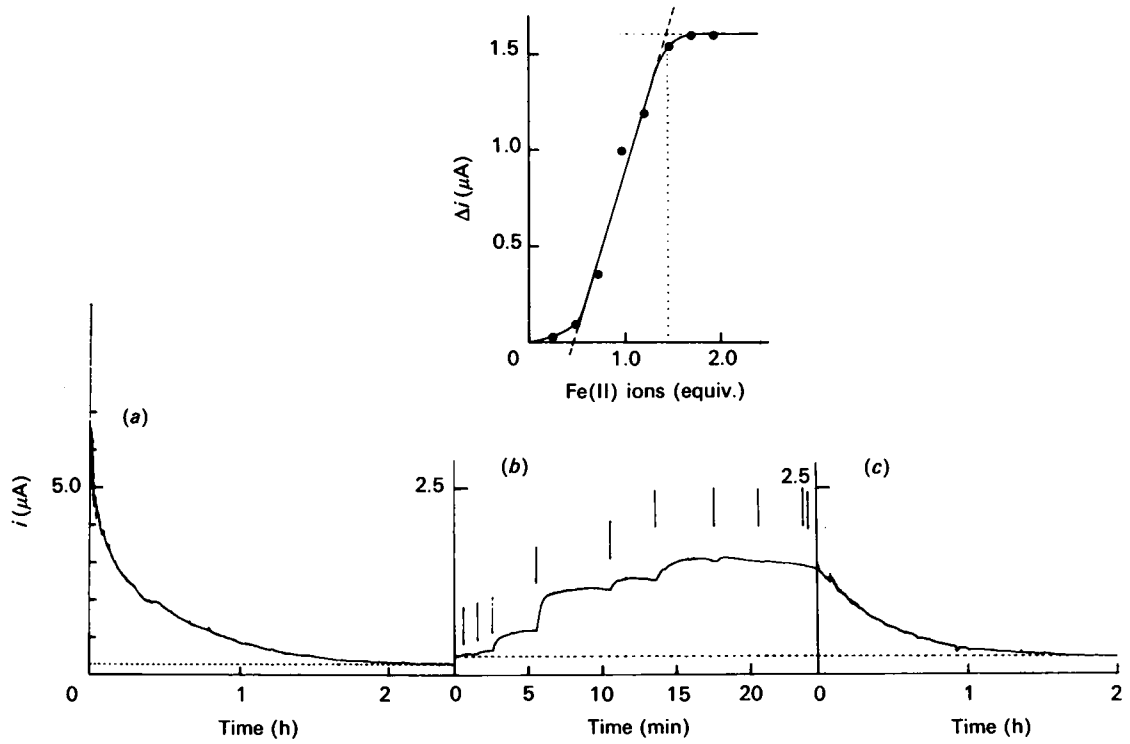


Fig. 2. Bulk electrolytic reduction of *D. africanus* Fd III and titration with Fe(II) ions

(*a*) Bulk electrolytic reduction of *D. africanus* Fd III at −610 V versus standard hydrogen electrode in stirred cell. The volume was 0.40 ml. Protein concentration was 0.10 mM in electrolytic medium of 20 mM-Hepes buffer, pH 7.4, containing 0.10 M-NaCl, 0.10 mM-EGTA and 1.5 mM-neomycin. The temperature was 3 °C. (*b*) Additions of 0.5 μl portions of 20 mM-Fe(II) ions to the fully reduced solution while holding it at a potential of −610 mV. The graph above shows the increase in current observed (connected by extrapolating the slowly decreasing maximum current value back to zero time) as a function of the number of mol of Fe(II) ions added per mol of protein. The line drawn through the central points corresponds to a predicted uptake of 1.0 equivalent of Fe(II) ions per protein molecule, following the initial lag due to proportional uptake of Fe(II) ions by EGTA. (*c*) Bulk electrolytic reduction at −610 mV following Fe(II) ions uptake (continuing from phase (*b*) on slow time scale).

general wave shape on increasing scan rate. Peak separations are close to the reversible limit, approx. 60 mV, at 16 $mV \cdot s^{-1}$ scan rate and increase to 85 mV at 800 $mV \cdot s^{-1}$. The wave shape is in excellent accordance with that expected for a one-electron process or two independent reversible one-electron processes that have an unresolvable small difference in reduction potentials. For example, the current measured at $E^{0'}$ is 83% and 85% of that measured at E_p, the peak potential, using scan rates of 8 and 80 $mV \cdot s^{-1}$ respectively. The theoretical value is 85.2% at 25 °C [11]. The electrode reaction of the product may therefore be described as reversible and diffusion-controlled. These data were collected from solutions at pH 7.4 in Hepes buffer, with 0.10 mM-$NaClO_4$ as the supporting electrolyte. Similar results were obtained for solutions containing 0.10 M-NaCl as supporting electrolyte at pH 6.3 in Hepes buffer (observed $E^{0'} = -398 \pm 5$ mV) and pH 8.4 in Taps buffer (observed $E^{0'} = -409 \pm 5$ mV).

These results suggest that, when reduced, the [3Fe-4S] centre reacts with Fe(II) ions to form a product having a redox potential very similar to that of the original [4Fe-4S] cluster. This product is capable of reduction and must therefore be generated in the oxidized state. This sequence of observations could be repeated many times for a single sample of Fd III plus Fe(II) ions, because cyclic voltammetry only acts upon the relatively small fraction of the bulk solution (the 'diffusion layer') that is under the influence of the electrode. Brief stirring replaces this with a fresh layer of unchanged protein.

