Reconstitution of *Azotobacter vinelandii* ferredoxin I as a {2[4Fe-4S]$^{1+/2+}$} protein

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As normally isolated, *Azotobacter vinelandii* ferredoxin I (Fd I) is a {[4Fe-4S],[3Fe-3S]} protein: (7Fe)Fd I. We report that anaerobic reconstitution of Fd I from its apoprotein yields a protein whose spectra are distinct from those of (7Fe)Fd I and typical of bacterial ferredoxins. We identify this new form of Fd I as a {2[4Fe-4S]} protein: (8Fe)Fd I. (8Fe)Fd I is unstable in air and decomposes to give a ~10% yield of (7Fe)Fd I. These results increase the probability that (8Fe)Fd I is the form of Fd I occurring in vivo and that (7Fe)Fd I results from oxidative degradation during purification.

Ferredoxin *Reconstitution* Azotobacter vinelandii *[3Fe] cluster* *O_2 lability* *Spectroscopy*

1. INTRODUCTION

Azotobacter vinelandii ferredoxin I (Fd I) [1] has recently been recharacterized by X-ray crystallography [2,3] and Mossbauer spectroscopy [4] and shown to contain two different Fe-S clusters. One is a [4Fe-4S] cluster, essentially identical structurally to those earlier characterized in *Peptococcus aerogenes* ferredoxin [5] and *Chromatium vinosum* HiPiP [6]. The other is a novel [3Fe-3S] cluster, of composition [Fe_3S_3(S-Cys)$_5$X], in which the Fe_3S_3 core is nearly planar and X is not firmly identified. Fd I is quite stable in air and has been isolated aerobically. As isolated, the protein is in the {[4Fe-4S]$^{2+}$, [3Fe-3S]$^{3+}$} oxidation level, denoted hereafter by (7Fe)Fd I_{ox}. Dithionite (DT) reduces the [3Fe-3S] cluster, leading to (7Fe)Fd I_{red}.

Fe-S clusters containing 3Fe and exhibiting similar spectroscopic features have also been characterized in *Desulfovibrio gigas* ferredoxin II [7] and beef heart aconitase [8]. As yet it is not established that these clusters are identical in structure to that in Fd I. An Fe–Fe distance of ~4.1Å is obtained by X-ray crystallography of Fd I. In contrast, a ~2.7Å distance has been deduced from EXAFS measurements on both *D. gigas* Fd II [9] and beef heart aconitase [10]. Also, a Fe_3S_4 core stoichiometry is reported for beef heart aconitase [10].

The [3Fe] clusters of both *D. gigas* Fd II [11] and beef heart aconitase [8] can be converted to [4Fe-4S] clusters, which, on oxidation, regenerate the initial [3Fe] clusters. This leads to the obvious possibility that the [3Fe] clusters are products of oxidative degradation during purification and that the physiological proteins contain [4Fe-4S] clusters. In the case of beef heart aconitase, only the [4Fe-4S] protein is enzymatically active [8].

We report here reconstitution of Fd I in a form containing two [4Fe-4S] clusters. As isolated, the [4Fe-4S] clusters are in the 2+ oxidation level; DT causes reduction to the 1+ level. This {2[4Fe-4S]$^{1+/2+}$} protein is thus essentially identical to the well-known bacterial ferredoxins,

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epitomized by *Clostridium pasteurianum* ferredoxin (CpFd). We refer to the oxidized and reduced levels of the reconstituted Fd I as (8Fe)Fd I_{ox} and (8Fe)Fd I_{red}, respectively. (8Fe)Fd I_{ox} is unstable in the presence of air, yielding, inter alia, the initial (7Fe)Fd I protein. These results increase the probability that (7Fe)Fd I as heretofore isolated is the result of oxidation during purification and that (8Fe)Fd I is the physiological form of this protein.

2. MATERIALS AND METHODS

(7Fe)Fd I_{ox} was obtained as a side-product of *Azotobacter vinelandii* nitrogenase purification [12]. After elution from the first DEAE-cellulose column of nitrogenase purification at ~0.4 M NaCl, further purification was carried out aerobically by DEAE cellulose (DE-52) and Sephadex (G-75) chromatography, ammonium sulfate precipitation and crystallization. (7Fe)Fd I_{ox} so obtained exhibits $A_{400}:A_{280} = 0.60$.

