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PURIFICATION AND CHARACTERIZATION OF A 7Fe-FERREDOXIN FROM *RHODOBACTER CAPSULATUS*

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Summary: A ferredoxin was purified anaerobically from *Rhodobacter capsulatus* grown photoheterotrophically with excess ammonia. This ferredoxin, called ferredoxin II (FdII), had a molecular weight of approximatively 15,000 by gel filtration and 14,000 by SDS polyacrylamide gel electrophoresis indicating that it is monomeric. Its absorption spectrum (oxidized form) exhibited maxima at 280 nm and 400 nm; the A_{400}/A_{280} ratio had a calculated value of 0.55. Chemical determination of its iron and sulfur atom content, the value of the extinction coefficient at 400 nm ($\varepsilon_{400} = 26.8\ mM^{-1}\ cm^{-1}$) and EPR spectra indicated that ferredoxin II contained one [3Fe-4S] and one [4Fe-4S] cluster. Upon reduction with excess dithionite only the [3Fe-4S] cluster became reduced. The reduction of both clusters was achieved by using 5-deazaflavin as photocatalyst. Ferredoxin II was also purified from bacteria grown under nitrogen limiting (*nif* derepressing) conditions. In *in vitro* assays, ferredoxin II catalyzed electron transport between illuminated chloroplasts and nitrogenase.

Bacterial ferredoxins are present in a wide variety of microorganisms and act as electron carriers in diverse metabolic pathways including fermentation, N_2 and CO_2 fixation, and sulfate reduction (1). Ferredoxins have been classified in different groups depending on the type of Fe-S cluster they contain, on the basis of amino acid sequence comparisons and available tridimentional structures (2, 3). Most of the known ferredoxins from photosynthetic bacteria were gathered in one group on the criterion of their amino acid sequence similarities. In this group, ferredoxins are 60 to 64 amino acids in length (81 for *Chromatium vinosum* ferredoxin) and contain 9 cysteines, 8 of which probably serve as ligands for two [4Fe-4S] clusters. However, previous studies brought evidence that several forms of unrelated ferredoxins exist in some species of photosynthetic bacteria. From *Rhodospirillum rubrum*, four ferredoxins were isolated, two of which were found to be membrane-bound (4, 5). None of these ferredoxins has yet been sequenced. In *Rhodobacter capsulatus*, four soluble ferredoxins have recently been identified (6). Ferredoxin I from the latter species was shown to consist of a polypeptide of 64 residues, the sequence of which resembled that of the known ferredoxins from photosynthetic bacteria (7). The gene encoding FdI, called *fdxN*, was cloned and localized in a group of *nif* genes on the chromosome (7). Like *nif* genes, *fdxN* was

Abbreviations: SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis; EPR: electron paramagnetic resonance.

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expressed only when bacteria were nitrogen-limited, thus indicating that FdI has a role in nitrogen fixation.

In this paper, we report the purification and characterization of a second ferredoxin, called ferredoxin II, from *R. capsulatus*. It is shown, based on biochemical evidence and EPR spectroscopic data, that this ferredoxin contains a [3Fe-4S] and a [4Fe-4S] cluster, like the well-characterized ferredoxin I from *Azotobacter vinelandii* (8).

MATERIALS AND METHODS

Purification

R. capsulatus B10 was grown photoheterotrophically as previously described (9) except that ammonium concentration in the culture medium was 10 mM. Purification was carried out under O_2-free argon, with sodium ascorbate (2 mM) and dithiothreitol (1 mM) present in all buffers.

Step 1: Preparation of the cell extract. Cells (300 g wet weight) were resuspended in 600 ml of 0.1 M Tris-HCl buffer, pH 8.0, containing 20 mM EDTA. Cells were treated with lysozyme (0.3 mg/ml at 30°C) and then broken by sonication. The extract was centrifuged 1 h at 40 000 g in a Kontron TGA50 ultracentrifuge.

