Isolation and overexpression in *Escherichia coli* of the flavodoxin gene from *Anabaena* PCC 7119

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The gene coding for flavodoxin from *Anabaena* PCC 7119 was cloned by using the polymerase chain reaction (PCR). The gene is transcribed into a 1250-base transcript. The expression of the flavodoxin gene was analysed and found to be regulated at the transcriptional level by the availability of iron. The PCR-amplified gene was cloned into the expression vector pTrc 99b and expressed in *Escherichia coli*. High concentrations of flavodoxin were found (20 % of total protein). The recombinant protein was purified from the cytosolic fraction of the cells and it exhibited properties identical with those of the wild-type *Anabaena* flavodoxin.

INTRODUCTION

Flavodoxins are small acidic proteins that transfer electrons at low potentials and contain a non-covalently bound flavin mononucleotide as their only redox centre. They have been isolated from different micro-organisms and extensively studied, since they represent the simplest model for the study of flavoprotein biochemistry.

In the non-photosynthetic nitrogen-fixing bacterium *Azotobacter* the flavodoxin-encoding gene is expressed constitutively [1]. However, in *Klebsiella* this protein is synthesized only under nitrogen-fixing conditions [2]. In both species the gene coding for flavodoxin (*nifF*) is clustered together with the genes coding for other proteins involved in the electron transport to nitrogenase, such as pyruvate:flavodoxin oxidoreductase (*nifJ* of *nif* gene cluster), and the expression of this cluster of genes is repressed when there is no demand for nitrogen fixation [3,4].

In the cyanobacterium *Anabaena* PCC 7119 the synthesis of significant amounts of flavodoxin occurs only during growth under iron-limited conditions. In those conditions flavodoxin functionally replaces ferredoxin in the photosynthetic electron transport from Photosystem I to NADP⁺, as well as in the reduction of nitrogenase [5,6], which, in this organism, takes place in differentiated cells called heterocysts.

It has been reported that heterocysts synthesize a unique plant-type ferredoxin that efficiently couples electron transport from different donors to nitrogenase; this protein has been characterized and its structural gene (*fdxH*) cloned and expressed at high levels in *Escherichia coli* [7,8]. Although partial mapping of the *nif* cluster in cyanobacteria has been performed [9,10] and different *nif* gene arrangements have been described for non-heterocystous and heterocystous cyanobacteria [11,12], the location of the gene coding for flavodoxin has not been defined yet, and little is known about the role and regulation of flavodoxin in the reduction of nitrogenase in cyanobacteria.

In order to improve the understanding of its function, the gene for flavodoxin has been amplified by using the polymerase chain reaction (PCR) [13,14]. The present paper describes a system for the overexpression in *E. coli* of this PCR-amplified flavodoxin gene, which produces a fully functional protein. The availability

of recombinant flavodoxin from *Anabaena* will allow the performance of structural studies aimed at the understanding of the mechanism of electron transport among flavoproteins. Isolation of large amounts of either the native or the mutant protein for X-ray-crystallographic or n.m.r.-spectroscopic studies will be possible.

MATERIALS AND METHODS

Materials and bacterial strains

All chemicals were reagent grade. Restriction endonucleases, alkaline phosphatase, T4 polynucleotide kinase, T4 DNA ligase and the Klenow fragment of DNA polymerase I were obtained from Pharmacia (Uppsala, Sweden); preparation of reaction buffers and reaction conditions followed the recommendations of the supplier. [γ -³²P]dATP (3000 Ci/mmol), [α -³²P]dCTP (3000 Ci/mmol) and [γ -³⁵S]thio[dATP] (> 600 Ci/mmol) were obtained from Amersham International (Little Chalfont, Bucks., U.K.).