Reconstitution of Fd I was modelled on a procedure for the reconstitution of clostridial ferredoxins [13]. Fd I apoprotein was prepared by trichloroacetic acid precipitation. (7Fe)Fd I_{ox} (~4 mg) in 0.1 M potassium phosphate buffer (pH 7.5) was exchanged into 0.1 M Tris buffer (pH 8.5) on a G-25 Sephadex (Pharmacia) column and then incubated with ~5% trichloroacetic acid (Baker) for 1 h at 0°C. The precipitate was centrifuged. The pellet was resuspended in 0.1 M Tris buffer (pH 8.5) and re-precipitated. The white pellet was then dissolved in 0.5 M Tris buffer (pH 8.6) and chromatographed on G-25 Sephadex, using 0.1 M Tris buffer (pH 8.5). Following concentration to ~2 mg/ml in an ultrafiltration cell (Amicon), using a YM5 membrane, dithiothreitol (Sigma) and guanidine hydrochloride (Sigma) were added to final concentrations of ~50 mM and ~5 M respectively and the solution incubated overnight. Sodium sulfide (Fisher, dissolved in 0.5 M Tris buffer, pH 8.6) and ferrous ammonium sulfate (Mallinckrodt, dissolved in water) were then added to a concentration ~25 times that of Fd I. After incubation for 20 min the dark brown-black solution was diluted ~5-fold with 0.02 M Tris buffer (pH 7.4) and chromatographed on DEAE cellulose (Whatman DE-52), using 0.02 M Tris buffer (pH 7.4). Black material was retained at the top of the column; a brown band passed through. The brown eluant was concentrated and diafiltered with 0.02 M Tris buffer (pH 7.4) in the Amicon cell. All stages of the above procedure were carried out anaerobically inside an O_2-free, N_2-flushed glove box (Vacuum Atmospheres, $O_2 < 0.5$ ppm) using carefully deoxygenated solutions, with the exception of the trichloroacetic acid precipitation which took place outside under O_2-scrubbed N_2.

Molar concentrations of (7Fe)Fd I_{ox} solutions were obtained by absorption spectroscopy, using $\epsilon_{400} = 29800$ [14]. Molar concentrations are converted to mg/ml, using an M_r of 12685 [3]. We argue below that the reconstituted protein obtained is (8Fe)Fd I_{ox}. Its molar concentration was obtained by absorption spectroscopy, assuming $\epsilon_{390} = 30600$, identical to ϵ_{390} of *Clostridium acidiurici* ferredoxin [15]. On this basis the reconstitution yield is 15–25%. The $A_{390}:A_{280}$ ratio of the reconstituted protein is 0.4–0.5, considerably lower than that of (7Fe)Fd I_{ox}. We attribute this to the presence of substantial Fd I apoprotein. However, because of the low reconstitution yield and because apoprotein does not contribute to the spectroscopic features of importance in the characterization of the reconstituted protein, we have not pursued further purification.

Absorption and circular dichroism (CD) spectra were measured using Cary 14 and JASCO J-500C instruments. Low volume cylindrical cells with fused quartz windows (Optical Cell Co.) were filled and loaded into an anaerobic holder with fused quartz windows inside the glove box. EPR spectra were obtained on solutions frozen under N_2 and in liquid N_2 in quartz tubes using a Varian E-12 spectrometer and an Oxford Instruments ESR-9 flow cryostat.