In order to quantify this reaction a series of bulk electrolysis experiments were carried out. These were designed to exploit the observation that the reaction product is capable of further reduction. A typical result is given in Fig. 2. A stirred solution of 0.10 mM-7Fe *D. africanus* Fd III containing 0.1 mM-EGTA and 1.5 mM-neomycin at pH 7.4 was reduced at −610 mV (Fig. 2*a*). The solution volume was 0.4 ml. After the reduction current had fallen to a constant low level, a cyclic-voltammetry scan was measured to ensure that the protein was chemically unchanged. Then, 0.5 μl portions of 20 mM-Fe(II) ion solution (each 0.25 equivalent to protein) were titrated into the electrochemical cell. The first two portions had little effect on cell current (Fig. 2*b*). With the next four portions, however, there was, in each case, an immediate and rapid (seconds) increase in cell current. Addition of further portions of Fe(II) ion solution produced little additional increase in the current, which eventually decayed to a constant low value (Fig. 2*c*). At any time after these additions of Fe(II) ions the cyclic voltammogram of the Fd had the form of Fig. 1(*c*). In some experiments the protein was subsequently re-oxidized at −256 mV. This had no immediate effect on the form of the cyclic voltammogram. In one typical experiment, the initial reduction of 7Fe Fd and the reduction subsequent to the addition of Fe(II) ions

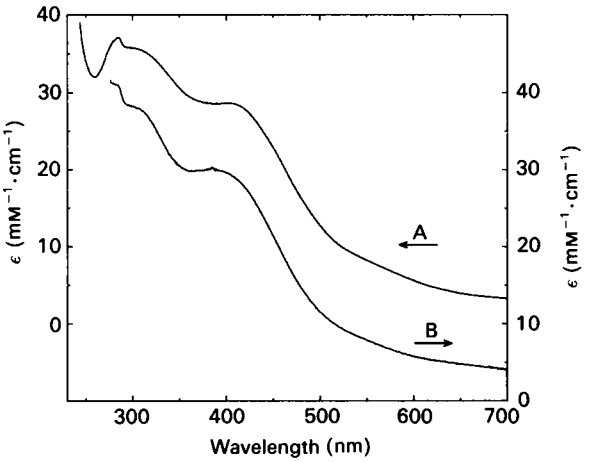


Fig. 3. Absorption spectra of fully oxidized (*a*) 7Fe and (*b*) 8Fe *D. africanus* Fd III

The spectra have been normalized to a common absorption coefficient scale and then offset. The ticks on the vertical axes indicate the zero line for spectrum A. Spectrum A, 370 μM-7Fe Fd in 40 mM-Hepes buffer, pH 7.4, containing 0.10 M-NaCl. The pathlength was 1 mm. Spectrum B, 103 μM-8Fe Fd in 20 mM-Hepes buffer, pH 7.4, containing 0.10 M-NaCl. The pathlength was 1 mm. The sample contained 1.5 mM-neomycin and had been electrochemically oxidized at −256 mV.

consumed 1.89 and 0.81 electron equivalent respectively (a ratio of 1:0.43). In a further experiment in which 7Fe Fd in the absence of EGTA was fully reduced, Fe(II) ions were added and the product was electrochemically oxidized, the initial reduction and subsequent oxidation required 2.30 and 1.10 electron equivalents respectively (a ratio of 1:0.48). We have previously shown that complete reduction of the 7Fe Fd consumes two electrons per monomer [7]. Thus, within the reasonable expectation of error, we find that the product of Fe(II) ion uptake by fully reduced 7Fe Fd can be oxidized or reduced by one electron per monomer.

Since the uptake of Fe(II) ions by the reduced [3Fe-4S] cluster is rapid compared with the reduction time of the product (approx. $t_{\frac{1}{2}}$ 20 min), we were able to use the current increase as a monitor of Fe(II) ion uptake in the titration. The result is shown in Fig. 2. In constructing this graph we have included small corrections for the current decreasing during the experiment because the sample was being reduced. The line drawn through the points corresponds to an uptake of 1.0 equivalent of Fe(II) ions per protein molecule, indicating that the Fe(II) ion–Fd III reaction has a 1:1 stoichiometry. The initial lack of response on the first additions of Fe(II) ions must be due to preferential uptake of Fe(II) ions by the chelator EGTA. This could be demonstrated by monitoring the Fe(III)–EGTA/Fe(II)–EGTA couple at +250 mV with cyclic voltammetry. That the EGTA only chelated approx. 0.5 equivalent of Fe(II) ions can be explained in terms of the EGTA having already chelated adventitious iron from the protein solution. Since each protein molecule may release seven iron ions, the EGTA may be effectively consumed during the course of minor degradation.

The most straightforward interpretation of these electrochemical results is that the reduced form of the 3Fe cluster, namely $[3Fe\text{-}4S]^0$, in *D. africanus* Fd III reacts with Fe(II) ions to produce a 4Fe cluster in the oxidation state $[4Fe\text{-}4S]^{2+}$. This new cluster has a similar redox potential to that of the 4Fe centre native to the 7Fe protein (Scheme 1 below). We term the product 8Fe Fd III. This proposal is of note since, as stated above, the sequence shows that the 3Fe cluster site contains only three cysteine residues [8] whereas a 4Fe cluster requires four ligands. The [4Fe-4S] centre formed from the 3Fe cluster must therefore include non-thiolate co-ordination. In the next section we describe spectroscopic evidence that suggests that the product is indeed a $[4Fe\text{-}4S]^{2+,1+}$ cluster, albeit with unusual magnetic properties in the reduced state.

E.p.r. and m.c.d. spectroscopy

We have used direct electrochemical reduction to prepare both reduced and oxidized samples of 8Fe Fd for optical and e.p.r. spectroscopies. For each sample it was possible to assess accurately and rapidly the extent of reduction, and, by measuring a cyclic voltammogram, to ensure that all the Fd is in the 8Fe form.

