Expression in *Escherichia coli* and characterization of a reconstituted recombinant 7Fe ferredoxin from *Desulfovibrio africanus*

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Desulfovibrio africanus ferredoxin III is a monomeric protein (molecular mass of 6585 Da) that contains one [3Fe-4S]^{1+/0} and one [4Fe-4S]^{2+/1+} cluster when isolated aerobically. The amino acid sequence consists of 61 amino acids, including seven cysteine residues that are all involved in co-ordination to the clusters. In order to isolate larger quantities of *D. africanus* ferredoxin III, we have overexpressed it in *Escherichia coli* by constructing a synthetic gene based on the amino acid sequence of the native protein. The recombinant ferredoxin was expressed in *E. coli* as an apoprotein. We have reconstituted the holoprotein by incu-

bating the apoprotein with excess iron and sulphide in the presence of a reducing agent. The reconstituted recombinant ferredoxin appeared to have a lower stability than that of wild-type *D. africanus* ferredoxin III. We have shown by low-temperature magnetic circular dichroism and EPR spectroscopy that the recombinant ferredoxin contains a [3Fe-4S]^{1+/0} and a [4Fe-4S]^{2+/1+} cluster similar to those found in native *D. africanus* ferredoxin III. These results indicate that the two clusters have been correctly inserted into the recombinant ferredoxin.

INTRODUCTION

The sulphate-reducing bacterium *Desulfovibrio africanus* expresses three ferredoxins, I, II and III [1–3], all of which function as electron carriers in the phosphoroclastic reaction and the sulphite reductase system. The two former ferredoxins each contain one [4Fe-4S]^{2+/1+} cluster [4]; the amino acid sequence of ferredoxin I has recently been revised and the X-ray structure reported [5,6]. Ferredoxin III contains one [3Fe-4S]^{1+/0} cluster and one [4Fe-4S]^{2+/1+} cluster per monomer when isolated aerobically [7]. Isolated ferredoxin III polypeptide has a monomeric molecular mass of 6585 Da, and the holoprotein contains 7–8 atoms of iron and 6–8 atoms of acid-labile sulphide ions per monomer [3]. The amino acid sequence of ferredoxin III has been determined chemically [3], showing 61 residues of which 7 are cysteines. All cysteine residues are involved in the binding of the [4Fe-4S]^{2+/1+} cluster and the [3Fe-4S]^{1+/0} cluster respectively (see Figure 1) [7]. Two modifications are observed compared with the conserved motif that is normally found for the binding of [4Fe-4S] clusters in ferredoxins [8]. The central residue usually conserved as a cysteine in the sequence Cys¹¹-Xaa-Xaa-Cys¹⁴-Xaa-Xaa-Cys¹⁷ is an aspartic acid residue in ferredoxin III, and the usual proline residue at position 52 after Cys⁵¹ has been changed to a glutamic

acid residue. Strikingly, the same two modifications (Asp¹⁴ and Pro⁵²) also appear in 7Fe *D. vulgaris* Miyazaki ferredoxin I, which overall shows 84% similarity with *D. africanus* ferredoxin III [9,10]. Three other ferredoxins, from *Pyrococcus furiosus*, *Sulfolobus acidocaldarius* and *Thermoplasma acidophilum*, contain the same consensus sequence of Cys-Xaa-Xaa-Asp-Xaa-Xaa-Cys and also bind a 3Fe cluster [11–13]. In spite of the presence of only seven cysteine residues in *D. africanus* ferredoxin III, the 7Fe form can rapidly and reversibly take up Fe²⁺ to produce an 8Fe ferredoxin, the reduced [3Fe-4S]⁰ cluster becoming a [4Fe-4S]²⁺ cluster [14]. The co-ordination of Asp¹⁴ as the fourth ligand to the resulting [4Fe-4S] cluster has been proposed but is not proven [7,14]. The binding of an exogenous thiolate ligand to this transformed cluster has been demonstrated and would require either the substitution of Asp¹⁴ or the increase of the co-ordination number of one Fe to five [15]. Other iron-sulphur proteins also show the ability to form a 4Fe cluster by uptake of Fe²⁺ into a 3Fe cluster, despite their incomplete set of cysteinyl ligands, such as activated aconitase, with water serving as a ligand to the fourth Fe of the transformed cluster, as was shown by electron nuclear double resonance studies [16,17]. The [3Fe-4S] cluster of *D. africanus* ferredoxin III can take up not only an Fe²⁺ ion, but has also been combined with other

Figure 1 Amino acid sequence of *D. africanus* ferredoxin III and proposed cluster binding

Abbreviations used: MCD, magnetic CD; IPTG, isopropyl β-D-thiogalactopyranoside; DTT, dithiothreitol.

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transition metal cations, such as Zn^{2+} , Cd^{2+} , Co^{2+} , Tl^{1+} and Cu^{1+} [18–20], to form heterometal $[\text{M}:3\text{Fe-4S}]^{2+/1+}$ clusters. The resulting heterometal clusters have been investigated with novel electrochemical methods and spectroscopy.