Anabaena PCC 7119 was grown in BG-11 medium [15] at 30 °C either in liquid media or on plates containing 0.75 % agar. The *E. coli* strain PC 2495, a *recA*[−], *hsdS*[−] derivative of strain JM101 [*supE*, *thi*[−], Δ (*lac*–*proAB*), [F', *traD* 36, *proAB*, *lacI* Δ Z M15]] (Phabagen Collection, University of Utrecht) was used for cloning and expression. Standard conditions for growth were at 37 °C in Luria broth.

Oligonucleotides ranging from 20 to 36 nucleotide residues in length were synthesized in a Biossearch 8600 solid-phase synthesizer and purified by h.p.l.c. when they were shorter than 30 nucleotides residues.

Southern-blot analysis

Chromosomal DNA from *Anabaena* was isolated by the procedure of Mazur & Haselkorn [9]. Restriction-enzyme-cleaved DNA was separated in agarose gel with Tris/borate/EDTA as electrophoresis buffer. Blotting of the DNA from the gel on to nitrocellulose filters was performed as described in ref. [16] and baked *in vacuo* at 80 °C for 30 min. Hybridization at low-stringency conditions (37 °C) was used for analysis with oligonucleotides. When blots were probed with homologous DNA, hybridizations were performed at low (50 °C) and high

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stringency (65 °C). Prehybridization for 1 h at the hybridization temperature was carried out in the following mixture: 5 × SSC (1 × SSC is 0.15 M-NaCl/15 mM-sodium citrate buffer, pH 7), 0.05 % sodium pyrophosphate, 10 × Denhardt's solution (1 × Denhardt's solution is 0.02 % Ficoll 400/0.02 % polyvinylpyrrolidone/0.02 % BSA), 0.1 % SDS and 0.1 mg of denatured salmon sperm DNA/ml (0.25 ml of hybridization mix/cm² of nitrocellulose) [16,17]. Hybridization was performed by adding denatured DNA probe labelled with ³²P to a high specific radioactivity (> 1.8 × 10¹⁶ Bq/g of DNA) by random priming [18] to the prehybridization medium and continuing the incubation for 16 h. The hybridized blots were washed three times for 15 minutes at a temperature 5 °C below the hybridization temperature in 5 × SSC and 0.1 % SDS and once with 5 × SSC. After washing, the filters were kept moist, wrapped in plastic foil and autoradiographed at -70 °C with the use of X-ray film and intensifying screens (Eastman Kodak, Rochester, NY, U.S.A.).

PCR amplification of flavodoxin DNA

PCR amplification was performed with the use of a Techne programmable Dri-Block PHC-1 (New Brunswick Scientific, New Brunswick, NJ, U.S.A.). The starting material was as follows: undigested chromosomal DNA, 1 µg; primer, 0.5 µg; dNTP mixture, 200 µM; 10-fold-concentrated amplification buffer, 5 µl; *Taq* polymerase (Perkin-Elmer Cetus, Norwalk, CT, U.S.A.), 1.5 units. The reaction was performed in a volume of 50 µl that was overlaid with 50 µl of mineral oil to prevent evaporation. Thirty cycles were run in the following conditions: denaturation at 91 °C, 1 min; annealing at 56 °C, 1 min; polymerization at 70 °C, 1.5 min (in the last cycle polymerization is carried out at 70 °C for 10 min). The product of the reaction was purified from an agarose-gel electrophoresis run with Tris/acetate/EDTA buffer by using the GeneClean™ kit (Bio101, La Jolla, CA, U.S.A.) and ligated to pEMBL8 [19]. This construct was used to transform *E. coli* PC 2495, and two of the transformed colonies containing the plasmid were chosen for DNA sequence analysis, which was performed in both directions by the dideoxy chain-termination method [20] with the use of the sequencing Kit T7 from Pharmacia.

RNA isolation and Northern-blot analysis

Total RNA was isolated from mid-exponential cultures of *Anabaena* PCC 7119 grown in BG-11 medium [15,21] in the presence of ammonium ferric citrate to a final [Fe³⁺] of 30 µM and in BG-11 medium without iron added. After denaturation with formamide/formaldehyde, RNA was analysed on formaldehyde/agarose gels run in 10 mM-sodium phosphate buffer, pH 6.5 [17]. Blotting of RNA on to nylon filters (Hybond; Amersham International) and hybridization with a homologous probe was carried out as described for the Southern blots.