Fig. 3 compares the absorption spectra of oxidized 7Fe *D. africanus* Fd III with the 8Fe product of Fe(II) ion uptake after electrochemical oxidation. Both spectra have similar overall intensities and comprise two broad bands centred approximately on 300 nm and 400 nm. The visible-absorption region of the 8Fe product, however, is shifted towards shorter wavelengths by some 15 nm. The shoulder at 408 nm in the 7Fe Fd has been replaced by a more rounded peak at 390 nm, with a shallow trough centred at 364 nm. The spectrum of the 8Fe protein is therefore similar to those observed from Fds containing only $[4Fe\text{-}4S]^{2+}$ clusters, such as those from *Bacillus stearothermophilus* [12] and *Clostridium acidi-urici* [13].

Figs. 4 and 5 present e.p.r. spectra recorded from 8Fe *D. africanus* Fd III at various levels of reduction. The bulk of our e.p.r. studies employed direct electrochemical reductions of relatively dilute protein (0.1 mM). The spectra presented in Figs. 4 and 5, however, were obtained from titrations of more concentrated protein (0.4–0.9 mM) with 0.1 M-$Na_2S_2O_4$ solutions. Apart from the improved signal-to-noise ratio arising from the greater protein concentrations, the dithionite-reduced samples generated spectra essentially identical with those produced electrochemically.

The e.p.r. spectrum of the oxidized 8Fe product is devoid of significant signal (Fig. 4*a*). As the Fd is progressively reduced, two sets of e.p.r. signals develop approximately in concert. These are a rhombic signal centred on $g = 1.93$ (Fig. 4*b*–4*d*) and a much weaker absorption shaped peak at $g = 5.27$ (Fig. 5). Reductive titrations monitored by e.p.r. indicate that, although the $g = 1.93$ species is slightly less reducing than the $g = 5.27$ species, their redox potentials are separated by no more than 8 mV and by probably substantially less. At no time after the addition of Fe(II) ions to reduced 7Fe Fd were the e.p.r. signals associated with the $[3Fe\text{-}4S]^{1+,0}$ cluster [7] observed at $g = 2.01$ or $g = 12$.

The $g = 1.93$ signal arises from a $S = \frac{1}{2}$ system. In the fully (two-electron-)reduced protein it has an integrated intensity of 1.0 ± 0.1 spins per protein monomer. Its general form and temperature-dependence are similar to those observed from many $[4Fe\text{-}4S]^{1+}$-cluster-containing proteins [14]. The observation of this signal is not surprising, as an almost axial '$g = 1.94$' signal arising

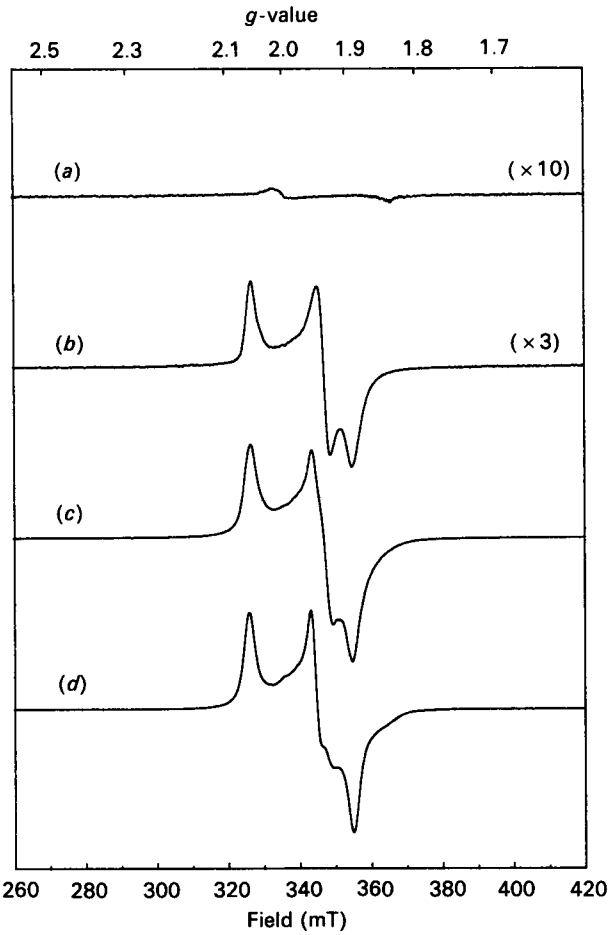


Fig. 4. X-band e.p.r. spectra of 8Fe *D. africanus* Fd III

All spectra were recorded at 12.5 K with microwave power 2 mW, microwave frequency 9.39 GHz and modulation amplitude 0.5 mT. Spectra have been normalized for concentration but no correction for variations in tube calibration have been made. (*a*) Oxidized. (*b*) Reduced by 0.45 electron equivalent. (*c*) Fully (two-electron-)reduced. (*d*) Fully (two-electron-)reduced. Spectra (*a*), (*b*) and (*c*) were recorded from 366 μM-Fd in 40 mM-Hepes buffer, pH 7.4, containing 0.10 M-NaCl. Samples were generated from 300 μl of 7Fe Fd by addition of 4.5 μl of 25 mM-Fe(II) ion solution and reduced by portions of 20 mM-$Na_2S_2O_4$ in a titration experiment. Spectrum (*d*) was recorded from 0.87 mM-Fd in 0.5 M-Tris/HCl buffer, pH 7.6, prepared in a similar way.