The stable $[\text{4Fe-4S}]^{2+/1+}$ cluster in *D. africanus* ferredoxin III has a midpoint redox potential $E^{\circ} = -410$ mV at pH 7.6. The oxidized 2+ state is diamagnetic, while the reduced 1+ cluster is paramagnetic with $S = 1/2$, as shown by EPR and magnetic CD (MCD) measurements [7]. The $[\text{3Fe-4S}]^{1+/0}$ cluster, with $E^{\circ} = -140$ mV, is paramagnetic in both oxidation states [7]. The ground-state spin for the oxidized 1+ state is $S = 1/2$ with a g value of 2.01, and $S = 2$ for the 0 state, with a small negative axial zero-field splitting [21]. The $[\text{4Fe-4S}]^{2+}$ cluster, resulting from the uptake of Fe^{2+} , has unusual electronic properties in the 1+ reduced state, with a spin ground state $S = 3/2$, while the oxidized 2+ state is diamagnetic [7,14].

The level of expression of native ferredoxins in wild-type bacteria is not always sufficient to isolate useful amounts of protein. Heterologous overexpression in *Escherichia coli* is now a frequently used method for the production of both native and mutated forms of ferredoxins. A number of efficient methods to maximize expression have been developed [22]. The expression in *E. coli* of 7Fe ferredoxins has previously been performed and consistently found to give poor yields, e.g. for *Rhodobacter capsulatus* and *Azotobacter vinelandii* ferredoxin [23,24], by contrast with high yields of up to 500-fold overexpression of holoferredoxin for $[\text{2Fe-2S}]$ ferredoxins [25,26].

Expression of ferredoxins in *E. coli* produces either apo- or holo-protein. The mechanism for *in vivo* iron-sulphur cluster incorporation in recombinant ferredoxins has not yet been elucidated. When the inclusion of iron-sulphur clusters does not take place during overexpression in *E. coli*, reconstitution of the apoferredoxin into holoferredoxin might be attempted. The general method for the incorporation of iron-sulphur centres involves the conversion of the apoprotein to its sulphhydryl form, using a thiol reductant, and the addition of either ferrous or ferric salts, in the presence of sodium sulphide. After incubation at pH ~ 8 , holoprotein yields of over 70% can be obtained [27]. Meyer and Moulis optimized the reaction conditions for the incorporation of $[\text{4Fe-4X}]$ clusters (with $\text{X} = \text{S}$ or Se) in *Clostridium pasteurianum* ferredoxin [28,29].

Previous experiments with ferredoxin III of *D. africanus* were limited by the rather low amounts of this protein that could be isolated, since the expression of ferredoxin III in *D. africanus* is very low, with yields of ~ 3 –15 mg of ferredoxin per kg of wet cell mass. As a consequence of this, another method to obtain ferredoxin III in large quantities is needed. We have used heterologous overexpression of ferredoxin III in *E. coli*. A ferredoxin III-encoding gene was made synthetically, since the amino acid sequence of ferredoxin III is known [3]. Hence, the primary structure could be back translated into codons using the preference of *E. coli*. This was a novel method, and furthermore it would enable the change of one or more amino acids in the recombinant ferredoxin by altering the necessary codons of the synthetic gene. Of special interest in this case is the change of Asp^{14} and Glu^{52} into Cys^{14} and Pro^{52} , respectively, generating the consensus sequence for many $[\text{2Fe-4S}]$ ferredoxins [8].

EXPERIMENTAL

Synthesis of the ferredoxin gene

The gene of *D. africanus* ferredoxin III was designed as two half genes, due to the limitations of synthesizer fidelity. The 5' half of the gene was designed with a flanking *EcoRI* restriction site, a ribosome binding site (based on the Shine–Dalgarno sequence

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GCT GAA TTC AGG AGA ATG AA
      ECORI
Met Gly Tyr Lys Ile Thr Ile Asp Thr Asp Lys Cys Thr Gly Asp Gly
ATG GGT TAC AAA ATC ACC ATC GAC ACC GAC AAA TGC ACC GGT GAC GGT

Glu Cys Val Asp Val Cys Pro Val Glu Val Tyr Glu Leu Gln Asp Gly
GAA TGC GTT GAC GTT TGC CCG GTT GAA GTT TAC GAA CTG CAG GAC GGT
      PstI
Lys Ala Val Ala Val Asn Glu Asp Glu Cys Leu Gly Cys Glu Ser Cys
AAA GCG GTT GCG GTT AAC GAA GAC GAA TGC CTG GGT TGC GAA TCT TGC

Val Glu Val Cys Glu Gln Asp Ala Leu Thr Val Glu Glu Asn
GTT GAA GTT TGC GAA CAG GAC GCG CTG ACC GTT GAA GAA AAC TAA

AAG CTT
      HindIII

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Figure 2 DNA sequence of the synthetic gene of *D. africanus* ferredoxin III

The *EcoRI*, *PstI* and *HindIII* restriction sites used in the construction of the gene are underlined. The ribosome binding site is shown in bold type.

