Expression in *Escherichia coli* and characterization of a recombinant 7Fe ferredoxin of *Rhodobacter capsulatus*

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The 7Fe ferredoxin of *Rhodobacter capsulatus* (FdII) could be expressed in *Escherichia coli* by cloning the *fdxA* gene coding for FdII downstream from the *lac* promoter. The expressed recombinant ferredoxin appeared as a brown protein which was specifically recognized in *E. coli* cell-free extracts by anti-FdII serum. The purified recombinant ferredoxin was indistinguishable from *R. capsulatus* FdII on the basis of its molecular, redox and spectroscopic properties. These results indicate that the [3Fe-4S] and [4Fe-4S] clusters were correctly inserted into the recombinant ferredoxin.

INTRODUCTION

Ferredoxins are small electron-carrier proteins which take part in various redox reactions in bacteria, plants and animals. They contain iron–sulphur centres that can be of three different types designated as [2Fe-2S], [3Fe-4S] and [4Fe-4S] clusters. Some bacterial ferredoxins, such as the well-characterized *Azotobacter vinelandii* ferredoxin I [1,2], are known to contain one [3Fe-4S] and one [4Fe-4S] cluster. On the basis of amino acid sequence similarities, ferredoxins from *Azotobacter*, *Pseudomonas*, *Bacillus* and thermophilic *Thermus* species have been classified into a distinct phylogenetic group [3]. Recently, new members of this group of 7Fe ferredoxins have been characterized, including a ferredoxin from *Streptomyces griseus* [4,5] and ferredoxin II (FdII) from the photosynthetic bacterium *Rhodobacter capsulatus* [6,7].

R. capsulatus provides a rare example of a bacterium capable of synthesizing four distinct ferredoxins [8]. FdI and FdIII are 2[4Fe-4S] ferredoxins [8,9], whereas FdIV contains a single [2Fe-2S] cluster [10]. Genetic evidence indicates that FdI, FdIII and FdIV are likely to be involved in nitrogen fixation [10–12]. On the other hand, FdII presumably plays a vital role in the bacterium since its structural gene could not be inactivated by insertional mutagenesis [13,14]. FdII was also shown to be constitutively expressed in *R. capsulatus* [15], although at a relatively low concentration [6]. The availability of an expression system allowing easy recovery of FdII should facilitate the further characterization of this ferredoxin, including structure determination.

In this report, we describe the construction and use of a vector directing the synthesis of FdII in *Escherichia coli*. A simple four-step purification protocol allowed us to recover the recombinant ferredoxin in good yield. On the basis of several criteria, the recombinant ferredoxin appeared to be identical with the native FdII of *R. capsulatus*.

MATERIALS AND METHODS

Restriction endonucleases, T4 DNA ligase and DNA polymerase I (Klenow enzyme) were from Boehringer-Mannheim France S.A. *E. coli* strain DH5 α and JM109 were purchased from Bethesda Research Laboratories, Inc. and Pharmacia France SA respectively.

Antiserum against FdII was obtained by immunization of a white rabbit, using purified *R. capsulatus* FdII [6] as antigen.

Subcloning of the *fdxA* gene

Plasmid pCD6 carrying *fdxA* on a 1276 bp *HindIII*–*EcoRI* insert [15] was cut at a unique *BssHII* site located 154 bp upstream of the initiation codon and treated with the Klenow enzyme to generate blunt ends (Fig. 1). Subsequent digestion with *EcoRI* generated a 960 bp fragment which was purified by agarose-gel electrophoresis [16] and cloned into the *EcoRI* and *SmaI* sites of pUC19. The resulting plasmid, pCD11, was digested with *MaeI*, blunt-ended and cut with *EcoRI* to generate a 840 bp fragment which was cloned into the *SmaI* and *EcoRI* sites of pUC19. The plasmid obtained, called pCD28, was introduced into *E. coli* strains DH5 α and JM109 by transformation.