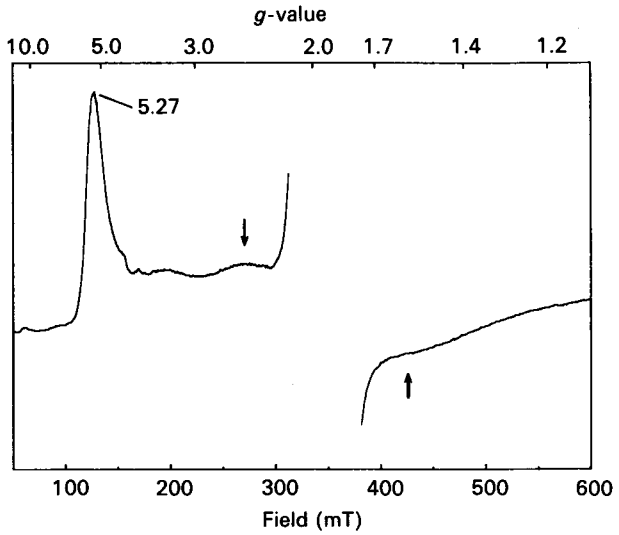


Fig. 5. X-band e.p.r. spectrum of reduced 8Fe *D. africanus* Fd III

The spectrum was recorded at 8 K with microwave power 80 mW, microwave frequency 9.39 GHz and modulation amplitude 1.0 mT. The sample was 0.87 mM-Fd in 0.5 M-Tris/HCl buffer, pH 7.6. It was generated from 250 μl of 7Fe Fd by addition of 5.5 μl of 50 mM-Fe(II) ion solution and was reduced by 10 μl of 100 mM-$Na_2S_2O_4$. The arrows indicate features tentatively assigned to the *g*-components as discussed in the text.

from a reduced $S = \frac{1}{2}$ [4Fe-4S]$^{1+}$ cluster is observed in two-electron-reduced 7Fe Fd III [7], and the electrochemical results show that the [4Fe-4S]$^{2+,1+}$ couple associated with this signal is not significantly affected by the reaction with Fe(II) ions. We can therefore assign this spectrum to a reduced [4Fe-4S]$^{1+}$ centre. The lineshapes of the e.p.r. spectra in Fig. 4, however, are distinctly different from those observed for the 7Fe Fd, and they vary as the protein is reduced. For example, the signal from protein reduced by approx. 0.45 electron equivalent (or less) has a rhombic e.p.r. spectrum of the classic '$g = 1.94$' type with apparent *g*-values of 2.051, 1.931 and 1.89 (Fig. 4*b*). In contrast, the spectrum from fully (two-electron-) reduced Fd is more complex, with additional features, slight shifts in *g*-values and broader linewidths (Fig. 4*c*). In addition, the lineshape of the spectrum of fully reduced Fd III is dependent on sample conditions. Compare, for example, Figs 4(*c*) and 4(*d*). This behaviour is well known in Fds containing two [4Fe-4S]$^{2+,1+}$ clusters, such as those from *Clostridium pasteurianum* and *Micrococcus lactilyticus*. When these proteins are reduced both clusters adopt a $S = \frac{1}{2}$ spin state. A magnetic interaction between these two $S = \frac{1}{2}$ [4Fe-4S]$^{1+}$ centres produces a complex e.p.r. lineshape [15]. Only if both the clusters are reduced in an Fd monomer is the interaction observed. Clearly, when the protein is reduced by 0.45 equivalent (out of a maximum of 2) this is a statistically unlikely event, so a simple non-interacting e.p.r. signal is seen. The lineshapes of these spectra have been known to vary with sample conditions [16]. Clearly a closely analogous phenomenon is occurring in 8Fe *D. africanus* Fd III. However, the intensity of the $g = 1.93$ signal in fully (two-electron-)reduced protein represents only a single spin per Fd monomer. This suggests that half the clusters in the protein must adopt a spin state other than $S = \frac{1}{2}$.

The e.p.r. signal at $g = 5.27$ is illustrated in Fig. 5. This signal is not saturating at 5 K with 100 mW microwave power. On increasing the sample temperature, this feature broadens and it is difficult to measure at 25 K. Also apparent in Fig. 5 are broad features above and below $g = 2$ (arrowed). These bands are not present in the oxidized 8Fe protein, and do not power-saturate with the $g = 1.93$ signal. The $g = 5.27$ signal is assigned to a species with a spin ground state $S = \frac{3}{2}$. We believe the broad features to higher field comprise the other two *g*-components of this spectrum. The origin of these bands can be understood by using a spin Hamiltonian [17], where g_0 is the *g* tensor and *D* and *E* are the axial and rhombic zero-field splitting parameters respectively. Solution of the spin Hamiltonian indicates that an $S = \frac{3}{2}$

system with an axial zero-field splitting (D) greater than the Zeeman interaction comprises two separate Kramer's doublets. If the zero-field splitting contains a rhombic component, e.p.r. transitions become allowed within both doublets. With an isotropic g_0 tensor = 2, a signal at $g = 5.27$ is obtained if $E/D = 0.27$, the other two g-values being observed at $g = 2.34$ and $g = 1.62$. Other combinations of g_0 values and E/D also yield signals at $g = 5.27$, but with different higher-field g-values. Unfortunately the $g = 1.93$ signal prevents measurement of all the g-values. In addition, we have not observed any other signals that can be assigned to transitions within the second doublet of the $S = \frac{3}{2}$ system. Therefore, with the current data, we cannot unambiguously assign the details of the ground state of the spectrum in Fig. 5. However, from its oxidation–reduction properties it is clear that this $S = \frac{3}{2}$ spectrum is associated with the clusters of the 8Fe Fd. That this spectrum does not arise from a minority species can be shown by single integrations of the isolated $g = 5.27$ peak [18], a procedure that requires estimation of the higher-field g-values. By using a variety of estimates consistent with the spin Hamiltonian and the observed spectrum, this gives intensities in the region of 1.5 to 0.5 spins per ferredoxin monomer. The $S = \frac{3}{2}$ paramagnet is therefore not a minority species, and hence we assign it to a reduced [4Fe-4S]$^{1+}$ cluster.