[30]) to aid transcription and the coding sequence for the first 30 amino acids, finishing with a *PstI* restriction site. The 3' end of the gene was designed with the same *PstI* restriction site followed by the coding sequence for the remaining 32 amino acids, a stop codon and a flanking *HindIII* restriction site (Figure 2). Each half gene was synthesized by annealing together two oligonucleotides, which contained a 15 bp complementary overlap, extending each oligonucleotide using the Klenow fragment of T4 DNA polymerase and then cloning the resulting blunt-ended fragment into plasmid pUC19 (Pharmacia/LKB), resulting in plasmids pJB01 and pJB02. The complete gene was then created by subcloning the *PstI*–*HindIII* fragment from pJB02 into pJB01 restricted with the same enzymes. The resulting plasmid, pJB17, was oriented such that the ferredoxin III gene would not be transcribed by the vector promoter, in case that this might be lethal to the host *E. coli* cells.

After DNA sequencing to determine the fidelity of the complete ferredoxin III gene sequence, expression of the gene was attempted in a number of vector systems, including pUC18 and pKK223 (Pharmacia/LKB) but the level of expression of ferredoxin III was low. We thus created an *NcoI* site (CCA ATG) around the ATG start codon, during PCR amplification of the ferredoxin gene, and then recloned the gene as an *NcoI*–*HindIII* fragment into the vector pET21-d, resulting in plasmid pJB17. Plasmid pET21-d (Novogen) has a T7 RNA polymerase promoter site immediately upstream of a ribosome binding site and the *NcoI* site. In *E. coli* BL21(DE3), which contains an inducible T7 RNA polymerase gene integrated in the chromosome, transcription from the vector T7 promoter is switched on by the presence of the inducer isopropyl β -D-thiogalactopyranoside (IPTG).

Overexpression

E. coli BL21(DE3) containing the plasmid pJB17 were grown at 37 °C in 5 l of Luria–Bertani medium [31], equally divided over ten 2 l baffled conical flasks and supplemented with 0.7 mM ampicillin (Beecham Research). When the cells reached an absorbance of 1.6 at 550 nm, 1 mM IPTG (Melford Laboratories) was added for induction, and harvesting was 3 h later.

Purification of apoferredoxin

Cells (~ 30 g) were suspended at 0 °C in 10 mM Tris/HCl, pH 7.6, containing 5 mM dithiothreitol (DTT) (Aldrich), 20 μM pepstatin, 20 μM leupeptin, 2 mM PMSF and ~ 20 μg of DNase I (all purchased from Sigma unless otherwise stated) and were

subsequently broken in a French-pressure cell press (SLM Aminco) at a maximum external pressure of 6.9 MPa (1000 lb/in²). The lysed cells were centrifuged at 30 000 *g* for 15 min to remove unbroken cells, and the remaining supernatant was centrifuged at 170 000 *g* for 4 h at 4 °C. The resulting clear supernatant was subjected to a 50–90 % ammonium sulphate precipitation [32], which contained the recombinant apoferredoxin. This precipitate was redissolved in 40 mM Tris/HCl (pH 7.6)/5 mM DTT and dialysed overnight in 1 cm-diam. tubing (molecular mass cut off of 3500 Da; Medicell). The dialysed fraction was loaded on to an anion-exchange column (2.5 cm × 20 cm) of Whatman DE 52 DEAE cellulose, equilibrated with 40 mM Tris/HCl (pH 7.6)/5 mM DTT and developed with a discontinuous gradient from 50–500 mM NaCl. SDS/PAGE was used to follow the chromophoreless ferredoxin during the chromatographic purification steps. The colourless ferredoxin-containing fractions, which eluted between 180 and 350 mM NaCl, were pooled. The volume was reduced using a 0–90 % ammonium sulphate precipitation, which was followed by dialysis against 40 mM Tris/HCl (pH 7.6)/5 mM DTT/10 mM EDTA. SDS/PAGE was performed in a small vertical slab-gel unit from Bio-Rad (Mini-Protan II), using a method adapted from Laemmli [33].

Reconstitution of apoferredoxin

The dialysed semi-purified ferredoxin III fraction (protein concentration ~ 10 mg/ml, determined using the Lowry method [34]), contained ~ 10 % of apoferredoxin III. Part of it was used for small-scale reconstitution experiments, while a reconstitution at optimized conditions was carried out with the remaining part. Reconstitution and all further treatments of the protein were performed in an anaerobic glovebox (Faircrest) operating under an N₂ atmosphere (O₂ < 1 p.p.m., monitored with a Systech EC 90 oxygen meter).

Reconstitution tests (1 ml total volume) were carried out with a final ferredoxin III concentration of 0.1 mg/ml, in 50 mM Tris/HCl buffer at pH 8.3. DTT and β -mercaptoethanol (BDH) were separately tested as reducing agents at various concentrations from 7 to 140 mM. Iron and sulphide were subsequently added as various equimolar quantities of FeCl₃·6H₂O and Na₂S·9H₂O (Aldrich) from 30 mM or 100 mM stock solutions to give final concentrations between 0.3 and 5 mM. Different incubation times were applied, ranging from 15 min to overnight incubation. The excess of FeCl₃ and Na₂S formed a fine black Fe/S precipitate that could not be precipitated by fast centrifuging. Owing to the small scale of the test experiments, elution over a small Sephacryl S-300 column (1 cm × 3 cm; Sigma) was sufficient to separate a brown-coloured fraction from the black precipitate that was eluted from the column first. The collected brown fraction was concentrated in an 8 MC Amicon cell equipped with a YM3 membrane and then loaded on non-denaturing PAGE gel. The electrophoresed gel was subsequently stained with ferricyanide [35] and Coomassie Blue, which stain for Fe and protein respectively. From the resulting protein-stained gel, the ratio of holo/apo ferredoxin was determined using densitometry (Ultrascan XL Enhanced Laser Densitometer LKB; Bromma).