Expression of flavodoxin

A new *Nco*I site at the beginning of the coding region and a *Hind*III site downstream of the stop codon were created by synthesizing suitable oligonucleotides and PCR amplification of the flavodoxin gene. The *Nco*I-*Hind*III fragment was inserted after the *trc* promoter of the expression vector pTrc99b [22], and the construct was used to transform *E. coli* PC 2495. Several colonies containing the pTrc-Fld99b plasmid were grown overnight in minimal medium [16] supplemented with 0.2 % glucose, 5 µg of thiamin/ml and 50 µg of ampicillin/ml. After 14 h of incubation the cultures were diluted 100-fold in Luria broth medium in the presence of 50 µg of ampicillin/ml and cells were grown to a concentration of 2 × 10⁸ cells/ml. At this point isopropyl β-D-thiogalactoside was added to a final concentration

of 1 mM and cells were allowed to grow at least 2 h after induction. The level of expression was monitored by taking samples at different times and analysing total protein content by electrophoresis on denaturing PAGE gels.

Purification of flavodoxin

Flavodoxin from *E. coli* was isolated and purified as follows. Cells were chilled on ice, pelleted and washed once with 0.15 M-NaCl. After centrifugation they were dissolved in 50 mM-Tris/HCl buffer, pH 8, containing 1 mM-2-mercaptoethanol, 1 mM-EDTA and 1 µM-phenylmethanesulphonyl fluoride. After a freeze-thaw cycle, lysozyme (Boehringer, Mannheim, Germany) was added to a final concentration of 0.5 mg/ml, and the suspension was stirred at 4 °C for 5 min. The cells were disrupted by ultrasonic treatment for four periods of 45 s, with 30 s intervals, or alternatively passed twice through a French pressure cell at 48 MPa (7000 lbf/in²). Debris and unbroken cells were removed by centrifuging twice at 18000 g for 30 min at 4 °C. FMN (50 µM) was added to the cell-free extract and the mixture was gently stirred in the dark at room temperature for 2 h. The remaining supernatant after precipitation with 65 %-saturated (NH₄)₂SO₄ was poured on to a DE-52 DEAE-cellulose column previously equilibrated with 65 %-saturated (NH₄)₂SO₄ in 50 mM-Tris/HCl buffer, pH 8. Flavodoxin was eluted with a reverse gradient of the equilibration buffer from 65 % to 0 % saturation with (NH₄)₂SO₄. After dialysis of the pooled flavodoxin fractions, purification was continued on a DE-52 DEAE-cellulose column previously equilibrated with 50 mM-Tris/HCl buffer, pH 8. A nearly purified flavodoxin was eluted with a linear gradient of 0.1–0.5 M-NaCl in 50 mM-Tris/HCl buffer, pH 8. Afterwards the flavoprotein was dialysed and concentrated on a small DE-52 DEAE-cellulose column and poured on to a Sephacryl G-200 column. The purified flavodoxin was eluted with 50 mM-Tris/HCl buffer, pH 8, and the purity of the protein was monitored by SDS/PAGE and by the *A*₄₆₅/*A*₂₇₄ ratio.

Determination of the N-terminal amino acid sequence

This was performed by the Biochemical Facility at Universitat Autònoma de Barcelona with the use of Applied Biosystems equipment.

Determination of kinetic parameters

Flavodoxin-dependent NADPH-cytochrome *c* reductase activity was determined as described previously [23]. Photo-reduction of NADP⁺ in the light/chloroplast assay was performed as described by Shin & Arnon [24].