The e.p.r. data indicate that, when reduced, 8Fe *D. africanus* Fd III contains approximately equal proportions of [4Fe-4S]$^{1+}$ clusters with an $S = \frac{1}{2}$ ground state and [4Fe-4S]$^{1+}$ clusters with an $S = \frac{3}{2}$ ground state. This situation can arise in one of two ways. First, each 4Fe centre in an Fd molecule could adopt both spin states in some form of equilibrium. Alternatively, the spin state could be an inherent property of each cluster site, with each Fd molecule containing one $S = \frac{3}{2}$ and one $S = \frac{1}{2}$ centre. For reasons outlined below in the Discussion section, we prefer the latter alternative.

In order to confirm the assignment of the 8Fe *D. africanus* Fd III from the electrochemical and e.p.r. data we have obtained low-temperature m.c.d. data for both the fully oxidized and the fully reduced protein. As detailed in the preceding paper [7], it has so far proved problematic to prepare samples for m.c.d. spectroscopy by using direct electrochemical manipulation. This is because the ethanediol glassing agent necessary for the low-temperature experiment substantially increases viscosity and concomitantly decreases the rate of reduction. Hence samples for m.c.d. spectroscopy were prepared with the use of either $Na_2S_2O_4$ and/or an appropriate organic redox dye to control the potential. The redox state and chemical condition of the samples were monitored by e.p.r. We note that the presence of ethanediol in the sample buffer has no significant effect on the e.p.r. properties of the 8Fe Fd.

M.c.d. samples of oxidized 8Fe Fd III were prepared by adding a small excess of Fe(II) ions to solutions of 7Fe Fd, and then reducing them, either with a dithionite titration monitored by e.p.r. spectroscopy, or with phenosafranine. The redox potential of this dye (approx. −252 mV) is sufficiently negative to reduce the [3Fe-4S]$^{1+,0}$ clusters so that Fe(II) ion uptake occurs, but it is too oxidizing to reduce either the newly formed 4Fe centre or the one already existent. Fig. 6(*a*) shows a typical m.c.d. spectrum of 8Fe Fd III recorded at 1.6 K and 50 K with a magnetic field strength of 5 T. At 1.6 K

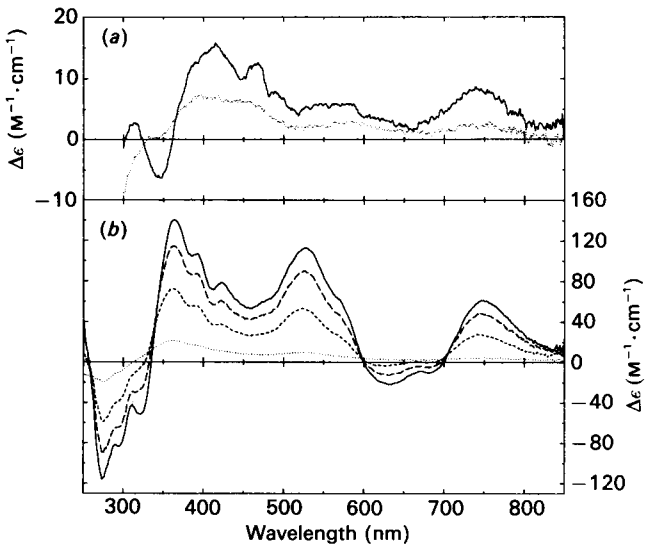


Fig. 6. Low-temperature m.c.d. spectra of 8Fe *D. africanus* Fd III recorded at 5.00 T and a number of temperatures

For both samples, 8Fe Fd was generated from 7Fe Fd by addition of 1.1 equivalents of Fe(II) ion solution before either partial or complete reduction with $Na_2S_2O_4$ solution. (*a*) Oxidized Fd recorded at 1.54 K (——) and 48.7 K (·····). The protein was 190 μM in 25 mM-Taps buffer, pH 8.0, containing 50% (v/v) ethanediol. The pathlength was 1.20 mm. (*b*) Reduced 8Fe Fd, reduced by $Na_2S_2O_4$ solution, recorded at 1.66 K (——), 4.2 K (– – –), 10 K (------) and 102 K (·····). The protein was 390 μM in 20 mM-Hepes buffer, pH 7.4, containing 0.10 M-NaCl and 50% (v/v) ethanediol. The pathlength was 1.00 mm.

the intensity is low with a maximum observed amplitude of 16 $M^{-1}\cdot cm^{-1}$ at 415 nm. It exhibits a weak temperature-dependence, which indicates the presence of a small amount of a paramagnetic species. However, the low intensity shows that the majority of the species present are diamagnetic. This spectrum is very different from that of the reduced cluster [3Fe-4S]^0, which has a maximum intensity at 401 nm of 213 $M^{-1}\cdot cm^{-1}$ at 1.6 K in a 5 T magnetic field, and which is strongly temperature-dependent [7]. The m.c.d. evidence therefore confirms that the [3Fe-4S]^0 centre has reacted with the Fe(II) ions to produce a diamagnetic cluster.