3. RESULTS AND DISCUSSION

The brown protein resulting from the reconstitution procedure documented above exhibits the visible-UV absorption and CD shown in fig.1; its EPR at 11 K is shown in fig.2. The corresponding spectra of (7Fe)Fd I_{ox} are also shown in fig.1,2. The absorption spectrum of the reconstituted protein is quite similar, although not identical, to that of (7Fe)Fd I_{ox}. However, the CD and EPR bear no resemblance to those of (7Fe)Fd I_{ox}, showing that the reconstituted protein is an entirely different

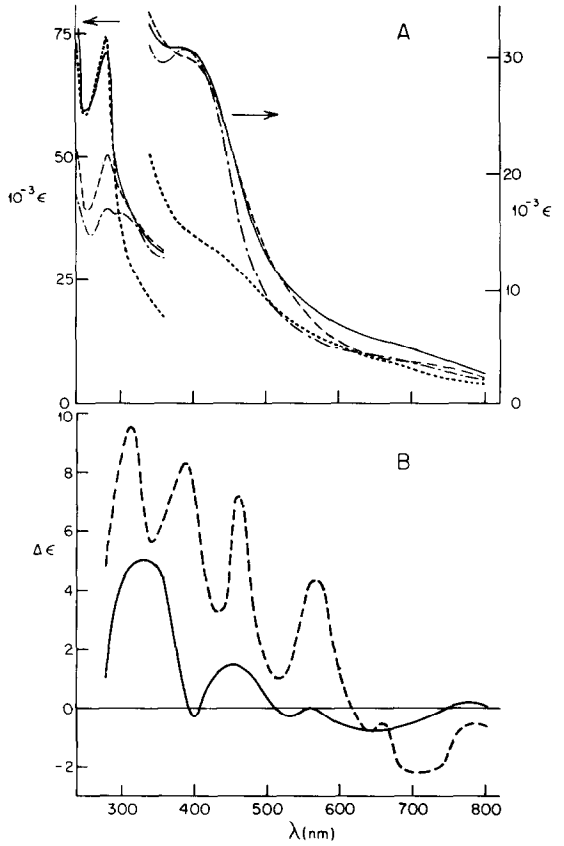

Fig.1. (A) Absorption spectra of (8Fe)Fd I_{ox} (——), (7Fe)Fd I_{ox} (– – –), oxidized CpFd (-·-·-) and air-exposed (8Fe)Fd I_{ox} (- - -). (B) CD spectra of (8Fe)Fd I_{ox} (——) and (7Fe)Fd I_{ox} (– – –). (8Fe)Fd I_{ox} is in 0.02 M Tris buffer, pH 7.4. (7Fe)Fd I_{ox} and oxidized CpFd are in 0.1 M potassium phosphate buffer, pH 7.5. All protein solutions were in the range 10–100 μM. Air-exposed (8Fe)Fd I_{ox} was the sample whose EPR is shown in fig.2B.

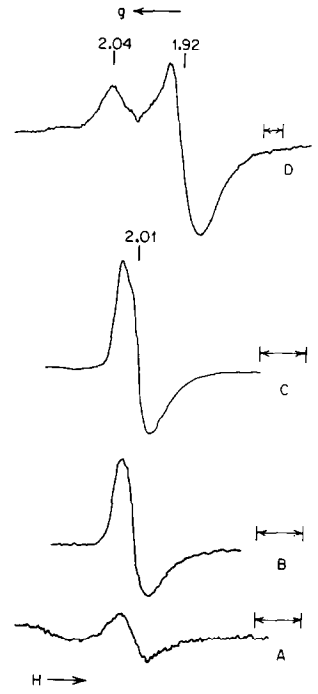

Fig.2. EPR spectra of: (A) (8Fe)Fd I_{ox}; (B) air-exposed (8Fe)Fd I_{ox}; (C) (7Fe)Fd I_{ox}; (D) (8Fe)Fd I_{red}. (8Fe)Fd I was 64 μM in 0.02 M Tris buffer (pH 7.4). (8Fe)Fd I_{red} was prepared by addition anaerobically of DT (Fisher) to a DT:(8Fe)Fd I molar ratio of ~10, followed by 5 min incubation. Air-exposed (8Fe)Fd I_{ox} was prepared by shaking a solution of (8Fe)Fd I_{ox} in an EPR tube at room temperature for a total of 76 min. At various intermediate times the sample was frozen and its EPR recorded. After ~30 min no significant change in EPR occurred. Sample temperature, microwave power, modulation amplitude, and gain were, respectively: (A) 11 K, 2 mW, 10 G and 5×10^4; (B) 11 K, 2 mW, 1 G and 4×10^4; (C) 11 K, 2 mW, 1 G and 5×10^3; (D) 13 K, 2 mW, 10 G and 4×10^4. Microwave frequency was 9.342 GHz. Horizontal arrows indicate 50 G.