The optimized and scaled-up reconstitution was carried out as follows. The semi-purified ferredoxin fraction was diluted ten times with 50 mM Tris/HCl, pH 8.3 (100 ml total volume). DTT was added to a final concentration of 7 mM (from a 700 mM stock solution), followed by slow addition of a 100 mM FeCl₃ solution to a final concentration of 2 mM. After 15 min of incubation, Na₂S (100 mM) was added to a final concentration

of 2 mM and the solution was further incubated for 2 h at room temperature. The solution was subsequently loaded onto a DEAE column (3 cm × 5 cm), equilibrated with 40 mM Tris/HCl, pH 7.6. The column was washed with 40 mM Tris/HCl (pH 7.6)/100 mM NaCl and then eluted with 450 mM NaCl. A fine black Fe/S precipitate remained at the top of the column, while brown holoferredoxin fractions were eluted. The fractions containing ferredoxin were pooled and diluted with 40 mM Tris/HCl, pH 7.6, to 100 mM NaCl and loaded onto a second DEAE column (1 cm × 15 cm), equilibrated with 40 mM Tris/HCl (pH 7.6)/100 mM NaCl. This column was eluted with a linear gradient of NaCl from 100 to 450 mM. Brown fractions that eluted at ~ 350 mM NaCl were collected. The most concentrated fractions were pooled and concentrated by dialysis against Sephadex G-25 (Pharmacia). The protein sample was transferred to 1 cm-diam. dialysis tubing (molecular mass cut off of 3500 Da; Medicell), which was covered with dry Sephadex G-25. Hydrated G-25 material was replaced several times by dry Sephadex G-25 until the protein sample was sufficiently concentrated. This concentrated reconstituted ferredoxin III fraction was loaded on to a Sephacryl gel-filtration S-100 column (1 cm × 12 cm; Pharmacia), equilibrated and eluted with 50 mM Tris/HCl (pH 8.3)/100 mM NaCl. Brown fractions were again collected and concentrated by dialysis against G-25.

Spectroscopy

UV/visible absorption spectra were recorded with a Hitachi U-3200 spectrophotometer. Holoferredoxin concentrations were determined by using the extinction coefficient $\epsilon = 28.6 \text{ mM}^{-1} \text{ cm}^{-1}$ at 408 nm, as reported for native *D. africanus* ferredoxin III [3].

EPR spectra were recorded on an X-band Bruker ER-200D SRC spectrometer, equipped with an Oxford Instruments ESR-900 helium-flow cryostat and a TE-102 microwave cavity. Field intensity was monitored by a Hall probe and the microwave frequency was measured using a microwave counter (Marconi Instruments, model 2440). Data were collected using a dedicated Bruker ESP-1600 computer. Spin densities of paramagnetic samples were estimated from integrations of EPR absorption spectra by comparison with a 1 mM Cu²⁺/10 mM EDTA standard solution [36].

Low-temperature MCD spectra were recorded on a modified JASCO J-500D CD spectrometer. The sample was placed inside an SM4 split-coil superconducting magnet (Oxford Instruments), with magnetic fields of up to 5 T and a minimum temperature of 1.6 K. The magnetic field and sample temperature were measured with a Hall probe (Lakeshore Cryogenics) and a carbon/glass resistor (Cryogenics Calibrations) respectively. Procedures for measuring MCD spectra have been described elsewhere [37].

RESULTS

The gene for overexpression of ferredoxin III was designed as two half genes, which were subcloned to form the complete gene. This was subsequently cloned into the vector pET21-d, which has a T7 RNA polymerase promoter site. Transcription from the vector T7 promoter was switched on in *E. coli* BL21(DE3) by the presence of the inducer IPTG. Expression of ferredoxin III was carried out on a 5 litre scale, with a maximum yield of 0.2 % of the total soluble proteins.

Harvested *E. coli* cells showed on SDS/PAGE a low-molecular-mass band that was not present in *E. coli* cells lacking the plasmid. The mobility of this band is similar to that of wild-type *D. africanus* ferredoxin III and I and is thus indicative of expression of recombinant ferredoxin III. Comparisons on native

Figure 3 Analysis of recombinant *D. africanus* ferredoxin III on polyacrylamide gels at different steps of purification and reconstitution