The m.c.d. spectrum of dithionite-reduced 8Fe Fd III (Fig. 6*b*) is considerably more intense than that of the oxidized 8Fe Fd and arises from predominantly paramagnetic species. The form of the spectrum bears a close resemblance to those of $S = \frac{1}{2}$ [4Fe-4S]$^{1+}$-containing proteins such as Fd I and Fd II from *D. africanus* [19] and the Fd from *C. pasteurianum* [20]. We note moreover that the intensity of the spectrum in Fig. 6(*b*) ($\Delta\epsilon_{max.}$ 140 $M^{-1}\cdot cm^{-1}$ at 364 nm, measured at 1.6 K and 5 T) is greater than that expected for two $S = \frac{1}{2}$ [4Fe-4S]$^{1+}$ centres (approximate $\Delta\epsilon_{max.}$ 100 $M^{-1}\cdot cm^{-1}$). Magnetization data have been recorded from a number of bands. Two examples are given in Fig. 7. It is clear that the magnetization varies with wavelength and that different temperature curves are not necessarily co-linear on a $\beta B/2kT$ plot. As discussed elsewhere [21], this indicates that the m.c.d. spectrum includes a contribution from paramagnets with spin $S > \frac{1}{2}$. We exclude any contribution to the m.c.d. spectra from a [3Fe-4S]^0 cluster. The

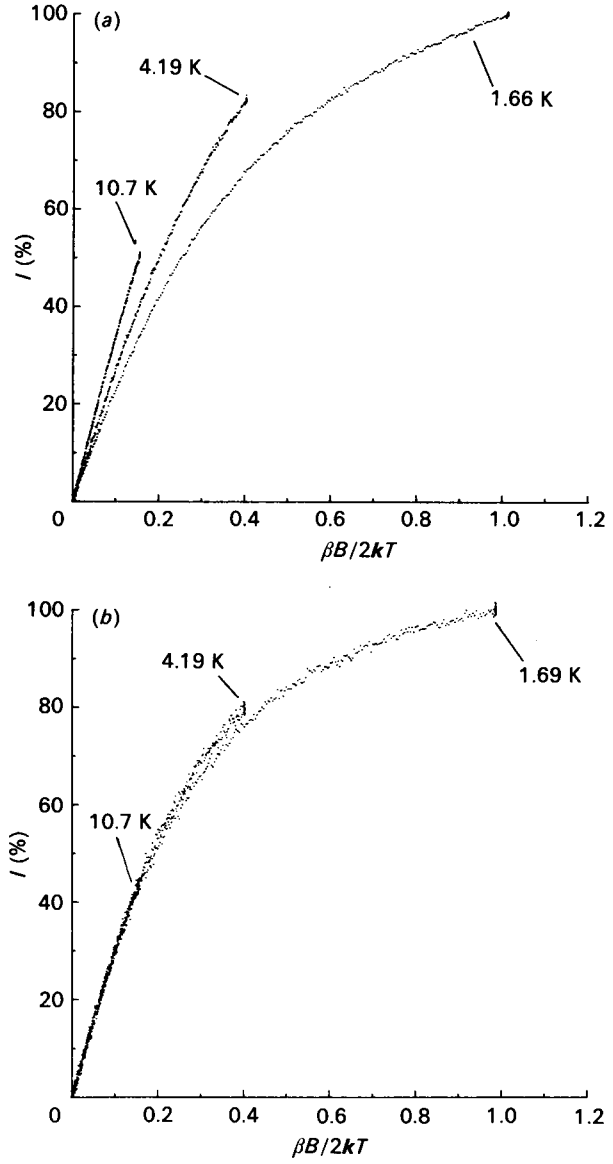
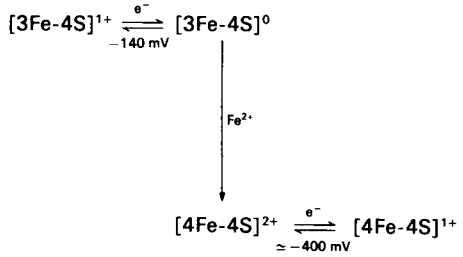


Fig. 7. Low-temperature m.c.d. magnetizations of reduced 8Fe *D. africanus* Fd III from the spectrum in Fig. 6(*b*)

Magnetizations were recorded at (*a*) 364 nm and (*b*) 749 nm, with approximate sample temperatures of 1.6 K, 4.2 K and 10 K. Exact temperatures are indicated on each panel.

latter has a ground-state spin $S = 2$ and characteristic m.c.d. magnetization curves [7,22]. The intensity of the m.c.d. spectrum of reduced 3Fe clusters at 1.6 K and 5 T is high: $\Delta\epsilon > 180\ M^{-1}\cdot cm^{-1}$ at 380 nm, 450 nm and 710 nm. Hence the contribution of even a small percentage of $[3Fe\text{-}4S]^0$ cluster to the m.c.d. spectrum (Fig. 6*b*) would be obvious.

The e.p.r. evidence strongly suggests that half the $[4Fe\text{-}4S]^{1+}$ clusters have spin state $S = \frac{3}{2}$. Because the m.c.d. spectrum (Fig. 6*b*) is a sum of the spectra from two $[4Fe\text{-}4S]^{1+}$ species that differ in their ground-state magnetic properties, it is not possible to determine the relative contributions of each. The form of the magnetization of the $S = \frac{1}{2}$ $[4Fe\text{-}4S]^{1+}$ cluster can be predicted, but the exact intensities and the form of the m.c.d. spectra vary somewhat between species from different proteins.



Scheme 1.

Analysis, using the approximate form of the spectrum calculated for the $S = \frac{1}{2}$ $[4Fe\text{-}4S]^{1+}$ cluster in two-electron-reduced 7Fe Fd III [7], indicates that the magnetizations are consistent with a mixture of $S = \frac{1}{2}$ and $S = \frac{3}{2}$ species (results not shown). The m.c.d. data therefore agree with the e.p.r. evidence that the reduced 8Fe Fd III contains two types of $[4Fe\text{-}4S]^{1+}$ clusters, one with an $S = \frac{1}{2}$ and the other an $S = \frac{3}{2}$ spin state.

