Recombinant expression of the *fdxD* gene of *Rhodobacter capsulatus* and characterization of its product, a [2Fe-2S] ferredoxin

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A gene called *fdxD* that could potentially code for a ferredoxin has recently been identified upstream of the nitrogenase structural genes in *Rhodobacter capsulatus* [Willison, Pierrard and Hübner (1993) *Gene* **133**, 39–46]. In the present study, the *fdxD* gene product has been overproduced in *Escherichia coli* in a soluble form. The recombinant protein, pink in colour, was purified to homogeneity, and biochemically characterized as a new ferredoxin. It represents the fifth ferredoxin so far identified in *R. capsulatus* and was designated FdV. Its N-terminal sequence is identical with that of the native ferredoxin isolated from

R. capsulatus. U.v.–visible-absorption spectra as well as results of c.d. and e.p.r. spectroscopy demonstrated that the *fdxD* product contained a [2Fe-2S] cluster correctly assembled and incorporated into the polypeptide. Although similar to plant-type ferredoxins, FdV appeared poorly competent in the photo-reduction of NADP⁺. On the basis of *in vitro* assays, FdV cannot serve as an electron donor for nitrogenase. The lack of reactivity of FdV in either of these assays may primarily be due to its relatively high mid-point redox potential ($E'_0 = -220$ mV, pH 7.5).

INTRODUCTION

Ferredoxins are small iron–sulphur proteins ubiquitously found in living matter. They function as electron carriers in a wide variety of physiological reactions. They contain one or two iron–sulphur centres linked to the polypeptide chain by cysteine residues. These prosthetic groups can be of three different types designated as [2Fe-2S], [3Fe-4S] and [4Fe-4S] clusters (Spiro, 1982; Beinert, 1990).

In the photosynthetic bacterium *Rhodobacter capsulatus*, four ferredoxins have been biochemically and genetically characterized so far (Jouanneau et al., 1991). They are encoded by the *fdxN* (FdI), *fdxA* (FdII), *fdxB* (FdIII) and *fdxC* (FdIV) genes. FdI (Schatt et al., 1989) and FdIII (Jouanneau et al., 1993) are two [4Fe-4S] ferredoxins, FdII contains one [3Fe-4S] and one [4Fe-4S] cluster (Jouanneau et al., 1990), whereas FdIV contains a [2Fe-2S] cluster similar to plant-type ferredoxins (Grabau et al., 1991).

Willison et al. (1993) identified a gene potentially coding for a ferredoxin upstream of the nitrogenase structural gene operon (*nif HDK*) of *R. capsulatus*. This gene, called *fdxD*, is preceded by a *nif* promoter consensus sequence.

The *fdxD* gene product has recently been identified in extracts of *R. capsulatus* after two-dimensional electrophoresis (Y. Jouanneau, C. Meyer, I. Naud and W. Klipp, unpublished work). Until now, the expected protein failed to be detected by its colour in purification fractions of nitrogenase or of other ferredoxins, suggesting that it is expressed at a relatively low level.

In order to facilitate the isolation and biochemical characterization of the product of *fdxD*, we have overexpressed *fdxD* in *Escherichia coli* using a vector carrying the T7 promoter. A simple three-step purification protocol allowed us to recover the recombinant ferredoxin in good yield. The biochemical and spectroscopic properties of this new [2Fe-2S] ferredoxin are presented.

MATERIALS AND METHODS

Construction of plasmid pAJ40

For cloning purposes, *Xba*I and *Bam*HI sites were introduced at the 5' and 3' ends of the *fdxD* gene respectively, using a PCR-based mutagenesis technique. Chromosomal *R. capsulatus* DNA, prepared by the method of Giuliano et al. (1988), was used as a template. Primers were designed as shown in Figure 1(a) and synthesized on an Applied Biosystems DNA synthesizer model 381A. PCR was performed using a Hybaid thermocycler with 50 pmol of each primer, 50 ng of genomic DNA, 200 μ M dNTPs and 2 units of *Taq* polymerase (Promega). The conditions for annealing, polymerization and denaturation were 45 °C for 2 min, 72 °C for 1 min and 95 °C for 1 min respectively. The amplified fragment obtained after 30 cycles was analysed on a 2% (w/v) agarose gel, then purified with the Magic PCR purification kit from Promega before direct ligation into pGEM-T plasmid (Promega).