Step 2: DEAE chromatography. The supernatant from centrifugation was diluted with half a volume of 25 mM Tris-HCl buffer, pH 7.5, (used as the buffer in later steps of purification) and applied to four columns (2.5 x 5 cm) of DEAE cellulose (Whatman DE52). The columns were washed with five bed volumes of buffer containing 0.17 NaCl. The ferredoxin fraction was eluted with buffered 0.4 M NaCl.

Step 3: Ammonium sulfate precipitation. The 0.4 M NaCl eluate was brought to 50% saturation with ammonium sulfate by slowly adding the salt while stirring. The suspension was centrifuged 15 min at 20 000 g and the supernatant was loaded on a column (2.5 x 10 cm) of phenyl Sepharose (Pharmacia). The column was washed with one bed volume of buffered ammonium sulfate (45% saturation) before eluting the ferredoxin in 25 mM Tris buffer.

Step 4: Hydroxylapatite chromatography. The phenyl Sepharose fraction was adsorbed onto a column (2.6 x 15 cm) of hydroxylapatite (HA ultrogel, IBF Biotechnics) equilibrated with Tris buffer. The column was washed with one bed volume of buffer and then eluted with a continuous gradient (200 ml) from 0 to 10 mM of potassium phosphate. The ferredoxin was directly concentrated on a small DEAE column (DEAE trisacryl, IBF Biotechnics) and eluted in a few ml of buffered 0.5 M NaCl.

Step 5: Gel filtration. The concentrated ferredoxin fraction was applied to a column (2.6 x 110 cm) of Sephacryl S100 HR (Pharmacia) equilibrated in buffered 0.4 M NaCl and eluted at a flow rate of 20 ml/h. The ferredoxin fraction was diluted four fold in Tris buffer and applied to a DEAE cellulose (Whatman DE52) column.

Step 6: DEAE chromatography. The DEAE cellulose column (2 x 15 cm) was developed with a linear gradient (400 ml) of 0.17 M to 0.4 M NaCl. The ferredoxin eluted at 0.3 M NaCl. It was diluted two-fold in Tris buffer, concentrated on a small DEAE trisacryl column and finally eluted in a small volume of buffered 0.4 M NaCl. The purified ferredoxin was kept frozen as pellets in liquid nitrogen.

Analytical methods

The molecular weight of ferredoxin II was estimated from its relative mobility by gel filtration on a Sephacryl S100HR column calibrated with the following markers: bovine serum albumin (65,000), ovalbumin (43,000), chymotrypsinogen (25,000), myoglobin (17,500), spinach ferredoxin (11,000) and *Clostridium pasteurianum* ferredoxin (6,800). The subunit molecular weight was determined by SDS-PAGE in the presence of urea (10). Purity was checked on SDS polyacrylamide gel (11) stained with Coomassie blue.

Absorption spectra were recorded on a Hewlett-Packard, model 8452 diode-array spectrophotometer. Redox titrations and preparation of samples for EPR spectroscopy were performed in a glove box maintained under purified argon ($O_2 < 2$ ppm). Ferredoxin was reduced chemically by excess dithionite or photochemically using 5-deazaflavin. The extent of reduction was measured spectrophotometrically at 450 nm.

EPR spectra were recorded on a Varian E 109 spectrometer equipped with a helium flow cryostat (Oxford Instrument, ESR 900). Spin concentrations were calculated by double integration of the signals using a 200 μM Cu(II)-EDTA solution as a reference.

Iron (12) and inorganic sulfide (13) were assayed according to published procedures. Amino acid analysis was performed as previously described (7) on protein samples hydrolysed in 6N HCl and derivatized with phenylisothiocyanate. Extinction coefficients of the ferredoxin were calculated from the absorbance values at the maxima and the protein concentration determined from quantitative amino acid analysis.