(A) 20% native polyacrylamide gel, stained subsequently with ferricyanide and Coomassie Blue. Lane 1, purified *D. africanus* ferredoxin I (holo fd); lane 2, semi-purified apo recombinant *D. africanus* ferredoxin III (apo fd); lane 3, semi-purified reconstituted recombinant *D. africanus* ferredoxin III, obtained after reconstitution of semi-pure apoferredoxin III (lane 2), using 14 mM β -mercaptoethanol/5 mM FeCl_3 /5 mM Na_2S , followed by elution over a 1 cm $\times$ 3 cm Sephacryl S-300 column and concentration in an Amicon cell. The two lower-molecular-mass bands, indicated with a filled arrow, were also visible after staining with ferricyanide. The open arrow indicates apoferredoxin III, which consists of several oligomers. (B) SDS/15% polyacrylamide gel, stained with Coomassie Blue. Lane 4, semi-purified apo recombinant (rec. fd) *D. africanus* ferredoxin III; lane 5, semi-purified reconstituted recombinant *D. africanus* ferredoxin III, after two subsequent DE 52 columns and an S-100 column; lane 6, purified reconstituted recombinant *D. africanus* ferredoxin III, obtained from semi-purified ferredoxin III (lane 5) plus an additional DE 52 column.

gels showed that the *E. coli* cells did not contain holoferreredoxin but only apoferredoxin, which was present as several bands of low molecular mass, with a mobility as indicated in Figure 3(A). Reconstituted holoferreredoxin III migrates faster than apoferredoxin on native gel; its mobility is compared with that of *D. africanus* ferredoxin I in Figure 3(A).

Although the purification of apoferredoxin was carried out in the presence of the reducing agent DDT, the ferredoxin formed different oligomers (see the low-molecular-mass bands indicated in Figure 3A). Owing to this oligomerization the ferredoxin behaved anomalously on the chromatography columns, trailing elution peaks. After the ferredoxin had been applied to a DEAE column, further purification steps, such as gel filtration or FPLC, were unsuccessful. Reconstitution was therefore undertaken with the semi-purified apoferredoxin fraction, as described in the Experimental section.

The reconstitution of recombinant ferredoxin III was carried out in 50 mM Tris/HCl buffer, pH 8.3, as Meyer had shown that a pH value close to 8 was optimum for the reconstitution of $2[4\text{Fe-4Se}]$ *Clostridium pasteurianum* ferredoxin [28]. By comparing the effects of DTT and β -mercaptoethanol on the reconstitution of ferredoxin III, the bifunctional DTT was found to be more efficient as a reducing agent than β -mercaptoethanol and gave the best result at 7 mM concentration. After variation of FeCl_3 and Na_2S concentrations we found the highest reconstitution rates when 2 mM final concentrations were used (i.e. a 130-fold excess to protein). A 15 min incubation time was included after FeCl_3 addition, followed by 2 h of incubation after Na_2S had been added. Both incubation times were sufficient, and longer incubation did not affect the reconstitution yields. Brown fractions were concentrated and loaded on native gels after the black Fe/S precipitate had been removed. On the native gels brown bands were clearly visible at low molecular mass, which had a mobility similar to *D. africanus* ferredoxin I (Figure 3). The

Figure 4 UV/visible absorption (Abs) spectra of oxidized recombinant and wild-type *D. africanus* ferredoxin III

Spectrum A (lower trace), recombinant (oxidized) *D. africanus* ferredoxin III (300 μM in 40 mM Tris/HCl, pH 7.6, containing 0.4 M NaCl). A_{408}/A_{280} ratio = 0.68. The pathlength was 1 mm. Spectrum B (upper trace), native (oxidized) *D. africanus* ferredoxin III (162 μM in 0.5 M Tris/HCl, pH 7.4). A_{408}/A_{280} ratio = 0.76. The pathlength was 1 mm.

Figure 5 X-band EPR spectra of oxidized recombinant and wild-type *D. africanus* ferredoxin III

Spectra were recorded with microwave power 2 mW, microwave frequency 9.40 GHz and modulation amplitude 10 G. (a) Recombinant (oxidized) *D. africanus* ferredoxin III. Protein concentration was 300 μ M in 40 mM Tris/HCl, pH 7.6, containing 0.4 M NaCl. A_{408}/A_{280} ratio = 0.68; temperature = 9 K; spectrometer gain = 5×10^4 . (b) Native (oxidized) *D. africanus* ferredoxin III. Protein concentration was 300 μ M in 0.2 μ M Hepes (pH 7.4)/100 μ M EGTA. Temperature = 8 K; spectrometer gain = 6.3×10^4 .

gel was stained with ferricyanide and Coomassie Blue to verify the presence of both iron and protein. The brown bands of ferredoxin I and the recombinant ferredoxin III were shown to be iron-containing protein bands (see Figure 3). This was the first proof that successful reconstitution was obtained for recombinant ferredoxin III. With our optimized conditions we obtained a yield of approximately 70% reconstitution, based upon densitometry results (not shown). The conditions are similar to those reported for the incorporation of 2[4Fe-4S] and 2[4Fe-4Se] clusters in *C. pasteurianum* ferredoxin [28,29] and the insertion of the F_A/F_B [4Fe/4S] clusters in *Synechococcus* sp. PCC 6301 Photosystem I [38,39], although our optimal reconstitution was obtained at higher concentration of Fe^{3+} and sulphide.

The larger scale reconstitution (100 ml) was carried out at the optimized conditions, using the semi-pure apoferredoxin III fraction, that, prior to reconstitution, had been dialysed against EDTA. In this way adventitious metal ions, such as Zn^{2+} , were removed, to prevent interference with the reconstitution reaction.