DISCUSSION

The results presented here provide clear evidence that the [3Fe-4S] cluster in 7Fe *D. africanus* Fd III readily reacts with Fe(II) ions to generate a [4Fe-4S] cluster. This chemistry is summarized in Scheme 1. The electrochemical data establish that the reduced 3Fe cluster will react rapidly and stoichiometrically with Fe(II) ions. No electrochemical or spectroscopic evidence for iron uptake by the oxidized state of the 3Fe cluster could be obtained. The electrochemical data also demonstrate the different redox properties of both the native $[3Fe\text{-}4S]^{1+,0}$ cluster and the product. The spectroscopic data provide good evidence that the product is a $[4Fe\text{-}4S]^{2+,1+}$ cluster, albeit one with unusual magnetic properties when in the reduced state.

The avidity of the reduced 3Fe cluster for iron is high. The presence of an Fe(II) chelator, such as EGTA, is required to prevent the [3Fe-4S] cluster from acquiring iron from its environment. For example, on standing in anaerobic $Na_2S_2O_4$ solution the 7Fe Fd III apparently slowly converts into the 8Fe Fd. The source of the acquired iron may be introduced by handling the protein, or alternatively by the slow breakdown of other Fd molecules. We note that this behaviour is very similar to that previously observed in the enzyme aconitase [2,23]. As isolated this enzyme is inactive and contains a [3Fe-4S] cluster. When reduced this cluster shows an analogous reactivity for iron, producing the active [4Fe-4S] containing enzyme.

The co-ordination of the 4Fe cluster generated by the reaction of *D. africanus* Fd III with Fe(II) ions is novel. As stated above, the polypeptide of this Fd contains only seven cysteine residues. As the two [4Fe-4S] centres in the 8Fe Fd require four ligands each, one of the clusters must include at least one non-cysteine ligand in its co-ordination. In the preceding paper [7] we examined the 7Fe form of Fd III by using direct electrochemistry together with e.p.r. and m.c.d. spectroscopies. It was thereby shown that the iron–sulphur centres were spectroscopically analogous with the [3Fe-4S](3Cys) and [4Fe-4S](4Cys) clusters defined by X-ray crystallography. In accordance with the detailed structural analysis by

Fukuyama *et al.* [9], we assigned the cysteine residues to the iron–sulphur clusters as indicated in Fig. 7 in the preceding paper [7]. The binding domain of the tri-co-ordinate [3Fe-4S] cluster comprises the sequences Cys^{11}-Thr-Gly-Asp^{14}-Gly-Glu-Cys^{17} and Cys^{51}-Glu. Comparison of this with the sequences of crystallographically defined Fds [9] shows that this is a typical binding sequence for [4Fe-4S] centres that undergo the 2+,1+ oxidation state change except that the expected cysteine ligand at position 14 has been replaced by an aspartic acid residue. Therefore, when a 4Fe cluster occupies this site, the carboxylate side chain of this aspartic acid residue is the most probable candidate for the non-cysteine ligand.

It should be emphasized that we currently have no positive proof that this aspartic acid residue is a ligand to the [4Fe-4S] centre in this site. However, because of the steric constraints of the polypeptide chain, it seems unlikely that any other residue can co-ordinate this cluster. The only other possibility, therefore, is that a small molecule such as water or OH^- ion binds the iron. The small pH-dependence of the effective $E^{0'}$ for the 8Fe Fd shows that if this were to be the case then no H_2O/OH^- equilibrium can be operative within the pH range used in our experiments. The possibility of Cl^- co-ordination becomes unlikely when we consider that identical results were obtained whether the background medium was Cl^- or ClO_4^-. In any case, this is the first well-authenticated report of non-cysteine co-ordination of a 4Fe cluster (see 'Note added in proof').

When the 8Fe Fd III is oxidized the magnetic properties of both 4Fe clusters as determined by e.p.r. and m.c.d. measurements are diamagnetic (spin $S = 0$) at liquid-He temperatures. In the reduced Fd half the clusters have a spin $S = \frac{1}{2}$ ground state, with a characteristic e.p.r. signal at $g = 1.93$, and the other half have a ground state of spin $S = \frac{3}{2}$, with an e.p.r. signal at $g = 5.27$. It is reasonable to assign the $S = \frac{1}{2}$ properties to the conventional $[Fe_4S_4(Cys)_4]$ centre as this cluster adopts this spin state in the 7Fe form of Fd III [7]. For this reason we are inclined to reject the possibility that both clusters in an Fd monomer may adopt either an $S = \frac{3}{2}$ and an $S = \frac{1}{2}$ spin state in some form of equilibrium. It also seems unlikely that such chemistry would consistently produce equal proportions of each spin state. We therefore assign the $S = \frac{3}{2}$ properties to the novel 4Fe cluster.

There are three precedents for $[4Fe\text{-}4S]^{1+}$ cores in proteins with $S = \frac{3}{2}$ ground states. These are the iron (Fe) protein of nitrogenase [24,25], amidotransferase from *Bacillus subtilis* [26] and hydrogenase II from *C. pasteurianum* [27]. In none of these cases are the ligands binding the 4Fe cluster established, although they are commonly assumed to be cysteine sulphur atoms. An important question is raised by the present work. To what extent is the $S = \frac{3}{2}$ ground state of the reduced [4Fe-4S] centre a direct consequence of non-cysteine (aspartic acid?) co-ordination? For example, the cluster in the Fe protein from nitrogenase can exist in either an $S = \frac{1}{2}$ or $S = \frac{3}{2}$ form [24,25]. The proportion of clusters in each spin state can be altered by changing the solvent of the enzyme. The majority of the clusters have an $S = \frac{3}{2}$ ground state in sample containing 0.5 M-urea, whereas the presence of 50% ethanediol or 15% hexamethylphosphoramide favours the $S = \frac{1}{2}$ form of the cluster. In addition, there is evidence that a small percentage of the clusters in MgATP-bound Fe protein have a ground state of $S = \frac{5}{2}$ [28]. One explanation for these different spin states, supported by the work presented here on *D. africanus* Fd III, is that changes in the ligands of the 4Fe cluster are taking place. Relevant here is a recent comparative e.x.a.f.s. study of the Fe protein from *Azotobacter vinelandii* [29]. Although the e.x.a.f.s. from both the $S = \frac{1}{2}$ and $S = \frac{3}{2}$ forms of the enzyme fitted best to a [4Fe-4S] centre with four sulphur ligands, an oxygen-for-sulphur substitution in the cluster co-ordination could not be excluded.