Escherichia coli DH5 α (Bethesda Research Laboratory) was made competent by treatment with CaCl₂, then transformed with the ligation product, and grown overnight at 37 °C on Luria–Bertani (LB) plates containing 100 mg/l ampicillin. Recombinant clones were screened by restriction analysis with *Pst*I to select for a plasmid carrying the *fdxD* gene in the right orientation with respect to the T7 promoter. One such plasmid, called pAJ40, was subjected to DNA sequencing by the chain-termination method (Sanger et al. 1977), using a Sequenase DNA-sequencing kit (United States Biochemical) and [³⁵S]thio[dATP] (1000 Ci/mmol; Amersham).

Expression of the *fdxD* gene

E. coli strain BL21(DE3)(pLysS) (Novagen), transformed with the recombinant plasmid pAJ40, was grown aerobically at 37 °C in LB medium containing 100 mg/l ampicillin and 50 μ M FeSO₄. When cultures reached an optimal absorbance value of 0.8

Figure 1 Construction of the ferredoxin expression plasmid (pAJ40)

(a) Sequence of the PCR-amplified fragment containing the sequence of the *fdx* gene, and the deduced amino acid sequence of FdV. The sequence of the primer AJ10 and the reverse primer AJ11 used for the PCR amplification of the fragment are underlined. The restriction sites introduced in the sequence for subsequent subcloning are indicated in italics. The potential ribosome-binding site of the *fdx* gene is shown in bold. A putative transcription termination signal is underlined by inverted arrows. * indicates codons significantly differing from those preferred by *E. coli*. (b) Expression vector pAJ40 derived from plasmid pGEM-T (Promega) by introducing the PCR-amplified *fdx* gene under the control of the T7 RNA polymerase promoter.

(A_{600}), induction was triggered by the addition of 0.5 mM isopropyl β -D-thiogalactoside (IPTG). Bacteria from 10 litres of culture (40 g of wet cells) were harvested 3 h later, washed with 500 ml of 20 mM Tris/HCl, pH 7.5, containing 10 mM EDTA, centrifuged at 12000 *g* for 10 min at 4 °C, and stored at -70 °C.

Purification of FdV

Cell paste was resuspended in 60 ml (final volume) of 50 mM Tris/HCl, pH 8.0, containing 10 mM EDTA and 2 mM dithiothreitol (DTT) and treated with lysozyme (0.2 mg/ml) before sonication. This and all subsequent steps of the purification were carried out at 4 °C.

The bacterial extract was supplemented with 10 μ g/ml leupeptin (Sigma), 125 μ M phenylmethanesulphonyl fluoride

(Sigma), 10 mM $MgCl_2$ and 250 units of Benzonase (Merck), then centrifuged for 14 min at 12000 *g*. The soluble fraction appeared pink and was applied to a 9 ml column of DEAE-cellulose (Whatmann DE-52), which was washed with 60 ml of 25 mM Tris/HCl, pH 7.5, containing 1 mM DTT. The ferredoxin fraction was eluted with 25 mM Tris/HCl, pH 7.5, containing 250 mM NaCl and 1 mM DTT, in a volume of 63 ml. This fraction was concentrated by ultrafiltration on a YM10 membrane (Amicon) to a volume of 5 ml and then loaded on to a gel-filtration column (AcA54, Sepharose; 2.5 cm $\times$ 110 cm), equilibrated with 25 mM Tris/HCl containing 200 mM NaCl and 1 mM DTT, and was eluted at a flow rate of 23 ml/h. The ferredoxin fraction (33 ml) was concentrated and dialysed against 25 mM Tris/HCl, pH 7.5, containing 1 mM DTT before further purification by h.p.l.c. The dialysed fraction was applied to a DEAE 5PW h.p.l.c. column (Waters), previously equilibrated with 25 mM Tris/HCl, pH 7.5, containing 1 mM DTT. The column was developed at a flow rate of 1 ml/min with a 30 min linear gradient from 0 to 0.25 M NaCl in the same buffer using a Waters h.p.l.c. system controlled by a Baseline M810 software. FdV was eluted at approx. 0.125 M NaCl, and was desalted on a small Sephadex G-25 column. Purified FdV was stored as pellets in liquid nitrogen.

