Electrochemical and spectroscopic studies of ferredoxins

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Abstract Fast diffusion-dominated electron transfer between bacterial ferredoxins and pyrolytic graphite 'edge' electrodes promoted by aminoglycosides permits detailed voltammetric studies and preparation of oxidation states inaccessible by chemical titration. Low temperature EPR and magnetic circular dichroism spectroscopy identifies the cluster types, and magnetic states at defined oxidation levels. In the 7Fe ferredoxin, Fd II, Azobacter chroococcum, the [3Fe-4S] cluster exhibits pH dependent $E_{1/2}$ values (30°C): $E_{1/2}$ (alkaline) = -460 ± 10 mV vs NHE, $dE_{1/2}/d(pH)$ = 55 mV, pK = 7.8. The [4Fe-4S] cluster is characterised by an unusually low reduction potential, -645± 10 mV vs NHE at pH 8.3. FdIII, Desulphovibrio africanus, contains one [3Fe-4S], and one [4Fe-4S] cluster when isolated aerobically. Cyclic voltammetry shows that when reduced the [3Fe-4S] centre reacts rapidly with ferrous ion to form a [4Fe-4S]$^{2+}$ cluster. Since the protein sequence contains only seven cysteines this cluster must possess non-cysteinyl ligation, which we propose to be the carboxylate side-chain of aspartic acid, residue 14. The magnetic properties of this cluster in the reduced state are $S = 3/2$.

INTRODUCTION

Most facultative or obligate anaerobic bacteria produce low molecular weight proteins, $M_r < 10$ kDa, called ferredoxins (Fd) which contain a pair of iron-sulphur clusters. The metal centres are redox active and may have the stoichiometry $[\text{Fe}_3\text{S}_4(\text{cys})_3]^{2-}$ or $[\text{Fe}_4\text{S}_4(\text{cys})_4]^{2-}$. Examples are known of ferredoxins containing two four-iron clusters, or one three-iron and one four-iron cluster. The structures of the two clusters are closely related being the cubane [4Fe-4S] core ligated by four thiol side-chains of cysteine and the [3Fe-4S] core, having the cubane structure with one iron atom missing from a corner, and consequently only three cysteine ligands (refs. 1, 2). In some cases there is facile interconversion of the clusters, [3Fe-4S] $\rightarrow$ [4Fe-4S] and in others none (ref. 3). Study of this process has been problematical given that the optical absorption spectra of the two forms are very similar to one another. We have therefore developed combined electrochemical and spectroscopic methods to enable these transformations to be studied (refs. 4-6). The methods have been applied to the study of two ferredoxins, namely Fd I, Azobacter chroococcum (Ac), which contains one [3Fe-4S] and one [4Fe-4S] cluster and a polypeptide sequence with nine cysteine residues, and Fd III, Desulphovibrio africanus (Da), which also possesses one of each of the cores [3Fe-4S] and [4Fe-4S], and a polypeptide sequence with only seven cysteine residues (ref. 7), insufficient to provide two [4Fe-4S] clusters with cysteinyl ligation only.

EXPERIMENTAL

Direct, unmediated, reversible electrochemistry of the Fd has been established using a pyrolytic graphite edge (PGE) electrode in combination with an aminoglycoside as a promoter. The PGE surface is rich in acidic oxides ($pK 5.6$) and in the presence of multi-charged cations, which provide an "ionic bridge", interacts with proteins bearing negatively charged surface domains. Fast, direct electron transfer can then take place between the protein and the electrode surface. Amino-glycosides have spatial distribution of cationic charges arising from the distribution of $-\text{NH}_3^+$ groups on the oligosaccharide (ref. 8). Using this method we have successfully carried out cyclic voltammetry (CV) and controlled potential bulk electrolysis to measure the number of electrons added to both Ac Fd I (ref. 4) and Da Fd III (refs. 5, 6).
RESULTS AND DISCUSSION

**Fd I, Azobacter chroococcum**

In the presence of 1-2 mM tobramycin or neomycin, this Fd shows well-defined CV at PGE electrodes, Fig. 1. Three quasi-reversible couples, 'A', 'B', and 'C' are revealed. No other waves are observed up to +600 mV vs NHE. Couple 'A' is assigned to the [3Fe-4S]_1^0 cluster. The half-wave potentials, $E_{1/2}$, show a marked break above pH 7.8, below which $E_{1/2}$ increases by 55 mV per pH unit. The relevant equilibria are as follows

$$[3Fe-4S]^+ + e^- \rightleftharpoons [3Fe-4S]^0 \quad E_{1/2} = -450 \pm 10 \text{ mV}$$

$$[3Fe-4S]^0 + H^+ \rightleftharpoons [3Fe-4S]^0H^+ \quad pK_a = 7.8 \pm 0.1$$

MCD studies have shown the existence of two distinct pH-interconvertible forms of the reduced three-iron cluster in Ac Fd I (ref. 11). The intimate $H^+/e^-$ coupling implied here is an indication that protonation may occur at the core itself, possibly on the open face close to the position normally occupied by the fourth iron.

Waves 'B' at pH 8.3 have $E_{1/2} = -636 \pm 10 \text{ mV}$ vs NHE and belong to the [4Fe-4S]^{2+/1+} couple. After exhaustive anaerobic bulk reduction of -835 mV an EPR signal characteristic of the [4Fe-4S]^{1+} couple is found, Fig. 1. The complex features of the spectrum most likely arise from spin-spin interactions between the [4Fe-4S]^{1+} core and the reduced $(S = 2)$ [3Fe-4S]^0 system. The $E_{1/2}$ value represents, we believe, the lowest redox potential yet determined for a biological Fe-S cluster.