RESULTS AND DISCUSSION

Amplification and cloning of the gene

The nucleotide sequence of the flavodoxin gene from *Anabaena* PCC 7120 was published in the course of this work [25]. This strain can also assimilate N₂ and is very similar to the PCC 7119 strain in morphology and physiology except in the ability to produce gas-vacuolated hormogonia [15]. Therefore, by using the information of the published sequence, two oligonucleotides, located complementary to the beginning and to the end of the flavodoxin-coding region, were synthesized. *Nco*I and *Hind*III restriction sites flanking the beginning and the end of the gene were linked to these probes (Fig. 1a) in order to simplify further expression studies (see below under 'Expression of the gene in *E. coli*'). Both oligonucleotides were used as probes in a Southern hybridization experiment with chromosomal DNA from *Anabaena* PCC 7119 digested with *Eco*RI, with *Hind*III and also with a mixture of both enzymes. A major signal of about 800 bp

Fig. 1. (a) Amplification of DNA encoding for flavodoxin by using the PCR, and (b) Southern-blot analysis of chromosomal DNA from *Anabaena* digested with *Eco*RI, with *Hind*III and with both enzymes and probed with the PCR-amplified gene

(a) Amplification of DNA encoding for flavodoxin by using the PCR. The starting material was as follows: chromosomal DNA, 1 μ g; primer 1 (5'-TTGAATTCAGATCAGGTGTCATAATGTC-3') (lane 1) or primer 2 (5'-GTTAATTCAGACTTAAACCAAA-CCTTAAGAT-3') (lane 2), 0.5 μ g of each; dNTP mixture, 200 μ M; 10-fold-concentrated amplification buffer, 5 μ l; *Taq* polymerase (Cetus), 1.5 units. The reaction was performed in a volume of 50 μ l as described in the Materials and methods section. (b) Southern-blot analysis of chromosomal DNA from *Anabaena* digested with *Eco*RI (lane 1), with *Hind*III (lane 2) and with both enzymes (lane 3) and probed with the PCR-amplified gene. Hybridization was carried out for 16 h at 65 $^{\circ}$ C and washing of the blot was performed as described in the Materials and methods section.

of *Hind*III-digested DNA was observed with both probes (results not shown), indicating that the sequences of those probes were sufficiently similar to the DNA sequence to expect a PCR amplification of the gene with negligible secondary products.

The PCR was performed as described in the Materials and methods section with 1 μ g of chromosomal DNA as template. After 30 cycles a single amplification product of about 550 bp could be seen when the PCR mixture was electrophoresed in an agarose gel (Fig. 1a). When this PCR-amplified DNA was hybridized to totally digested chromosomal DNA from *Anabaena* PCC 7119, only one major signal was detected, even at low-stringency conditions (Fig. 1b). With chromosomal DNA digested with *Hind*III, the size of this signal was about 800 bp, suggesting that only one copy of the flavodoxin gene is present in the chromosome. This also indicates that if there is a different flavodoxin gene coding for the protein present in heterocysts, as occurs with ferredoxin, the degree of sequence similarity of the putative gene to the amplified flavodoxin gene is too low to be detected.

DNA sequence analysis

The amplified DNA was cloned into pEMBL8, and susceptible *E. coli* cells were transformed with this plasmid. This was done because in the direct sequencing of the PCR product a high percentage of the templates re-annealed during the reaction even in the presence of detergents such as Tween 20 or Nonidet P-40. Two of the colonies containing the plasmid with the flavodoxin insert were chosen for sequence analysis, giving the same in both cases (Fig. 2). This sequence of the flavodoxin gene from *Anabaena* PCC 7119 was found to be identical with that of the

Fig. 2. Alignment of the sequence of the flavodoxin gene from *Anabaena* PCC 7119 to the oligonucleotides that were used to create *Nco*I and *Hind*III sites for its overexpression in *E. coli*

The translated region is from nucleotide 16 to nucleotide 525.