Shortly after the present work was completed, Carney *et al.* [30] published a detailed study of the electronic properties of 16 compounds of the form $[Fe_4S_4(SR)_4]^{3-}$, the synthetic analogue of the protein-bound $[4Fe\text{-}4S]^{1+}$ core. When these compounds are polycrystalline, four categories of spin-state behaviour can be identified: (i) pure $S = \frac{1}{2}$, (ii) pure $S = \frac{3}{2}$, (iii) physical mixtures of $S = \frac{1}{2}$ and $S = \frac{3}{2}$ states, and (iv) spin-admixed ($S = \frac{1}{2} + \frac{3}{2}$) ground states. When frozen in solution with dimethylformamide or acetonitrile, all these compounds existed as physical mixtures of $S = \frac{1}{2}$ and $S = \frac{3}{2}$ spin states, regardless of which category they belong to when polycrystalline. Carney *et al.* [30] concluded that conformational variations, such as different ligand orientations, were probably responsible for the spin-state variability in the polycrystalline compounds. It is therefore possible that, in *D. africanus* Fd III, the spin $S = \frac{3}{2}$ ground state arises from differences in cluster conformation induced by the novel polypeptide of the binding site instead of being a direct consequence of ligand change. It may be relevant that *D. africanus* Fd III and the closely homologous *Desulfovibrio vulgaris* (Miyazaki) Fd I [31] are the only Fds reported in which the proline residue at position 51 has been substituted by a glutamic acid residue. Proline is well known as an important residue in determining polypeptide conformation.

The ease of conversion of the 7Fe ferredoxin into an 8Fe form raises the question of whether the 7Fe protein is physiologically relevant. In order to answer this question further work is required to establish the conditions required to reconvert the 8Fe ferredoxin into the 7Fe form.

One additional observation that deserves comment is that, despite the differences in their cluster sites and magnetic properties, the two $[4Fe\text{-}4S]^{2+,1+}$ centres in 8Fe *D. africanus* Fd III have similar redox potentials. We note that, in the limiting case, redox potentials for two identical non-interacting redox centres on one molecule would differ only by the statistical amount $(2RT/F)\cdot\ln 2$ (33 mV at 2 °C) [32]. This difference has no chemical significance and the measured, average $E^{0'}$ value is identical with that appropriate for the single isolated centre. In the case of 8Fe *D. africanus* Fd III, the observed single $E^{0'}$ value is some 5–10 mV more positive than that of the original single $[4Fe\text{-}4S]^{2+,1+}$ cluster. This result may be accommodated within either of two situations: the observed $E^{0'}$ is a composite value for two one-electron processes occurring with $E^{0'} = -410$ mV (the original cluster) and $E^{0'} = -390$ mV (the transformed cluster); or the redox potentials of the two clusters are indeed identical, with the potential of the original $[4Fe\text{-}4S]^{2+,1+}$ couple being increased slightly on the transformation of the other centre. The evidence from reductive titrations of the 8Fe Fd monitored by e.p.r. suggests that the latter case is true, with the redox potentials of the two

$[4Fe\text{-}4S]^{2+,1+}$ couples being indistinguishable within 8 mV. There is therefore a striking similarity of the redox properties of 8Fe *D. africanus* Fd III and those of many 8Fe Fds containing solely $[Fe_4S_4(Cys)_4]^{2-,3-}$ centres such as that from *C. pasteurianum* [33].

In conclusion, we have demonstrated that, as aerobically isolated, *D. africanus* Fd III is a 7Fe Fd containing one $[3Fe\text{-}4S]^{1+,0}$ and one $[4Fe\text{-}4S]^{2+,1+}$ cluster. The co-ordination and magnetic properties of both these clusters are very similar to those familiar from crystallographic characterization. When reduced, the 3Fe cluster reacts readily and rapidly with a stoichiometric quantity of Fe(II) ions, generating an additional oxidized $[4Fe\text{-}4S]^{2+,1+}$ cluster. This centre must include one non-cysteine ligand in its co-ordination, and the most probable candidate is an aspartic acid residue. This is the first well-authenticated example of a biological [4Fe-4S] cluster with a non-cysteine ligand (see 'Note added in proof'). When reduced, one of the two $[4Fe\text{-}4S]^{1+}$ centres in the 8Fe product has a spin state of $S = \frac{3}{2}$, whereas the other has an $S = \frac{1}{2}$ ground state. The balance of probabilities suggest that the cluster with the novel co-ordination also has the $S = \frac{3}{2}$ spin state. Whether this is cause and effect is unclear.

Finally, we note that Moura and co-workers [34,35] have shown that when reduced, the $[3Fe\text{-}4S]^{1+,0}$ centre in *Desulfovibrio gigas* Fd II not only will react with Fe(II) ions, but also will combine with metal ions such as Zn(II) and Co(II) ions to form mixed-metal clusters of the form [3FeM-4S]. The data in the present paper indicate that the 3Fe centre of *D. africanus* Fd III shows much greater reactivity towards Fe(II) ions than that reported for *D. gigas* Fd II [36]. We believe that our combined electrochemical and spectroscopic approach is the technique of choice when studying metal ion uptake in these systems.

Note added in proof (received 18 September 1989)

A recent X-ray-crystallographic analysis of activated pig heart aconitase [37] has shown that this enzyme contains a [4Fe-4S] cluster co-ordinated by three cysteine residues and one water molecule.

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