RESULTS

Approximatively 4 to 5 mg of pure ferredoxin II was obtained per 300 g (wet weight) of *R. capsulatus* grown with excess ammonium. FdII could also be purified from bacteria grown under nitrogen-limiting conditions but the yield was consistently lower (2-3 mg FdII per 300 g cells). The ferredoxin preparation was judged homogeneous by visualization of a single band upon SDS-PAGE (Fig.1) and by N-terminal amino acid sequencing. The purification was performed anaerobically to prevent possible oxidative damage of the ferredoxin Fe-S clusters. A slight decrease in the chromophore absorption was observed upon exposure of the protein to air. The ferredoxin polypeptide migrated with an apparent molecular weight of 23 000 by SDS-PAGE in the Laemmli system (11) (Fig.1A). Under more stringent denaturing conditions (in the presence of urea), ferredoxin II appeared as a 14 kDa polypeptide (Fig.1B) which is close to the value of 12 540 calculated from the deduced amino acid sequence of the corresponding gene product (14). The atypical slow migration of ferredoxin II upon regular SDS-PAGE may reflect charge interactions with the polymer matrix or incomplete unfolding

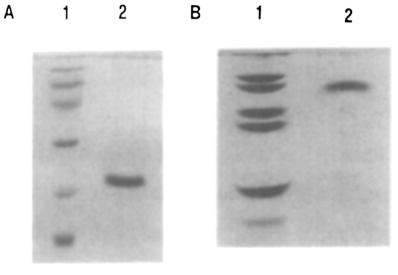


Figure 1. Analysis of purified FdII by polyacrylamide gel electrophoresis.
A: Electrophoresis was performed in the presence of 0.1% SDS as described in (11). Well 1: molecular weight markers were phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100) and α-lactalbumin (14,400) ; well 2: purified FdII (1.5 μg). **B:** Electrophoresis was performed in the presence of 8 M urea and 0.1% SDS (10). Well 1: molecular weight markers were CNBr-fragments of myoglobin having the following size (Da): 16,949, 14,404, 8,159, 6,214, 2,512. Well 2: purified FdII (0.8 μg).

Table 1. Amino acid composition of *R. capsulatus* ferredoxin II

Amino acid	mol/mol protein[a]	
Aspartate + asparagine	16.5	16
Glutamate + glutamine	14.1	12
Serine	2.4	2
Threonine	8.2	9
Glycine	7.9	7
Cysteine[b]	6.3	9
Alanine	5.6	4
Proline	10.1	11
Leucine	1.9	1
Isoleucine	4.7	6
Valine	6.7	9
Methionine	1.4	2
Tyrosine	4.2	6
Phenylalanine	3.1	3
Tryptophan	nd[c]	2
Histidine	2.0	2
Arginine	3.1	3
Lysine	7.3	7

[a] Results from amino acid analysis (left) are the means from three determinations. Whole numbers in the right column were deduced from the amino acid sequence of the ferredoxin II gene product (14).

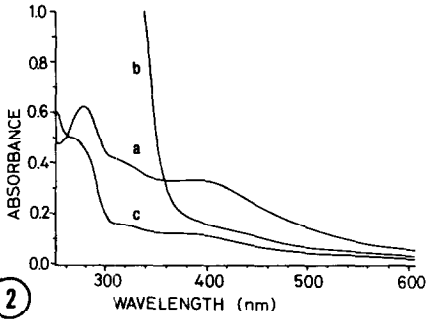
[b] Analyzed as carboxymethylcysteine.

[c] nd : not determined.

of the polypeptide chain in SDS. The native holoferredoxin has an estimated molecular weight of 15 000 by molecular sieve chromatography, indicating that it is monomeric.

The amino acid composition found after acidic hydrolysis of ferredoxin II is compared to that deduced from the DNA sequence in Table 1. The experimental values closely matched theoretical whole numbers except for relatively labile amino acids (cysteine, tyrosine) which were underestimated by chemical analysis. The protein contained a 3-fold molar excess of acidic over basic residues. Nine cysteines were found in the ferredoxin polypeptide, which is indicative of the presence of two [XS-4S] (X = 3 or 4) clusters per molecule.