Analytical methods

U.v.-visible-absorption spectra, e.p.r. spectroscopy and amino acid analysis were performed as previously described (Jouanneau et al., 1990, 1992). Iron was assayed spectrophotometrically as the bathophenanthroline complex after release from the protein by acid treatment (Moulis and Meyer, 1982). C.d. spectra were recorded at ambient temperature with a Jobin Yvon CD6 spectropolarimeter, using a 0.1 cm light-path cuvette.

Proteins were assayed as described by Bradford (1976). Samples were analysed by electrophoresis on 10% polyacrylamide gels in an SE200 small vertical slab-gel unit (Hoefer Scientific Instruments) following a procedure adapted from Schagger and von Jagow (1987), and stained with Coomassie Blue.

The N-terminal sequence of the *fdx* gene product was determined either directly on an h.p.l.c.-purified fraction or after electrophoretic blotting (Ploug et al., 1989) on ProBlott membrane (Applied Biosystems). An Applied Biosystems model 477 A protein sequencer, fitted with an on-line model 120A phenylthiohydantoin analyser was used for detection of phenylthiohydantoin-amino acid residues. Isoelectric point was determined by using the first dimension of O'Farrell's (1975) system.

Measurement of redox potentials

Redox titration of FdV was performed spectrophotometrically using 5-deazaflavin as a photoreductant and phenosafranin as dye indicator [$E'_0 = -252$ mV (normal hydrogen electrode)]. Experiments were carried out at 25 °C inside a glove-box (Jacomex) containing argon gas purified to contain less than 1 p.p.m. of O_2 . Spectrophotometric measurements were taken inside the glove-box, using a cuvette holder connected through optic fibres to a Hewlett-Packard 8452A spectrophotometer (Jouanneau et al., 1993). Cuvettes of 0.2 cm light-path contained, in a 375 μ l volume, 8 μ M 5-deazaflavin, 16 mM sodium oxalate, 52.6 μ M phenosafranin and 97 μ M FdV in 25 mM potassium phosphate, pH 7.5. The mixture was exposed to the light of a slide projector for short intervals of time (5–30 s), and absorbance changes were monitored after 30 s of equilibration in darkness. The percentage reduction of FdV was determined from absorbance readings at 410 nm (an isosbestic point

between the oxidized and reduced forms of phenosafranin), using $\epsilon_{410(\text{ox.})} = 9.25 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ and $\epsilon_{410(\text{red.})} = 4.95 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ for the oxidized and reduced forms of FdV respectively. The extent of phenosafranin reduction was calculated from the decrease in absorbance at 520 nm after correction for absorption by ferredoxin at this wavelength ($\epsilon_{520(\text{ox.})} = 5.88 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ and $\epsilon_{520(\text{red.})} = 3.33 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ for oxidized and reduced FdV respectively).

Electron-transfer assays

The electron-carrier activity of ferredoxins was tested in the photoreduction assay of NADP⁺ using spinach chloroplasts thylakoids as a source of reductants (Hallenbeck et al., 1982). Ferredoxin activity was also assayed in an *in vitro* system that coupled the generation of reducing equivalent by illuminated chloroplasts to the activity of nitrogenase, as previously described (Jouanneau et al., 1992). Ferredoxin from spinach leaves, purified as previously reported (Grabau et al., 1991), was used as a reference in these assays.

RESULTS

Construction of the expression plasmid

On the basis of nucleotide sequence analysis, the *fdxD* gene is preceded by a typical *nif* promoter and a potential Shine-Dalgarno sequence 5 bp upstream from the initiation codon (Willison et al., 1993). For the biochemical characterization of this gene product, our strategy was to overexpress the *fdxD* gene with a bacteriophage T7 expression system (Studier et al., 1990). As the translational signals from *R. capsulatus* are recognized in *E. coli* (Grabau et al., 1991; Jouanneau et al., 1992), efficient translation of the *fdxD* messenger was expected from its own natural ribosome-binding site.