Purification of recombinant FdII

E. coli strains carrying pCD28 were grown aerobically at 37 °C in Luria broth medium containing 100 mg of ampicillin/l and 50 μ M-FeSO $_4$. Cultures of strain JM109 were supplemented with 1 mM-isopropyl thio- β -D-galactoside (Gerbü Trading G.m.b.H., Gaiberg, Germany) when absorbance at 600 nm reached approx. 0.6. After overnight incubation on a rotary shaker, cultures were harvested by centrifugation. Purification of recombinant FdII was performed from about 68 g of cells (wet mass), equivalent to 15 litres of culture. Cells were washed and resuspended in 90 ml of 0.1 M-Tris/HCl buffer, pH 8.0, containing 20 mM-EDTA. All subsequent steps were carried out under argon, with 25 mM-Tris/HCl buffer, pH 7.5, supplemented with 2 mM-sodium ascorbate (TA buffer). Cell-free extract was prepared according to a previously published protocol [6] involving lysozyme treatment, sonication and a 1 h centrifugation at 40 000 g. Isolation of the recombinant ferredoxin was accomplished by DEAE-cellulose chromatography, (NH $_4$) $_2$ SO $_4$ fractionation and chromatography on phenyl-Sepharose CL-4B (Pharmacia France SA), as previously described [6] with the following modifications. The ferredoxin was recovered from the phenyl-Sepharose column by gradient elution from 50 % to 0 % satd. (NH $_4$) $_2$ SO $_4$ in TA buffer (2 $\times$ 100 ml). The ferredoxin fraction was diluted 12-fold in TA buffer and applied to a DEAE-cellulose column (1.6 cm $\times$ 10 cm). The column was developed with a linear gradient from 0.2 to 0.4 M-NaCl in TA buffer (2 $\times$ 150 ml). The recombinant ferredoxin eluted at approx. 0.3 M-NaCl, was concentrated on a small DEAE-cellulose column (approx. 2 ml), eluted in 2–3 ml of

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TA buffer containing 0.4 M-NaCl and kept as pellets in liquid nitrogen.

Analytical methods

SDS/PAGE was performed in an SE200 small vertical slab-gel unit (Hoefer Scientific Instruments, San Francisco, CA, U.S.A.) using a method adapted from [17]. The resolving gel contained 18 % (w/v) polyacrylamide.

For Western blotting, proteins were transferred to PH79 nitrocellulose membranes (0.1 μ m pore size; Schleicher und Schüll, Dassel, Germany) using a TE22 Mini Transphor unit (Hoefer Scientific Instruments). Electrophoretic transfer was carried out at 200 mA for 30 min at 4 °C in the buffer system recommended by Towbin *et al.* [18]. Membranes were processed by an enzyme-linked immunostain procedure [18], with polyclonal anti-FdII rabbit serum (4:1000 dilution) and horseradish peroxidase-goat anti-rabbit IgG conjugate (Bio-Rad S.A. France) as first and second antibodies respectively. The staining solution contained 50 mg of *o*-dianisidine/l (Sigma Chimie S.a.r.l., L'Isle d'Abeau, France), 0.32 ml of 30 % hydrogen peroxide/l and 9 g of NaCl/l in 10 mM-Tris/HCl, pH 7.4.

Proteins were assayed as described by Bradford [19]. Concentration of the purified recombinant FdII was estimated from the absorbance at 400 nm by using an absorption coefficient of 26.8 mM⁻¹·cm⁻¹ [6]. Amino acid analysis and N-terminal-sequence determination were performed as previously described [11].

Spectroscopic analyses

U.v.-visible absorption spectra were recorded on an HP8452A diode-array spectrophotometer (Hewlett-Packard). E.p.r. spectra were recorded on a Varian E109 spectrometer equipped with a helium-flow cryostat (Oxford Instruments, ESR 900). Spin concentrations were determined by double integration of the signals.