Fig. 3. Northern-blot analysis of *Anabaena* cells grown with or without Fe^{3+}

Total RNA was denatured, electrophoresed on a 1.1% agarose gel, blotted and hybridized with the PCR-amplified flavodoxin gene. Lane 1, RNA from cells grown in BG-11 medium containing 30 μ M- Fe^{3+} ; lane 2, RNA from cells grown in BG-11 medium without Fe^{3+} .

gene from the 7120 strain. This also means that the PCR is a reliable method for cloning short genes.

Regulation of the flavodoxin gene in *Anabaena*

The synthesis of considerable amounts of flavodoxin in *Anabaena* occurs when the concentration of iron in the medium becomes limiting [5,6]. To detect flavodoxin mRNA, total RNA was isolated from *Anabaena* cells grown without added iron and in standard medium (iron 30 μ M). Hybridization with the PCR

product containing the entire flavodoxin gene showed a 1.25 kb transcript that was only present in cells grown without iron (Fig. 3), which means that expression of flavodoxin is regulated by iron at transcriptional level. Similar studies have been carried out for *Anacystis nidulans* [26], and expression of flavodoxin was also found to be regulated by iron at the mRNA level; however, in the same conditions a larger transcript of 1900 bp was detected.

Expression of the gene in *E. coli*

For the analysis of the complexes between flavodoxin and other redox proteins and especially the role and the contribution of different amino acid residues to the stability and functionality of those complexes, the availability of site-directed-mutated proteins would be extremely helpful. An essential step in our research is therefore the expression of those proteins in an organism easily transformable and fast-growing, such as *E. coli*.

A number of expression systems have been designed to overproduce foreign proteins in this organism; a very effective one uses the strong hybrid *tac* promoter described by Amman *et al.* [22]. We have used the pTrc99b expression vector, which carries the *trp/lac* (*trc*) promoter, the *lacZ* ribosome-binding site, the multiple cloning site of pUC18 and the *rrnB* transcription terminators. For the expression of non-fused proteins, the multiple cloning site has been inserted behind an *NcoI* site, and the *lacP* allele of the *lac* repressor gene has been added to the vector to ensure complete repression of the *trc* promoter during growth in the host strain.

A new *NcoI* site was created in the gene at position 16 (Fig. 2) in order to be able to ligate the amplification product into the expression vector. This creates a mutation in the second codon of the flavodoxin gene, and a serine residue is changed into alanine. From the X-ray structures that have been determined from several flavodoxins [27–29] it is well known that the first amino acid residue of this protein is not involved in the binding of the apoprotein to the flavin cofactor, and thus significant changes in the structure and activity of the mutated flavoprotein are not expected. Moreover, it has been reported that in some systems alanine is one of the amino acid residues that stabilize the protein when it is present at the *N*-terminal position [30].

For the expression of flavodoxin, *E. coli* PC 2495 was transformed with the construct. When expression of the flavoprotein was induced upon addition of isopropyl β -D-thiogalactoside, a high level of synthesis of flavodoxin was observed by SDS/PAGE, as shown in Fig. 4. Densitometric scans (results not shown) indicate that after isopropyl β -D-thiogalactoside induction the flavoprotein is expressed to at least 20% of the total protein content. An increase in the amount of recombinant flavodoxin can already be observed 5 min after the addition of isopropyl β -D-thiogalactoside and the maximum amount is reached after 2 h. When induction is performed in the presence of 2 mM-isopropyl β -D-thiogalactoside and the cells are harvested after 2 h, considerable amounts of apoprotein are observed, according to the A_{465}/A_{274} ratio of the partially purified protein. When induction is carried out overnight at 37 °C in the presence of 0.5–1 mM-isopropyl β -D-thiogalactoside, however, the holoprotein is produced. In those conditions, just as in the cases in which other flavodoxins have been overexpressed [31–33], *E. coli* cells produced, in addition to the flavodoxin peptide, large amounts of cofactor: both liquid cultures and colonies grown on agar plates showed the characteristic orange colour of *Anabaena* flavodoxin.

