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Crystallization of a flavodoxin involved in nitrogen fixation in *Rhodobacter capsulatus*

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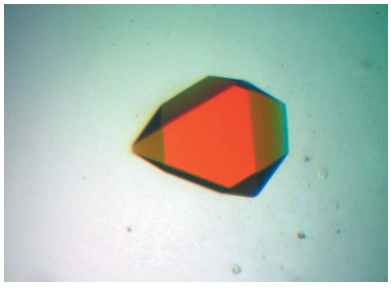
Flavodoxins are small electron-transfer proteins that contain one molecule of noncovalently bound flavin mononucleotide (FMN). The flavodoxin NifF from the photosynthetic bacterium *Rhodobacter capsulatus* is reduced by one electron from ferredoxin/flavodoxin:NADP(H) reductase and was postulated to be an electron donor to nitrogenase *in vivo*. NifF was cloned and overexpressed in *Escherichia coli*, purified and concentrated for crystallization using the hanging-drop vapour-diffusion method at 291 K. Crystals grew from a mixture of PEG 3350 and PEG 400 at pH 5.5 and belong to the tetragonal space group $P4_12_12$, with unit-cell parameters $a = b = 66.49$, $c = 121.32$ Å. X-ray data sets have been collected to 2.17 Å resolution.

1. Introduction

Rhodobacter capsulatus is a purple photosynthetic bacterium that is capable of reduction of atmospheric dinitrogen (N_2) when ammonium ion levels in the medium are limiting (Weaver *et al.*, 1975). Nitrogen fixation is carried out by the enzyme nitrogenase, which transfers electrons originating from low-potential electron carriers, such as flavodoxin or ferredoxin molecules, to molecular N_2. In this work, we have centred our attention on the flavodoxin NifF, which has been identified as a candidate for nitrogenase reduction in *R. capsulatus*. NifF is the product of a single gene from the *nif* operon (where *nif* stands for 'nitrogen fixation') and is a 181-amino-acid polypeptide with a predicted molecular weight of 19.8 kDa (Gennaro *et al.*, 1996). It carries a noncovalently bound flavin mononucleotide (FMN) molecule as the prosthetic group responsible for its redox properties. FMN is able to adopt three redox states: a fully oxidized state (quinone), a one-electron semi-reduced state (semiquinone) and a two-electron reduced state (hydroquinone). Flavodoxins behave as a mono-electron carrier and, in the case of NifF from *R. capsulatus*, the semiquinone and hydroquinone have been proposed to be the relevant redox states involved in electron exchange (Nogués *et al.*, 2005).

The proposed electron donor to NifF is the enzyme ferredoxin/flavodoxin:NADP(H) oxidoreductase (FPR), as described by Bittel *et al.* (2003). *Rhodobacter* FPR belongs to the plant-type ferredoxin NADP(H) reductase family (EC 1.18.1.2) and contains a noncovalently bound FAD molecule as a prosthetic group. FPR is able to accept two electrons from the NADPH molecule and eventually transfers them to two NifF molecules in a reversible mechanism (Nogués *et al.*, 2005; Bittel *et al.*, 2003). The crystallization and determination of the three-dimensional structure of FPR has been reported recently by our group (Pérez-Dorado *et al.*, 2004; Nogués *et al.*, 2005). Here, we describe the initial results obtained for the crystallization of NifF and X-ray diffraction data to 2.17 Å resolution.



2. Experimental

2.1. Protein expression and purification

After amplification by the polymerase chain reaction, the *nifF*-coding sequence from *R. capsulatus* was cloned into pET-32a

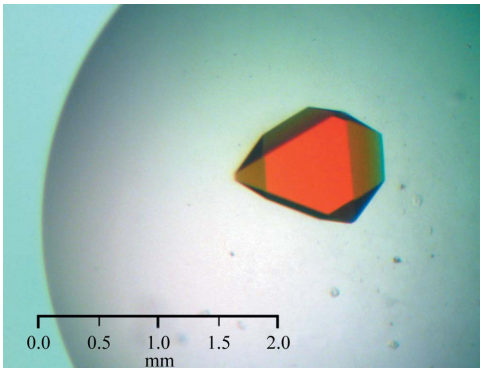


Figure 1
Crystal of the flavodoxin NifF obtained with 20–25%(*w*/*v*) PEG 3350, 2–5%(*v*/*v*) PEG 400 and 100 m*M* sodium acetate pH 5.5. The approximate dimensions of the crystal are 1.0 × 0.7 × 0.7 mm.

(Novagen), producing plasmid pET32-Fld, which was used to transform *Escherichia coli* BL21 (DE3) cells as described previously (Bittel *et al.*, 2003). After transformant cultures reached exponential growth at 310 K, they were induced with 0.5 m*M* IPTG for 2 h. After centrifugation, the cleared cell lysate was applied onto an Ni–nitriloacetic acid (NTA) fast-flow affinity column (Qiagen). The fusion product carrying a His_6-Trx tag (Novagen) was eluted with 300 m*M* imidazole in 50 m*M* Tris–HCl pH 7.0, digested with enterokinase to remove the tag and purified as recommended by the supplier (see Bittel *et al.*, 2003). Quantification of the isolated flavodoxin was performed spectrophotometrically using the absorption maximum of the FMN at 450 nm ($\varepsilon_{450\,\mathrm{nm}}$ = 11.7 $\mathrm{m}M^{-1}\,\mathrm{cm}^{-1}$) reported by Yakuning *et al.* (1993).