To facilitate subsequent subcloning of *fdxD*, a PCR-mediated mutagenesis was performed to create *Xba*I and *Bam*HI sites

conveniently located at the 5' and 3' ends of the gene respectively. PCR was performed using chromosomal *R. capsulatus* DNA as a template. A 490 bp fragment containing the ribosome-binding site, the *fdxD*-coding region and a putative termination signal of transcription (Figure 1a) was amplified and directly cloned into the pGEM-T vector. This vector contains a T7 promoter allowing overexpression of the cloned gene. A clone with the *fdxD* gene in the same orientation as the T7 promoter was selected from the positive clones. The resulting plasmid, pAJ40 (Figure 1b), was subjected to DNA sequence analysis, which confirmed that the sequence of the insert was not altered.

Expression of the *fdxD* gene and purification of FdV

fdxD gene expression from plasmid pAJ40 was performed using *E. coli* strain BL21(DE3)(pLys). This strain contains the T7 RNA polymerase gene integrated into the chromosome under the *lacUV5* promoter (Studier et al., 1990). The T7 RNA polymerase is therefore inducible by IPTG. It contains in addition a plasmid that directs the synthesis of the T7 lysozyme, a natural inhibitor of the T7 RNA polymerase. Hence the basal expression of the gene of interest in BL21(DE3)(pLysS) is negligible in the absence of inducer. Analysis of cell extracts from cultures induced by IPTG for 3 h revealed the presence in the soluble fraction of an additional pink protein that was found to bind to DEAE-cellulose. This protein could be purified to near-homogeneity by a simple three-step purification protocol, as described in the Materials and methods section. The purity of the recombinant ferredoxin was checked by running the proteins on a 15% polyacrylamide/SDS gel (Figure 2). An apparent molecular mass of 14 kDa was determined. This estimation is in good agreement with the expected size, 13 309 Da, deduced from the sequence of the *fdxD* gene after removal of the mass of the initial methionine. The purification yielded approx. 2.8 mg of purified protein/l of culture.

Characterization of FdV

Biochemical characterization

The N-terminal amino acid sequence of the purified FdV polypeptide was determined up to the 20th residue, and found to be as expected from the known DNA sequence except that the initial methionine residue was lacking. This information indicates that the methionine had been removed in *E. coli*, as it had in *R. capsulatus*. Efficient processing has previously been observed in *E. coli* even at high levels of expression [up to 50 mg of processed protein/l of culture (Ta and Vickery, 1992)].

The amino acid composition determined after acid hydrolysis of recombinant FdV was found to be in close accord with the theoretical composition deduced from the *fdxD* gene (results not shown). Estimation of the isoelectric point of urea-denatured FdV yielded an experimental value of 5.8, significantly higher than the theoretical value of 4.8 deduced from amino acid sequence analysis (Pierrard, 1993). FdV was found to contain 2.05 ± 0.05 Fe atoms per molecule on the basis of the chemical assay of iron. This value was calculated taking the ferredoxin concentration as the protein content of the samples (Bradford, 1976) divided by the molecular mass of the FdV polypeptide, 13 309 Da. This result suggested that FdV contained a [2Fe-2S] cluster, as confirmed by the spectroscopic data presented below.

Spectroscopic properties

The pink colour of the recombinant ferredoxin suggested that a [2Fe-2S] cluster had been assembled and incorporated into the

Figure 2 Purification of recombinant FdV as analysed by SDS/PAGE

Protein samples from each step of the purification were separated by SDS/PAGE on a 15% polyacrylamide gel and stained with Coomassie Blue. Lanes 1 and 6, protein standards (phosphorylase b 97.4 kDa, BSA 67 kDa, ovalbumin 46 kDa, carbonic anhydrase 30 kDa, trypsin inhibitor 21.5 kDa and α -lactalbumin 14.4 kDa); lane 2, BL21(DE3)(pLysS)(pAJ40) whole cells before induction; lanes 3–5, purification fractions of FdV: DEAE-cellulose (3); AcA54 gel filtration (4) and pool from h.p.i.c. ion-exchange chromatography (5).

Figure 3 U.v.-visible-absorption spectra of *R. capsulatus* ferredoxin V

Spectra of *R. capsulatus* FdV in the oxidized and dithionite-reduced states were recorded using a Hewlett-Packard diode-array spectrophotometer, model HP8452A. FdV was made 57 μM in 50 mM Tris/HCl, pH 8.0. The cuvette had a 0.2 cm light-path.