species. The spectra are consistent with the presence of $[4Fe\text{-}4S]^{2+}$ clusters and we conclude that the protein is Fd I $\{2[4Fe\text{-}4S]^{2+}\}$:(8Fe)Fd I. The absorption spectrum is typical of $[4Fe\text{-}4S]^{2+}$ clusters. For comparison, the spectrum of oxidized CpFd, which contains $[4Fe\text{-}4S]^{2+}$ clusters, is also shown in fig.1. The CD spectrum is very weak, a feature common to all [4Fe-4S] clusters [16]. The shape is unlike that observed in other proteins; this is consistent with the previously observed sensitivity of the CD of [4Fe-4S] clusters to protein environment [16]. The strong $g \sim 2.01$ EPR of (7Fe)Fd I_{ox} is essentially absent, consistent with the fact that $[4Fe\text{-}4S]^{2+}$ clusters are diamagnetic in their ground state. The EPR spectrum does exhibit an extremely weak feature at $g \sim 2.01$. This is most easily attributed to either (7Fe)Fd I_{ox} surviving the reconstitution procedure or, more likely, to (7Fe)Fd I_{ox} produced by oxidation of (8Fe)Fd I (see below). The intensity of the $g \sim 2.01$ signal corresponds to a (7Fe)Fd I_{ox}:(8Fe)Fd I_{ox} molar ratio of ~0.003.

Addition of a 10-fold excess of DT to

(8Fe)Fd I_{ox} causes bleaching of the visible-near-UV absorption spectrum, radical change in the CD spectrum and development of an anisotropic 'g = 1.94' EPR spectrum; the latter is shown in fig.2. Integration of this EPR spectrum, using DT-reduced CpFd as a standard, gives ~0.5 spins/molecule. The spectrum evidences some multiplicity of structure, which can be attributed to inter-cluster spin-coupling, as observed in other {2[4Fe-4S]$^{1+}$} proteins [17].

Exposure of (8Fe)Fd I_{ox} to air leads to bleaching of the absorption spectrum (fig.1) and development of a strong g 2.01 EPR signal at 11 K (fig.2). Under the experimental incubation conditions employed, reaction is essentially complete in ~30 min. The g value, line shape and temperature-dependence of the EPR signal induced are identical to those of (7Fe)Fd I_{ox} and we attribute the EPR to this species. The CD of the air-exposed protein also shows clearly the features at ~460 and ~570 nm of (7Fe)Fd I_{ox}. Both EPR and CD lead to a (7Fe)Fd I_{ox} yield of ~10%. Absorption, CD and EPR spectra also show evidence of other products besides (7Fe)Fd I_{ox}. The absorption and CD spectra decrease in identicality to those of (7Fe)Fd I_{ox} at $\lambda \leqslant 400$ nm; the EPR spectrum includes $g \sim 4.3$ intensity. We conclude therefore that air oxidation produces multiple products, only one of which is (7Fe)Fd I_{ox}; it is likely that Fe^{3+} and apoprotein are among the other products. The identification of (7Fe)Fd I_{ox} as an oxidation product of the reconstituted protein strongly reinforces the characterization of the latter as a {2[4Fe-4S]} protein; (7Fe)Fd I_{ox} would not be expected to result from oxidation of a protein containing a single [4Fe-4S] cluster.

Azotobacter vinelandii Fd I now provides an additional example of a protein able to support more than one type of Fe-S cluster and the first example of conversion of a {2[4Fe-4S]} protein to a {[4Fe-4S],[3Fe-3S]} protein. $Fe(CN)_6^{3-}$ oxidation of CpFd yields a product containing a [3Fe] cluster [18], but the exact nature of this protein is not yet established.

The demonstration that (8Fe)Fd I exists and on exposure to air yields (7Fe)Fd I significantly increases the probability that (7Fe)Fd I results, in the purification procedures used heretofore, from oxidative degradation of (8Fe)Fd I and that the latter is the in vivo protein form. Anaerobic purification is now under study to examine this possibility directly.

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