Biological assays

Ferredoxin activity was assayed in electron-transfer experiments coupling the reducing power generated by illuminated chloroplasts to nitrogenase activity. Assays were performed under argon in 7.2 ml vials stoppered with rubber septa. The reaction mixture contained in a final volume of 0.475 ml: spinach chloroplast fragments equivalent to 84 μ g of chlorophyll *a*, 100 μ l of an ATP-generating system [20], 7.5 mM-sodium ascorbate, 50 μ M-2,6-dichlorophenol-indophenol, 15 μ M-dichlorophenyldimethylurea and ferredoxin as indicated. Acetylene was injected in the gas phase to a final proportion of 10 %. Vials were incubated at 30 °C in a shaking water-bath illuminated from below at a light intensity of about 10000 lux. The reaction was started by injection of 25 μ l of nitrogenase preparation, equivalent to 0.136 mg of protein. Nitrogenase was reconstituted by mixing the two enzyme components purified from *R. capsulatus* [21] at a molar ratio (Rc2/Rc1) of 8. The reaction was stopped after 20 min with 25 μ l of 25 % trichloroacetic acid, and the ethylene formed was assayed by gas chromatography on a GC121DFL chromatograph equipped with a 1.5 m column of Porapak R (Delsi Nermag Instruments, Argenteuil, France).

RESULTS

Expression and purification of recombinant *R. capsulatus* FdII

The *fdxA* gene coding for *R. capsulatus* FdII has been previously sequenced as part of the 1276 bp *Hind*III-*Eco*RI insert of pCD6 [7]. The *fdxA* promoter was localized on a 160 bp sequence preceding the start of the gene [15]. To direct the synthesis of FdII in *E. coli*, a vector was constructed by subcloning

in two steps an 840 bp *Mae*I-*Eco*RI fragment of pCD6 in plasmid pUC19 (Fig. 1). In the resulting plasmid, pCD28, the promoter of *fdxA* was replaced by the inducible *lac* promoter, but the original ribosome-binding site contained within the 30 bp left upstream from the initiation codon was conserved. Plasmid pCD28 was introduced into *E. coli* strains DH5 α and JM109. In the latter strain carrying the *lacI*^q mutation on episome F' [22], transcription from the *lac* promoter depends on induction by isopropyl thio- β -D-galactoside. Cell extracts prepared from either strain of *E. coli* harbouring pCD28 were found to contain a polypeptide cross-reacting with anti-FdII antibodies on Western-blot analysis. In strain JM109, synthesis of this polypeptide was apparently triggered on induction by isopropyl thio- β -D-galactoside (results not shown). It is noteworthy that some basal concentration of the polypeptide was also detected in uninduced cells at a late stage of growth, which probably resulted from the titration of the *lac* repressor on accumulation of a high copy number of pCD28. For purification of the recombinant ferredoxin, strain DH5 α was preferred to strain JM109, because it consistently gave higher yields in terms of recovered recombinant protein (approx. 0.16 mg/l of culture). Furthermore, we noticed that cultures of strain JM109 produced in the extracellular medium a form of recombinant protein that was denatured, as judged from its absorbance spectrum (results not shown). Purifi-

Fig. 1. Construction of the pCD28 expression vector used for synthesis of *R. capsulatus* FdII in *E. coli*

pUC DNA is represented by thick lines, *R. capsulatus* DNA inserts by thin lines. The *Mae*I(M) cleavage sites of pUC19 are not shown. Other restriction sites indicated are: Bs, BssHII; E, *Eco*RI; H, *Hind*III; Sm, *Sma*I.

Fig. 2. Analysis of proteins at different steps of the purification of recombinant FdII

(a) SDS/PAGE analysis and Coomassie Blue staining. (b) Western blot revealed by immunostaining. Lane 1, soluble bacterial extract, 65 μ g; lane 2, first DEAE-cellulose column eluate, 27 μ g; lane 3, supernatant after $(\text{NH}_4)_2\text{SO}_4$ precipitation (desalted), 10.6 μ g; lane 4, Phenyl-Sepharose eluate, 0.7 μ g; lane 5, second DEAE-cellulose column eluate, 1.3 μ g in (a), 0.12 μ g in (b); lane 6, (a) molecular mass markers of 94, 67, 43, 30, 20.1 and 14.4 kDa, (b) *R. capsulatus* FdII, 0.1 μ g.