The incubated reconstituted solution was applied to a DEAE column to remove the Fe/S precipitate and was further purified in two subsequent chromatography steps. After both chromatography columns the diluted holoferredoxin containing fractions were concentrated. During anaerobic concentration of the recombinant holoferredoxin degradation of iron-sulphur clusters occurred and apoferredoxin was consequently formed. Concentration of these fractions was therefore carried out by dialysis against a liquid-absorbing resin, Sephadex G-25 (Pharmacia), which was a gentler method than, for example, ultrafiltration in Amicon or Centricon units. Various protein contaminants were removed by chromatography, except apoferredoxin that was formed during concentration of purified holoferredoxin fractions. The samples of the reconstitution tests had been concentrated by

Figure 6 X-band EPR spectra of reduced 8Fe recombinant and wild-type *D. africanus* ferredoxin III

Spectra were recorded with microwave power 2 mW, microwave frequency 9.40 GHz and modulation amplitude 10 G. (a) Recombinant *D. africanus* ferredoxin III, reduced with excess sodium dithionite. Protein concentration was 300 M in 40 mM Tris/HCl, pH 7.6, containing 0.4 M NaCl. A_{408}/A_{280} ratio = 0.68; temperature = 18 K; spectrometer gain = 1×10^5 . (b) Native *D. africanus* 8Fe ferredoxin III. Sample contained 640 μ M Fe^{2+} and was reduced with excess of sodium dithionite. Protein concentration was 611 μ M in 20 mM Tris/HCl, pH 8.6. Temperature = 20 K; spectrometer gain = 3.2×10^4 .

ultrafiltration in an Amicon cell, which must have caused degradation of reconstituted clusters in the recombinant ferredoxin, as it did in the scaled-up reconstitution. However, all samples had been treated in the Amicon in a similar way, so that this should not have had large effects on the overall results, when comparing holo/apo ratios. Similar treatments with wild-type ferredoxin III did not result in decomposition of the iron-sulphur clusters, which implies that the recombinant ferredoxin has a stability lower than the native protein.

The absorption spectrum of oxidized reconstituted recombinant *D. africanus* ferredoxin III has the same features and overall intensities as wild-type ferredoxin III with maxima at 280 and 408 nm, and a shoulder at 305 nm (Figure 4) [1]. The purity index (A_{408}/A_{280}) of purified recombinant ferredoxin III was typically 0.68, which is considerably lower than the purity index of wild-type ferredoxin III, which has been reported as 0.78 [3]. The presence of apoferredoxin in the purified reconstituted recombinant ferredoxin III results in the low purity index.

EPR spectra of oxidized reconstituted recombinant ferredoxin III contain an anisotropic signal at $g = 2.01$, with a low-field sharp positive peak with a shoulder and a broad negative 'tail' at high field (Figure 5). On increasing the temperature the $g = 2.01$ signal is significantly broadened at 15 K. The line shape and temperature dependence of this signal is identical to the signal

Figure 7 X-band EPR spectrum of reduced 8Fe recombinant *D. africanus* ferredoxin III

The spectrum was recorded at 8 K with microwave power 82 mW, five scans, microwave frequency 9.40 GHz and modulation amplitude 10 G. The protein concentration was 300 μ M in 40 mM Tris/HCl, pH 7.6, containing 0.4 M NaCl. A_{408}/A_{280} ratio = 0.68. It was reduced by an excess of sodium dithionite. Spectrometer gain = 1×10^5 .

observed for oxidized wild-type *D. africanus* ferredoxin III, which represents an oxidized $[3\text{Fe-4S}]^{1+}$ cluster with a ground-state spin of $S = 1/2$ [7]. A similar signal is also observed in other proteins containing a $[3\text{Fe-4S}]^{1+}$ cluster [40]. Integration of the $g = 2.01$ signal gave 0.9 ± 0.1 spin per monomer of recombinant ferredoxin III, which proves the presence of one $[3\text{Fe-4S}]^{1+}$ cluster per monomer. The EPR spectrum of oxidized recombinant ferredoxin III also contained an anisotropic signal at $g = 4.3$, typical of adventitious iron, and unlikely to arise from a rubredoxin-type signal.

The X-band EPR spectrum of recombinant ferredoxin III, reduced with an excess of dithionite, shows a rhombic signal (Figure 6). The line shape and temperature dependence of this signal resemble the signal of two-electron reduced 8Fe wild-type ferredoxin III (Figure 6) [7], which is typical of a reduced $[4\text{Fe-4S}]^{1+}$ cluster with $S = 1/2$. The shape of both signals depends on sample conditions, such as buffer, pH and salt concentration, causing small variations in the signal line shape [7]. Double integration of this signal resulted in 0.88 ± 0.1 spin per monomer, thus indicating the presence of one $[4\text{Fe-4S}]^{1+}$ cluster per monomer with $S = 1/2$.