The absorption spectra of ferredoxin II, in the oxidized and reduced forms, are presented in Fig.2. The spectrum of the oxidized sample exhibited a broad shoulder at about 400 nm, like many bacterial ferredoxins. The extinction coefficient at 400 nm was 26.8 mM^{-1} cm^{-1} and the absorbance ratio A_{400}/A_{280} reached a maximum value of 0.55 in our best preparations. Upon exposure to air, this ratio dropped to about 0.50, the shoulder at 400 nm becoming less pronounced. However, the air-treated ferredoxin remained active as electron carrier to nitrogenase (data not shown). Upon reduction with excess dithionite, bleaching of the chromophore absorption occurred slowly over a 30 min period of time. Ferredoxin II was reduced to a greater extent by photoreaction with 5-deazaflavin, since the chromophore absorption decreased to a lower value than when reduced by dithionite (Fig.2). Hence, the



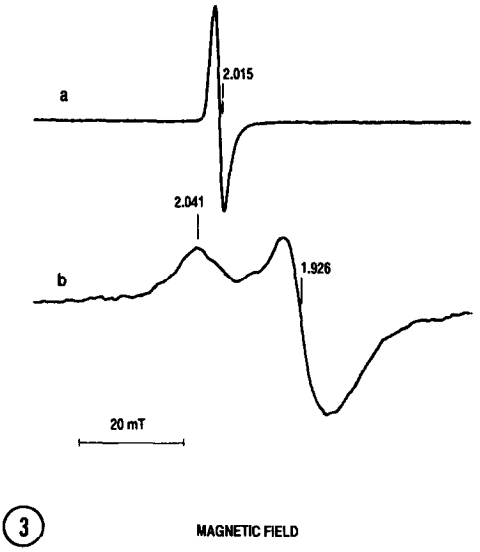


Figure 2. UV-visible absorbance spectra of *R. capsulatus* FdII.
Ferredoxin concentration was 56 μM in 0.1 M Tris-HCl buffer, pH 8.0. Spectra were recorded in cuvettes of 0.2 cm light path under argon (see Methods). **(a)** Sample of FdII as isolated (oxidized). **(b)** Dithionite-reduced FdII - the spectrum was recorded after 30 min incubation with 1.5 mM sodium dithionite. **(c)** Photoreduced FdII. The cuvette also contained 10 μM 5-deazaflavin and 25 mM sodium oxalate. It was illuminated for 30 min with a 150 W projector lamp before recording the spectrum.

Figure 3. EPR spectra of *R. capsulatus* FdII.
Spectra were recorded at 10°K with microwave frequency 9.212 GHz, modulation frequency 100 kHz, and modulation amplitude 1 mT. g-values are indicated on the figure. **(a)** Native FdII (oxidized) 62 μM in 25 mM Tris-HCl pH 7.5. Microvawe power 1 mW. **(b)** Photoreduced FdII. Protein concentration was 95 μM in 50 mM Tris-HCl. The sample also contained 25 mM oxaloacetate and 10 μM 5-deazaflavin. Photoreduction was monitored spectrophotometrically as in Figure 2. After reduction had gone to completion, the sample was transferred to an EPR tube. Gain was 62 fold higher than in **(a)**. Microwave power 2 mW.

ferredoxin appeared difficult to reduce, suggesting that its iron sulfur clusters have very low mid-point redox potentials. The nature of these clusters was investigated by EPR spectroscopy.

In the oxidized state, the EPR spectrum showed a nearly axial signal centered at g = 2.015, which had a pronounced tail at high field and was optimally observed between 10 and 15°K (Fig.3). Such a signal is characteristic of a [3Fe-4S] cluster. Integration of this signal yielded an estimated 1.0 spin/mol consistent with the presence of one [3Fe-4S] per molecule. Reduction by dithionite resulted in the disappearance of the g = 2.015 signal and led to an EPR silent state (data not shown). A more reduced form of ferredoxin II was obtained by using 5-deazaflavin as photocatalyst (Fig.2). EPR analysis of photoreduced FdII revealed a rhombic signal with g values at 2.041 and 1.926 (Fig.3), providing evidence for a second Fe-S cluster. The signal was found to have a fast relaxation time at temperatures above 30°K. These data indicate the presence of a reduced [4Fe-4S] cluster. Spin quantitation gave approximately 0.5 spin/mole of ferredoxin suggesting that the [4Fe-4S] cluster was only

partially reduced. The absence of other EPR signals at low field and the difficulty to reduce the ferredoxin, both agree with this assumption.