cation of the recombinant ferredoxin was performed under anaerobic conditions in the presence of ascorbate to prevent possible oxidative damage of the Fe-S clusters. The procedure was simplified from that previously described for the purification of FdII from *R. capsulatus* [6]. The recombinant ferredoxin, followed as a brown protein fraction during purification, showed binding properties similar to those of *R. capsulatus* FdII when chromatographed on DEAE-cellulose and hydrophobic interaction columns (Phenyl-Sepharose). Analysis of the purified recombinant protein by SDS/PAGE and Coomassie Blue staining revealed a single band that had the same relative mobility as the *R. capsulatus* FdII polypeptide (Fig. 2a). This protein band was specifically recognized by antibodies raised against *R. capsulatus* FdII (Fig. 2b).

Properties of recombinant FdII

Data presented in Fig. 2 indicated that the protein product purified from *E. coli* had the immunochemical and electrophoretic

Fig. 3. Absorbance spectra of recombinant FdII on photoreduction with 5-deazaflavin

Purified recombinant FdII was made 33 μ M in 25 mM-Tris/HCl, pH 8.5 containing 22.5 mM-sodium oxalate and 5 μ M-5-deazaflavin. The ferredoxin sample was kept under argon in a 0.2 cm light-path cuvette. A spectrum was recorded (upper trace: oxidized form). Spectra were then recorded after successive 5-min photoirradiation periods of the cuvette using the light source of a 150 W slide projector. A 30 s equilibration time was allowed before the recording of each spectrum. Acquisition time was 1 s. Contribution of 5-deazaflavin to total absorbance was less than 5% at $\lambda_{\text{max}} = 394$ nm.

properties expected for the *fdxA* gene product. Furthermore, the recombinant protein appeared to be identical with *R. capsulatus* FdII, on the basis of amino acid composition and the sequence of the 13 N-terminal residues (results not shown). Interestingly, the initial methionine predicted from the *fdxA* nucleotide sequence was missing, indicating that it was processed in *E. coli*, just as in *R. capsulatus*.

The brown colour of the recombinant ferredoxin suggested that it contained Fe-S centres, the nature of which was investigated by spectroscopic analysis. The u.v.-visible absorbance spectrum shown in Fig. 3 is qualitatively similar to that of 7Fe ferredoxin II of *R. capsulatus* [6]. Recombinant FdII was slowly and only partially reduced by excess dithionite at pH 7.5 but became extensively reduced by light-activated 5-deazaflavin (Fig. 3). The kinetics of reduction by 5-deazaflavin were apparently biphasic since the chromophore absorption quickly dropped within the initial 5 min and then slowly declined over 30 min. These data suggested that recombinant FdII had two redox centres with distant midpoint potentials. E.p.r. spectroscopy confirmed the existence of two such centres and revealed signals typical for a [3Fe-4S] and a [4Fe-4S] cluster for the oxidized and reduced ferredoxin respectively (Fig. 4). The signals for the oxidized [3Fe-4S] cluster and the reduced [4Fe-4S] cluster had comparable intensities (reduced/oxidized ratio = 0.76) which were consistent with the presence of one cluster each per molecule. Hence, the recombinant ferredoxin produced in *E. coli* was indistinguishable from the native 7Fe ferredoxin on the basis of molecular and spectroscopic criteria.

The biological activities of the native and recombinant FdII were compared in an electron-transfer assay coupled to nitrogenase activity. Titration of nitrogenase by increasing concentrations of ferredoxin indicated that the recombinant protein promoted lower acetylene-reduction rates than did native FdII. Double-reciprocal plots suggested, however, that the two proteins have a similar affinity for nitrogenase (Fig. 5). The lower activity of recombinant versus native FdII might be explained merely by the inhibitory effect of an impurity in the recombinant ferredoxin preparation rather than by a difference in its intrinsic properties. By assaying electron-transfer activity at each step of the purification, we indeed observed a strong inhibition in the crude