Figure 4 E.p.r. spectra of FdV and FdIV of *R. capsulatus*

FdV was 57 μM in 50 mM Tris/HCl, pH 8.0. FdIV was 62 μM in 25 mM Tris/HCl, pH 7.5. Both ferredoxin samples were reduced with excess dithionite (1 mM). The experimental conditions were 10.5 K, 0.5 mW microwave power, 9.225 GHz microwave frequency, 100 kHz modulation frequency and 1 mT modulation amplitude.

polypeptide. The nature of this centre was investigated by spectroscopic analysis. The absorption spectra of the oxidized and reduced ferredoxin are shown in Figure 3. A broad spectrum

Figure 5 C.d. spectrum of FdV

The concentration of FdV was 59 μM in 10 mM Tris/HCl, pH 8.0. The spectrum is presented as the degree of elliptical polarization against wavelength in the visible-near-u.v. region, and is an average of two scans.

with maxima at 336, 420 and 460 nm, characteristic of [2Fe-2S] ferredoxins, was observed for the oxidized form (Buchanan and Arnon, 1971; Cammack, 1980). The protein peak around 280 nm was not very pronounced, in accord with the relatively low abundance of aromatic residues in the FdV sequence. The absorbance ratio A_{420}/A_{280} was close to 1.0 for our best preparations, compared with 0.49 for spinach ferredoxin (Buchanan and Arnon, 1971). The absorption coefficient at 420 nm, based on protein estimation by the method of Bradford (1976), was calculated to be 10 $\text{mM}^{-1}\cdot\text{cm}^{-1}$, which falls within the range of values reported for [2Fe-2S] ferredoxins. The FdV spectrum also showed a shoulder at 540 nm, which became a peak of absorption on reduction. Such spectral features have previously been observed for the *E. coli* ferredoxin (Knoell and Knappe, 1974), but not for plant-type ferredoxins.

E.p.r. spectroscopy revealed a characteristic rhombic signal with g values at 2.032, 1.943 and 1.908 after reduction with dithionite. The spectrum is typical of a [2Fe-2S] cluster and similar to that found for plant ferredoxins. It differs from those observed for adrenodoxin-type [2Fe-2S] ferredoxins (Knoell and Knappe, 1974) which have a significantly more axial g tensor. A comparison of the e.p.r. spectra of the two [2Fe-2S] ferredoxins from *R. capsulatus* (Figure 4) shows slight differences, very noticeable at high field strength, which might reflect subtle changes in the geometry of their iron-sulphur clusters.

A c.d. spectrum of the oxidized ferredoxin FdV in the visible and near-u.v. regions is presented in Figure 5. At wavelengths above 300 nm, the spectrum is attributable to the [2Fe-2S] cluster of FdV and the spectrum shape is a characteristic of the type of Fe-S cluster (Stephens et al., 1991). The general features of the spectrum resemble those of the plant-type [2Fe-2S] ferredoxins. In particular, the 400–700 nm region of the spectrum is very similar, in terms of wavelength values at the extrema, to that of maize [2Fe-2S] ferredoxins, FdI and FdIII (Hase et al, 1991). Like these two ferredoxins, FdV displays $[\theta]_{420}$ values around 8×10^4 $\text{degrees}\cdot\text{cm}^2\cdot\text{dmol}^{-1}$, representing a molar ellipticity of $25 \text{ M}^{-1}\cdot\text{cm}^{-1}$. However, in the 300–400 nm region of the spectrum, the band pattern observed for FdV differs slightly from that of maize ferredoxins. The FdV c.d. spectrum is significantly more different from that displayed by adrenodoxin-type [2Fe-2S] ferredoxins (Ta and Vickery, 1992). These data confirm that FdV belongs to the plant-type [2Fe-2S] ferredoxin family.

Figure 6 Redox titration of FdV

Reductive titration of FdV was carried out by stepwise photoreduction using 5-deazaflavin as a catalyst (see the Materials and methods section). Intermediate redox potentials (E_m) were determined from the extent of phenosafranin reduction, measured at 520 nm. The ratio of the oxidized (Fd_{ox}) and reduced (Fd_{red}) forms of FdV was calculated from the absorbance at 410 nm. Data points could be correlated through a log curve fit with a calculated regression coefficient R of 0.977. The deduced E'_0 of FdV is -220 mV at pH 7.5.