Purification of recombinant flavodoxin

Although the treatment with the French press proved to be more effective for the extraction of cytosolic proteins than sonication, the latter procedure was preferred since the main

Fig. 4. SDS/PAGE analysis of the induction of the expression of flavodoxin from *Anabaena* PCC 7119 in *E. coli*

Cells were induced with isopropyl β -D-galactoside at a final concentration of 2 mM and samples were collected at different times. Bacterial pellets were washed with 0.2 M-NaCl and resuspended in the same solution. This material was used for total protein quantification by the method of Lowry *et al.* [34], with BSA as standard, and for electrophoretic analysis in a 15% acrylamide gel. Lanes 1–6, samples collected at times 0 min, 5 min, 15 min, 30 min, 60 min and 120 min after induction; lane 7, cells cultured in the absence of isopropyl β -D-galactoside; lane 8, markers; lane 9, flavodoxin purified from *Anabaena* PCC 7119.

Fig. 5. Isolation of recombinant flavodoxin from *E. coli*

After induction with isopropyl β -D-galactoside, cells were broken by ultrasonic treatment and centrifuged twice at 18000 *g* for 30 min. Lane 1 shows the proteins present in the pellet and lane 2 shows the supernatant, which contains nearly homogeneous flavodoxin. Lane 3 shows the supernatant fraction when cells were treated in the French press and centrifuged twice at 18000 *g* for 30 min. The electrophoresis was performed on polyacrylamide gel [15% (w/v) acrylamide] with the Laemmli discontinuous buffer system [35].

component of the cytosolic fraction isolated after sonication was the soluble flavoprotein (Fig. 5). When induction of expression with isopropyl β -D-galactoside was performed overnight, our purification procedure yielded about 83 mg of pure flavodoxin from a 10-litre culture. In those conditions, the spectrum of the

Fig. 6. Absorption spectrum of the recombinant flavodoxin holoprotein expressed in and isolated from *E. coli*

The buffer used was 50 mM-Tris/HCl, pH 8. The inset shows the characteristic flavoprotein maxima at 377 and 465 nm.

recombinant flavodoxin was identical with the spectrum of the cyanobacterial flavoprotein (Fig. 6). When induction with isopropyl β -D-galactoside was performed for 2–4 h, although FMN was added to the crude extract the purification procedure yielded a mixture of holo- and apo-flavodoxin (20–40% of apoprotein on the basis of the A_{465}/A_{274} ratio). It was possible to reconstitute the apo-flavodoxin present in this mixture by adding an excess of FMN and incubating all together for 2 h in the dark and at room temperature. After this treatment the free FMN was removed by gel-filtration chromatography and the A_{465}/A_{274} ratio was 0.17, which corresponds to that of the pure holo-flavodoxin.

Properties of the recombinant flavodoxin

The *N*-terminal amino acid analysis of the recombinant flavodoxin showed that the intact protein is produced. Truncated forms of flavodoxin were not detected, as occurs when other recombinant flavoproteins such as ferredoxin-NADP⁺ reductase are expressed in *E. coli* [36]. The sequence obtained from the analysis was identical with the sequence described for the protein purified from *Anabaena* [5] except that the first amino acid residue (serine) has been changed into alanine in the protein isolated from *E. coli*. Therefore the recombinant protein contained 169 amino acid residues, and a molecular mass of 18811 Da was estimated on the basis of the sequence of the DNA.

Values of flavodoxin-dependent NADPH-cytochrome *c* reductase activity and photoreduced NADP⁺ in the light/chloroplast assay were similar for both the *Anabaena* flavodoxin and the recombinant flavodoxin. The antigenicity of the recombinant protein to cyanobacterial flavodoxin antibodies was equal to the antigenicity showed for the native protein (results not shown). This fact and the kinetic data obtained with the recombinant protein support the idea that both flavodoxins are identical. Therefore the system described here is suitable for

the production of substantial amounts of recombinant flavodoxin.

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