![Figure 1(a)](image)

**Figure 1(a)** The redox properties of *Azotobacter chroococcum* Fd I as revealed by cyclic voltammetry. The pH dependence of $E_{1/2}$ values, determined in a separate experiment, is shown.

![Figure 1(b)](image)

**Figure 1(b)** X-band EPR spectrum of A.c. Fd I electrochemically reduced at -835 mV. Effective g values are indicated.

**Fe III, Desulphovibrio africanus**

Cyclic voltammetry of Da, Fd III, has been established using a PGE electrode and promoters, neomycin or tobramycin, Fig. 2. It was important to sequester all extraneous iron from the protein and from the glass apparatus with EDTA or EGTA solutions. Three waves marked 'A', 'B', and 'C' are observed. Waves 'A' at $E_{1/2} = -140 \pm 10 \text{ mV}$ correspond to the reduction of the [3Fe-4S]_1^0 cluster. In contrast to that of Ac Fd I, the $E_{1/2}$ value is pH independent. Waves 'B' correspond to the reduction of the [4Fe-4S]^{1+} core at $E_{1/2} = -410 \pm 5 \text{ mV}$ at 2°C. Both are one-electron reduction processes. Low-temperature MCD and EPR spectra identify the waves with cluster type.
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Figure 2. Cyclic voltammograms of Desulfovibrio africanus Fd III showing the effect of adding Fe(II).
I. Prior to addition of Fe(II).
II. After hold at +200 mV for addition of Fe(II) and stirring by 'microflea'.
III. Continued scan from II, with no re-stirring; waves associated with [3Fe-4S] have disappeared completely.

The power of the CV method can be seen from Fig. 2 in which the redox-initiated uptake of Fe(II) ion by the three-iron cluster is observed. Whilst holding the potential at +200 mV and stirring the solution by 'microflea' an aliquot of Fe(II) was added. Stirring was stopped and the CV scan commenced, first restricting the electrochemical perturbation to between -260 mV and +100 mV, the region of the [3Fe-4S]+,0 couple. On the first scan reduction of the [3Fe-4S]+ centre, wave 'A' was observed but the anodic wave, corresponding to oxidation of the [3Fe-4S] cluster, was much reduced in intensity. Subsequent restricted range scan showed that both the cathodic and anodic waves rapidly disappear. Extending the CV scan to the full potential range showed that both waves 'A' and 'C' had completely disappeared while waves 'B' had increased in amplitude by a factor of 2. The three-iron cluster has gained a Fe(II) ion when reduced and only when reduced and converted to a [4Fe-4S]2+ cluster. This cluster can accept one further electron.

The scheme summarizes the chemistry observed.

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\begin{align*}
[3\text{Fe-4S}]^{1+} + e^- & \xrightarrow{-140 \text{ mV}} [3\text{Fe-4S}]^0 \\
\text{Fe}^{2+} & \xrightarrow{e^-} [4\text{Fe-4S}]^{2+} \\
[4\text{Fe-4S}]^{2+} + e^- & \xrightarrow{-400 \text{ mV}} [4\text{Fe-4S}]^{1+}
\end{align*}
\]

The identification of the nature of the clusters has been made by EPR and low-temperature MCD spectroscopy (refs. 5,6).

The EPR spectrum of the fully reduced 8Fe Fd III reveals a set of rhombic signals centred on \( g = 1.93 \) region which integrate to \( 1 + 0.1 \) spins per protein monomer, plus a much weaker absorption shaped peak at \( g = 5.27 \). The latter signal is difficult to integrate but we estimate it corresponds to between 0.5 and 1.5 spins per protein monomer. Hence this signal is assigned to the [4Fe-4S]1+ core cluster formed by addition of Fe(II) plus one electron to the three-iron cluster and belongs to a spin state \( S = 3/2 \).

The low-temperature MCD spectrum, Fig. 3, of the fully reduced 8Fe Fd III has the form characteristic of [4Fe-4S] core clusters only. The MCD magnetisation properties (not shown) are consistent with the presence of a cluster with \( S = 3/2 \) subject to an axial zero-field splitting.
Since the sequence of Fd III, Da, contains only seven cysteines and the ligation of a pair of [4Fe-4S] cores requires eight ligands it is clear that one non-cysteiny ligand must coordinate one of the [4Fe-4S] cores. Because the magnetic properties of one of the reduced clusters are unusual, being $S = 3/2$, it is natural to assume that this is the cluster with a non-cysteiny ligand. The sequence (ref. 7) contains an aspartic acid residue at position 14, where a cysteine residue would normally be found. We therefore propose that the carboxylate side-chain of aspartate 14 is the fourth ligand.

There are three precedents for [4Fe-4S]$^{1+}$ cores in proteins with $S = 3/2$ ground states. These are the iron (Fe) protein of nitrogenase (ref. 12), amidotransferase from Bacillus subtilis (ref. 13) and hydrogenase II from Clostridium pasteurianum (ref. 14). In none of these cases is the ligand binding the four iron cluster established although they are commonly assumed to be cysteine sulphurs. An important question raised by this work is the extent to which the $S = 3/2$ ground state of the reduced [4Fe-4S] centre is a direct consequence of non-cysteine (aspartate?) coordination.

**REFERENCES**