Table 1 Comparison of the electron-carrier activities of *R. capsulatus* FdV and spinach ferredoxin (SpFd)

Numbers in parentheses indicate the ferredoxin concentration or range of concentrations used in the assays (μ M).

Ferredoxin	NADP ⁺ reduction (μ mol/min per μ mol of Fd)	Nitrogenase activity (nmol of ethylene/min per μ mol of Fd)
FdV	0.64 (5.0) 0.71 (10)	< 4 (1.7–6.8)
SpFd	5.0 (2.2)	64 (8.8)

Determination of the mid-point redox potential

Figure 6 shows the redox titration curve of FdV. The potentials were determined spectrophotometrically using phenosafranin as a dye indicator, as described in the Materials and methods section. The midpoint potential of FdV was calculated from these data using the Nernst equation. The value obtained at 25 °C and pH 7.5 was $E'_0 = -220$ mV versus a normal hydrogen electrode.

Electron-transfer assays

The activity of FdV was tested in two separate electron-carrier assays, both involving photosystem I of plant chloroplasts as a reductant. In the NADP⁺-reduction assay, ferredoxin mediates the light-driven electron transfer between photosystem I and the ferredoxin–NADP⁺ reductase. FdV did substitute for spinach ferredoxin in the *in vitro* photoreduction of NADP⁺ but with an efficiency almost 10-fold lower (Table 1). In the second electron-carrier assay, photoreduced ferredoxin is used as an electron donor to nitrogenase. Whereas spinach ferredoxin promoted significant enzyme activity, FdV did not show any detectable electron-transfer activity at least at the concentrations used. FdV was reduced by chloroplast photosystem I, as it mediated

NADP⁺ photoreduction and also as indicated by the absorbance decrease in the ferredoxin chromophore on incubation with illuminated chloroplasts (results not shown). Therefore, the lack of reactivity of FdV in the acetylene-reduction assay suggests that it cannot serve as an electron donor for the nitrogenase complex.

DISCUSSION

The *fdxD* gene of *R. capsulatus* can be expressed to a high level in *E. coli* by using an expression system derived from phage T7. The expression vector was constructed by simple cloning of a PCR fragment encompassing the *fdxD*-coding region as well as the translational signal into plasmid pGEM-T. Results presented in this study demonstrate that the recombinant protein was overproduced in *E. coli* as a soluble [2Fe-2S] ferredoxin. The protein was purified to high yields by following a three-step procedure. The preparation was apparently homogeneous on the basis of SDS/PAGE and N-terminal sequence analysis. The heterologous expression of several other [2Fe-2S] ferredoxins from different organisms (including *R. capsulatus*) has been reported using *E. coli* as the host, and most of the ferredoxins were produced in holoform (Böhme and Haselkorn, 1989; Coghlan and Vickery, 1989; Bourdineaud et al., 1990; Grabau et al., 1991; Fujinaga and Meyer, 1993). In the case of *R. capsulatus* FdV, the presence and correct insertion of a [2Fe-2S] cluster as the prosthetic group was demonstrated using e.p.r. and c.d. spectroscopy.

The amino acid sequence of FdV, as deduced from the *fdxD* sequence, demonstrates limited similarities (less than 20% residue identity) to [2Fe-2S] ferredoxins of bacterial and cyanobacterial origin (Pierrard, 1993). FdV also exhibited low identity with FdIV (16%) another [2Fe-2S] ferredoxin present in *R. capsulatus* (Grabau et al., 1991). Like FdIV, FdV contains four conserved cysteine residues that are required to ligate the [2Fe-2S] cluster. The ligand cysteines are found in the spatial arrangement, Cys-Xaa-Xaa-Xaa-Xaa-Cys-Xaa-Xaa-Cys followed by a more distant cysteine, which is typical of plant-type ferredoxins. These sequence features of FdV together with the e.p.r. and c.d. spectroscopic data provided conclusive evidence that FdV belongs to the class of plant-type [2Fe-2S] ferredoxins. Nevertheless, slight differences were noticeable when comparing the u.v.-visible and c.d. spectra of FdV and plant ferredoxins. The absorption spectrum of the FdV displays, in addition to the commonly found maxima around 330, 420 and 460 nm, a band at 540 nm which is generally absent from plant ferredoxins. A similar absorption band was observed for adrenodoxin-like proteins (Kimura and Suzuki, 1967), and such a feature was formerly proposed to be characteristic of this class of proteins (Knoell and Knappe, 1974). Since then, [2Fe-2S] ferredoxins distinct from the adrenodoxin-type group have been shown, like FdV, to display an additional band near 540 nm, including FdIV from the same bacterium (Y. Jouanneau, unpublished work), a [2Fe-2S] ferredoxin from *Clostridium pasteurianum* (Meyer et al., 1984) and two [2Fe-2S] proteins from *Azotobacter vinelandii* (Shethna et al., 1968).