Fig. 4. E.p.r. spectra of oxidized and reduced recombinant FdII

Spectra were recorded at 14 K under the following conditions: Microwave frequency, 9.220 GHz; modulation frequency, 100 kHz; modulation amplitude, 1 mT; microwave power, 1 mW. (a) Oxidized FdII, 15.6 μM in 10 mM-Tris/HCl, pH 7.5. (b) Reduced FdII, 169 μM in 25 mM-Tris/HCl, pH 8.5, containing 25 mM-sodium oxalate and 20 μM -5-deazaflavin. Photoreduction was conducted directly into the e.p.r. tube previously flushed with argon. The tube was illuminated for 30 min with a 150 W slide projector and then immediately transferred to liquid nitrogen. Gain was 6.25 times that for spectrum (a). Relevant g values are indicated.

Fig. 5. Comparison of the electron-transfer activity of recombinant and *R. capsulatus* FdII

Each point refers to an independent assay performed as described in the Materials and methods section. v represents the acetylene-reduction rate expressed as nmol/min per assay. $[\text{Fd}]$ is the micromolar ferredoxin concentration. ●, Recombinant FdII; ▲, FdII purified from *R. capsulatus*.

extract of *E. coli* which was gradually relieved in the following purification steps (results not shown).

DISCUSSION

This paper demonstrates for the first time that a 7Fe ferredoxin can be readily expressed in *E. coli* as a stable holoprotein. Expression of *R. capsulatus* FdII in *E. coli* was obtained after simple cloning of the structural gene, *fdxA*, under the control of the *lac* promoter in plasmid pUC19. The purified recombinant protein displayed the same molecular, spectroscopic and redox

properties as the native ferredoxin. These results imply that *E. coli* cells provide the conditions required for the correct assembly and insertion of one [3Fe-4S] and one [4Fe-4S] in the recombinant ferredoxin polypeptide. Besides, since no endogenous 7Fe ferredoxin is known in *E. coli*, it is inferred that the information specifying the type of cluster to be inserted in the recombinant polypeptide is contained in the amino acid sequence of *R. capsulatus* FdII. So far, a few ferredoxins have been expressed in *E. coli* in their holoform, including various [2Fe-2S] ferredoxins [10,23,24] and the 2[4Fe-4S] ferredoxins of *Clostridium pasteurianum* [25] and *R. capsulatus* (FdI) [10]. It is noteworthy that ferredoxins containing a single [2Fe-2S] cluster were expressed to higher concentrations than 2[4Fe-4S] ferredoxins. The [2Fe-2S] ferredoxins of human [23] and cyanobacterial [24] origin accumulated up to about 10% of the *E. coli* soluble proteins whereas the 2[4Fe-4S] ferredoxin of *C. pasteurianum* represented only 0.7% of the soluble extract [25]; in the present study, an estimated 0.12% of the proteins was recovered as the 7Fe ferredoxin of *R. capsulatus*. The observed concentrations of recombinant ferredoxin might primarily reflect differences in the efficiency of the various expression systems used. Nevertheless, using a vector allowing the simultaneous expression in *E. coli* of a 2Fe ferredoxin (FdIV) and an 8Fe ferredoxin (FdI) of *R. capsulatus* [10], we have estimated the 2Fe ferredoxin to be synthesized in a 6–7-fold greater amount than the 8Fe ferredoxin (Y. Jouanneau & C. Grabau, unpublished work). Hence, insertion or assembly of the iron-sulphur cluster(s) might be a rate-limiting step in the synthesis of recombinant holoferredoxins in *E. coli*. The level of expression of FdII in *E. coli*, although 2–3-fold as high as that reported in the natural host, *R. capsulatus* [6], is still relatively low. Possible limiting factors other than that discussed above include strength of the promoter, mRNA stability and rate of mRNA translation. In this respect, the vector used in this study lacked the consensus sequence found at most ribosome-binding sites in *E. coli* [26]. This might have precluded efficient translation of the *fdxA* message in *E. coli*. Construction of a vector designed to optimize the expression of the recombinant ferredoxin is required. We think that such a vector would help to characterize further the structure and function of the 7Fe ferredoxin of *R. capsulatus* and related ferredoxins.

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