At lower field the spectrum of the reduced protein shows a signal at a g value of 5.27 (Figure 7). A similar signal is present in the EPR spectrum of reduced 8Fe wild-type ferredoxin III, assigned to a $[4\text{Fe-4S}]^{1+}$ cluster with ground-state spin $S = 3/2$, the product of the reaction of the $[3\text{Fe-4S}]^0$ cluster with Fe^{2+} [14]. Double integration of this signal is not straightforward, but the intensity of the signal under the spectrum conditions indicates that this species is present in concentrations similar to those in wild-type ferredoxin III. Also, the absence at low field of a ' $g = 12$ ' signal, indicative of reduced $[3\text{Fe-4S}]^0$ clusters, shows that the majority of the 3Fe clusters must be converted into this 4Fe form

upon reduction with dithionite, by the uptake of adventitious Fe^{2+} , as in wild-type ferredoxin III. A signal at $g = 4.3$ is evidence of a small amount of residual adventitious Fe^{3+} ion.

The low-temperature MCD spectrum of oxidized recombinant ferredoxin III is given in Figure 8 (upper panel) and compared with that of the wild-type protein. The MCD spectrum shows many peaks and troughs and is very similar to the MCD spectrum of wild-type ferredoxin III [7], which both show MCD spectra characteristic of oxidized $[3\text{Fe-4S}]^{1+}$ clusters [21]. Magnetization curves of recombinant ferredoxin III at 349 and 469 nm are shown in Figure 8 (lower panel). Again, the close similarity of the magnetization curves of recombinant and native ferredoxin III can be noted [7]. At both wavelengths the intercept of the magnetization curves is 0.5, which confirms the ground-state spin $S = 1/2$ for the oxidized $[3\text{Fe-4S}]^{1+}$ cluster [7,41,42]. Unlike native oxidized ferredoxin III the magnetization curves of recombinant ferredoxin III at 349 nm do not overlap completely at different temperatures. This difference with the wild-type protein may be due to diamagnetic contributions underlying the band, most likely arising from apoferredoxin. The absence of a rubredoxin-type signal, which is very strong for the oxidized protein, confirms that the $g = 4.3$ signal observed in the EPR spectrum arises from adventitiously co-ordinated ferric ions rather than Fe^{3+} bound to protein cysteine residues.

The manipulation of reconstituted recombinant ferredoxin III samples (e.g. chromatography, concentration) caused degradation of the clusters and therefore formation of apoferredoxin, as was shown on native gels and by absorption spectroscopy. No intermediate form containing one cluster only has been observed. The instability of the recombinant holoferredoxin is in contrast to wild-type ferredoxin III, which, when handled anaerobically with care, did not show cluster destruction. Because of the inevitable presence of apoferredoxin in the reconstituted holoferredoxin samples, we were not able to further characterize the ferredoxin, as the quantity of the material suitable to obtain good spectra was too low.

DISCUSSION

The construction of a synthetic gene for recombinant *D. africanus* ferredoxin III and its successful expression in *E. coli* have been demonstrated in this report. Although iron-sulphur clusters were not present in the ferredoxin upon expression in *E. coli*, they were incorporated using reconstitution experiments. The low expression of apoferredoxin that we obtained, maximally up to 0.2% of the total cell proteins, is in accordance with a trend that can be observed for the expression of ferredoxins in *E. coli*, namely that $[2\text{Fe-2S}]$ ferredoxins are expressed in *E. coli* in higher amounts than 7Fe and 8Fe ferredoxins; compare, for example, yields of up to 30% of the total cell proteins for 2Fe *E. coli* ferredoxin [26] and 0.12% for the 7Fe ferredoxin II of *R. capsulatus* [23]. The favoured expression of a 2Fe ferredoxin was shown by the simultaneous expression in *E. coli* of a $[2\text{Fe-2S}]$ and $2[4\text{Fe-4S}]$ ferredoxin, ferredoxin IV and ferredoxin I of *R. capsulatus*, respectively, which produced the $[2\text{Fe-2S}]$ ferredoxin in 6–7-fold higher amounts than the 8Fe ferredoxin [23,43]. The assembly of iron-sulphur clusters might be a rate-limiting step for the biosynthesis of recombinant holoferredoxins in *E. coli*. An important factor, therefore, seems to be the recognition of metal-binding sites, such as cysteine residues co-ordinating the iron-sulphur clusters. They usually occur in conserved motifs [8]. An increase in the number of cysteine ligands involved in binding clusters seems to influence the total amount of (apo)ferredoxin expressed in *E. coli*. Apart from the co-ordinating cysteine

Figure 8 Low-temperature MCD spectra of oxidized recombinant and wild-type *D. africanus* ferredoxin III and magnetization curves of recombinant ferredoxin III

(Upper panel) MCD spectra were recorded at 1.6 K, magnetic field 5 T. Recombinant *D. africanus* ferredoxin III (—) concentration was $230 \mu\text{M}$ in 40 mM Tris/HCl, pH 7.6, containing 50% (v/v) glycerol. A_{408}/A_{280} ratio = 0.55. The pathlength was 1 mm. Native *D. africanus* ferredoxin III (---) concentration was $200 \mu\text{M}$ in 25 mM Taps buffer, pH 8.0, containing 50% (v/v) ethanediol. The pathlength was 1.17 mm. (Lower panel) Magnetization curves of recombinant *D. africanus* ferredoxin III at 349 and 469 nm; sample conditions as in upper panel.

residues, the rest of the protein environment of the cluster has an influence on the type of cluster that is incorporated as the region around a [2Fe-2S] cluster in ferredoxins is consistently found to be more hydrophobic than around a [4Fe-4S] cluster [44]. However, it is likely that other factors play a role in successful cluster formation during expression, such as the availability of Fe and S in the proper redox state. Recently, a *nifS* gene product (NIFS), which is involved in the synthesis of the FeMo-cofactor of nitrogenase, has been shown to have cysteine desulphurase activity to mobilize S from L-cysteine [45]. Furthermore, the level of expression of ferredoxins in *E. coli* is not only determined by

cluster insertion, but other factors are important, such as the efficiency of the expression system, strength of promoter and mRNA stability.