From the chemical determination of iron and sulfur atoms, we calculated that FdII contained an average of 6 atoms each of iron and inorganic sulfur. Taking into account the fact that such chemical analyses often underestimate the actual Fe-S content of ferredoxin, these results are consistent with the finding of one [3Fe-4S] and one [4Fe-4S] cluster per FdII as revealed by EPR spectroscopy.

The biological activity of ferredoxin II was tested in an *in vitro* assay coupling the reducing power generated by illuminated chloroplasts to the reduction of acetylene catalyzed by nitrogenase (9). Its activity was compared to that of ferredoxin I from *R. capsulatus* under standardized conditions, using a nitrogenase preparation with a specific activity of 39 units/mg protein (1 unit = 1 nmol C_2H_2 reduced per min). FdII had a specific activity of 0.62 units/nmol FdII, compared to 0.60 units/nmol FdI when FdI was used as electron carrier. Moroever, saturation curves of nitrogenase activity, obtained by increasing the concentration of electron carrier, were similar for both ferredoxins (data not shown). Hence, FdI and FdII from *R. capsulatus* appeared to be equally reactive towards nitrogenase at least under our *in vitro* experimental conditions.

DISCUSSION

A ferredoxin, called FdII, was purified to apparent homogeneity from *R. capsulatus* grown with an excess of fixed nitrogen. It differed from the previously characterized ferredoxin I (7, 9) by its size, amino acid composition and the type of iron-sulfur clusters that it contained. FdII exhibited molecular and spectroscopic properties very similar to those of *Azotobacter vinelandii* ferredoxin I, the structure of which has been described in detail (8). *A. vinelandii* FdI is a 106 residue polypeptide containing one [3Fe-4S] and one [4Fe-4S] cluster. Two such clusters have been identified in *R. capsulatus* FdII by EPR spectroscopy (Fig.3). We have recently established the primary structure of FdII as deduced from the nucleotide sequence of its gene (14). FdII is composed of 111 residues. Alignment of FdII and *A. vinelandii* FdI sequences showed that the two proteins share 65 identical amino acids. Such a high degree of homology further supports the conclusion that FdII is a 7Fe ferredoxin structurally very similar to *A. vinelandii* FdI.

A previous work reported the existence of two ferredoxins in *R. capsulatus* (15). These proteins, called ferredoxin I and ferredoxin II, probably correspond to the 2[4Fe-4S] FdI (7) and to the presently described 7Fe ferredoxin, respectively. Ferredoxin II was apparently synthesized under all tested growth conditions, although at different levels. Assays of ferredoxin activity performed in crude extracts from cells grown under various conditions indicated that nitrogen or carbon limitation promoted the accumulation of FdII inside the cells (15). We confirmed that FdII was present both in dark- and light-grown cells with excess or limiting amounts of fixed nitrogen. However, we found that the levels of expressed FdII were similar irrespective of the growth conditions. A detailed analysis of the expression of the FdII gene (called *fdxA*), using an in frame *fdxA-lacZ* fusion cloned in *R. capsulatus*, has recently

given evidence that FdII is constitutively expressed at a constant level (Duport C., Jouanneau Y., & Vignais P.M., submitted). The function of FdII is presently unknown. It is capable of transferring electrons *in vitro* to nitrogenase as efficiently as FdI. However, in cells derepressed for *nif* genes, FdII was estimated to be 5 to 10 fold less abundant than FdI on a molar basis. Hence, FdII is likely to have a minor role, if any, in the electron transport to nitrogenase. Unlike FdI, which is synthesized only in nitrogen-limited cells (7), FdII appeared to be expressed constitutively, suggesting that it could be involved in some essential metabolic pathway.

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