Considering its relative similarity to plant ferredoxins, it was of interest to determine whether FdV could replace spinach ferredoxin in the photoreduction of NADP⁺. FdV was not very active in this assay, probably because its mid-point redox potential ($E'_0 = -220$ mV) is too high to promote efficient reduction of NADP⁺ via ferredoxin–NADP⁺ reductase. Alternatively, a defective interaction with ferredoxin–NADP⁺ reductase may explain the lack of reactivity of FdV. A recent study identified two residues, Phe-65 and Glu-94, in the *Anabaena* 7120

ferredoxin sequence, as being crucial for this interaction (Hurley et al., 1993). These residues, which are strictly conserved among plant and cyanobacterial ferredoxins (Matsubara et al., 1980), are replaced by methionine and glycine respectively in *R. capsulatus* FdV. Therefore it may be inferred that FdV has a function different from that fulfilled by ferredoxins in plants, consistent with the results derived from the genetic analysis (Willison et al., 1993).

The *fdxN* gene is located next to the *nif* HDK operon coding for the nitrogenase polypeptides and preceded by a *nif* promoter consensus sequence, indicating that it may play a role in nitrogen fixation. Furthermore, it has recently been discovered that an *fdxN* deletion mutant of *R. capsulatus*, lacking FdI, accumulated FdV in greater amounts than wild-type cells (Y. Jouanneau, C. Meyer, I. Naud and W. Klipp, unpublished work). The *fdxN* mutation significantly reduced N_2 fixation. As FdI acts as an electron donor for nitrogenase (Hallenbeck et al., 1982) and FdV is overproduced in the *fdxN* mutant apparently to compensate for the lack of FdI, it was of interest to check whether FdV could donate electrons to nitrogenase in an *in vitro* system. Our results demonstrated that FdV was in fact inefficient as an electron donor, probably because its mid-point potential is too high to promote significant reduction of the nitrogenase. The properties of FdV are worth comparing with those of a [2Fe-2S] ferredoxin isolated from *A. vinelandii* and designated Fe-S protein II (Shethna et al., 1968). Like FdV, this protein was found to be ineffective in NADP⁺ photoreduction and *in vitro* electron transfer to nitrogenase (Shethna et al., 1968). The Fe-S protein II of *A. vinelandii* was found tightly associated with the nitrogenase complex which conferred increased stability of the enzyme with respect to oxidative denaturation by oxygen (Haaker and Veeger, 1977). We are now investigating whether FdV might have a similar role in *R. capsulatus*.

Many micro-organisms, including *Rhodospirillum rubrum* (Yoch et al., 1977; Carithers et al., 1977), *Streptomyces griseolus* (O'Keefe et al., 1991), *Clostridium thermoaceticum* (Elliott and Ljungdahl, 1982) and *Anabaena variabilis* (Yakunin et al., 1993b), contain multiple ferredoxins. The cyanobacteria and some higher plants have long been known to contain multiple forms of plant-type [2Fe-2S] ferredoxins but the reason for such diversity in the cell remains to be explained (Yakunin et al., 1993b). In this respect, *R. capsulatus* is a micro-organism that contains one of the widest arrays of small redox proteins, including several cytochromes (Meyer and Cusanovich, 1989), at least five distinct ferredoxins (Jouanneau et al., 1991; this study), a Rieske protein (Britt et al., 1991; Gurbel et al., 1991) and at least one flavodoxin (Yakunin et al., 1993a). This diversity probably reflects the versatile metabolism of this photosynthetic organism, which can grow by using photosynthesis, respiration or anaerobic fermentation and can fix both CO₂ and molecular N₂ [see Vignais et al. (1985) for review]. The function of this fifth ferredoxin from *R. capsulatus* remains unknown, and physiological characterization is in progress to determine the factors that regulate its expression.

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