We have established the reconstitution of a $[3\text{Fe-4S}]^{1+/0}$ and $[4\text{Fe-4S}]^{2+/1+}$ cluster in recombinant *D. africanus* ferredoxin III. Early reports on reconstitution of ferredoxins date from the late 1960s and were performed on Clostridial $2[4\text{Fe-4S}]$ ferredoxins [46,47]. Cluster incorporation has now been reported for various ferredoxins, with yields between 70 and 100% [28,29]. Attempts to reconstitute $[3\text{Fe-4S}]$ clusters have been undertaken, for example with *D. gigas* ferredoxin II [48]. Reconstitution of the

apoform of this 3Fe ferredoxin, using a slight excess of iron and sulphide, resulted in monomeric ferredoxin that contained a [4Fe-4S] cluster. When the equimolar additions of Fe and S were decreased, applying a Fe^{2+} /protein monomer ratio of 3:1, approximately equal amounts of [3Fe-4S] and [4Fe-4S] centres were formed. However, reconstitution of 100% 3Fe clusters has not been reported for *D. gigas* [48]. Reconstitution of aconitase from the apoenzyme has been performed and mainly resulted in incorporation of a [3Fe-4S] cluster, which could be readily converted to the active 4Fe cluster [49]. Upon the preparation of apoenzyme RSS_xSR polysulphide bonds (with $x = 1$ or 2) were formed. The S^{2-} ligands from the Fe/S cluster were hence trapped in the apoenzyme, and maintained the tertiary structure of aconitase. Under appropriate conditions iron-sulphur clusters were readily reconstituted without S^{2-} addition [49]. The reconstitution of another 7Fe ferredoxin, the apoform of *A. vinelandii* ferredoxin I, had mainly resulted in the insertion of two [4Fe-4S] clusters, such that a 4Fe cluster was formed instead of a 3Fe cluster upon incubation with Fe and S [50].

Hence, this is the first report of the successful reconstitution of a 7Fe ferredoxin, resulting in the incorporation of a [3Fe-4S] $^{1+/0}$ and [4Fe-4S] $^{2+/1+}$ cluster. The reason that a 3Fe cluster is included in ferredoxin III rather than a 4Fe cluster, under the applied reconstitution conditions, is probably due to the lability of the fourth ligand, Asp^{14} , which appears not to stabilize the 4Fe cluster. However, the 3Fe cluster in recombinant ferredoxin III could be converted into a 4Fe cluster. This was visible in the EPR spectrum of reduced ferredoxin (Figure 8), where adventitious Fe had been taken up into the [3Fe-4S] $^{1+/0}$ cluster, showing a g value at 5.27, that originates from the converted [4Fe-4S] $^{2+/1+}$ cluster, and has a ground-state spin of $S = 3/2$. With direct electrochemistry, using thin-film experiments, the conversion of the 3Fe cluster into a 4Fe cluster was confirmed when we applied conditions that were similar to the uptake of Fe^{2+} in wild-type ferredoxin III [14]. Owing to the presence of apoferredoxin we could not obtain cyclic voltammograms of good quality (results not shown).

We can conclude from the spectroscopic data that a [3Fe-4S] $^{1+/0}$ and a [4Fe-4S] $^{2+/1+}$ cluster have been incorporated into the recombinant protein and that the cluster type and electronic properties are similar to those of wild-type ferredoxin III. This shows that the direct protein environment of the clusters in recombinant ferredoxin is similar to wild-type protein. Owing to the low stability of recombinant ferredoxin III, we could not obtain additional structural data, such as CD and NMR, from this protein. As the amount of native ferredoxin III was also low, we could not perform comparative studies on wild-type and recombinant ferredoxin, involving reconstitution and stability experiments. The high instability of the recombinant ferredoxin shows a difference from the native ferredoxin. We had speculated that the synthetic gene possibly contained one or two incorrect amino acids originating from errors in the published amino acid sequence of *D. africanus* ferredoxin III. We have recently cloned the gene of *D. africanus* ferredoxin III to check the amino acid sequence, and have confirmed it to be identical to the published amino acid sequence (J. L. H. Busch, J. L. J. Breton, R. James and A. J. Thomson, unpublished work). This eliminates the possibility that this instability results from an incorrect polypeptide chain. However, upon reconstitution of the apoferredoxin III the holoferreredoxin might partly fold incorrectly, causing a decreased stability of the holoprotein. We are currently investigating this possibility. We are also examining a mutant form of ferredoxin III that has Asp^{14} mutated to a cysteine residue, for which we are using a strategy similar as that described in this report.

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