2.2. Crystallization

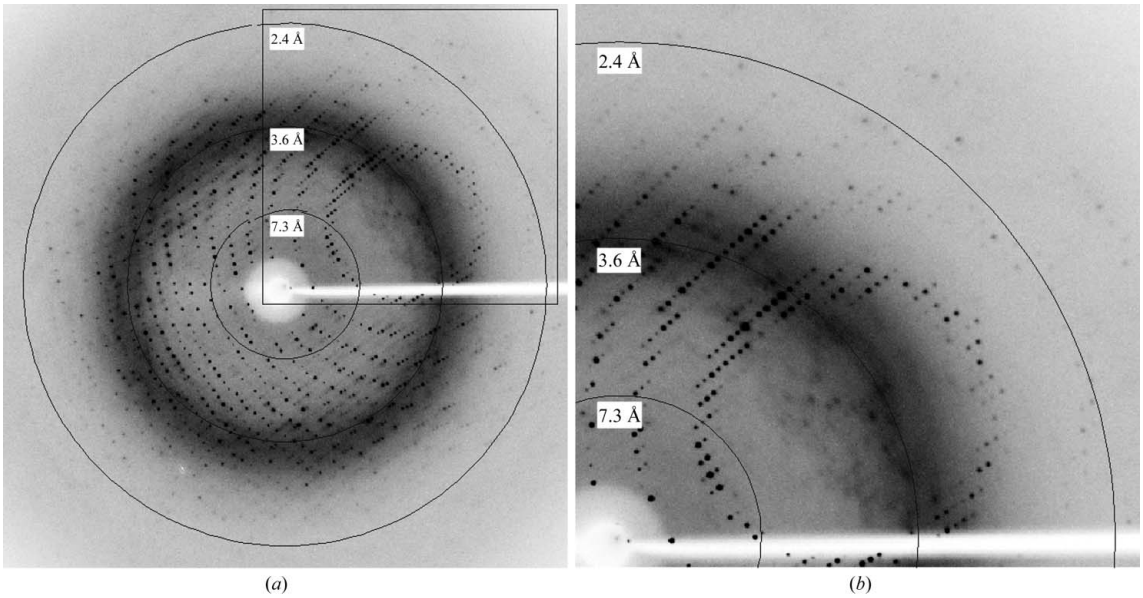
The protein was concentrated to 20 mg ml^{-1} in 50 m*M* Tris–HCl pH 8.0 using an Amicon ultrafiltration system with Amicon YM-10 membranes. Crystallization assays were carried out using the hanging-drop vapour-diffusion method at 291 K. The search for initial crystallization conditions was performed using Crystal Screens I and II and Index Screen from Hampton Research. Droplets formed by mixing 1 µl protein solution with 1 µl precipitant solution were equilibrated against 500 µl well solution. Microcrystals of NifF grew under several conditions containing polyethylene glycol (PEG) as the main precipitant agent. Consequently, we focused the search for crystallization conditions using the Synergy Screen (Majeed *et al.*, 2003), which contains an extensive combination of different PEGs, salts and other organic compounds. The best crystals grew in one of these conditions, which consisted of 25%(*w*/*v*) PEG 3350, 5%(*v*/*v*) PEG 400 and 100 m*M* sodium acetate pH 5.5. After several optimization steps, we obtained large NifF crystals which grew in 5 µl droplets formed by mixing 2.5 µl of a solution containing 20 mg ml^{-1} protein buffered in 50 m*M* Tris–HCl pH 8.0 and 2.5 µl precipitant solution containing 20–25%(*w*/*v*) PEG 3350, 2–5%(*v*/*v*) PEG 400 and 100 m*M* sodium acetate pH 5.5. Crystals reached maximum dimensions of 1.0 × 0.7 × 0.7 mm in two weeks (Fig. 1).

2.3. X-ray data collection and processing

X-ray diffraction data were measured using Cu *Kα* X-rays (λ = 1.5418 Å) generated by an in-house Enraf–Nonius rotating-anode generator operated at 40 kV and 90 mA and equipped with a double-mirror focusing system. Images were collected on a MAR Research 345 image-plate detector. Crystals were soaked for 10 s in a cryoprotectant solution consisting of mother liquor with 15%(*v*/*v*) glycerol and then flash-cooled at 100 K in a nitrogen stream using a cryogenic system. Despite the fact that the NifF crystals reached large dimensions (around 1 mm in length), they were extremely fragile and cracked very easily when mounted in the cryo-loops. For this reason, high-quality X-ray data sets were difficult to obtain. The collected images were processed and scaled using the programs *MOSFLM* (Leslie, 1992) and *SCALA* from the *CCP*4 package (Collaborative Computational Project, Number 4, 1994).

3. Results

An X-ray data set was collected to 2.17 Å resolution from a single NifF crystal and displayed an intense and good-quality pattern (Fig. 2). Preliminary X-ray data processing showed that the crystal belonged to the tetragonal crystal system, with unit-cell parameters



(*a*) (*b*)

Figure 2
(*a*) Typical X-ray diffraction pattern of NifF crystals; (*b*) enlargement showing resolution rings.

Table 1
Data-collection statistics for NifF crystals.

Values in parentheses are for the highest resolution shell.

Crystal data	
Space group	$P4_12_12$
Unit-cell parameters (Å)	
a	66.49
b	66.49
c	121.32
Data processing	
Temperature (K)	100
Wavelength (Å)	1.5418
Resolution (Å)	25.65–2.17 (2.35–2.17)
Unique data	24830
Redundancy	22.4 (22.9)
Data completeness (%)	99.9 (100)
Average $I/\sigma(I)$	26.0 (8.2)
Molecules per ASU	1
Matthews coefficient ($Å^3$ Da^{-1})	3.4
Solvent content (%)	64
R_{merge}†	0.07 (0.49)

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the ith measurement of reflection hkl and $\langle I(hkl)\rangle$ is the weighted mean of all measurements.

$a = b = 66.49$, $c = 121.32$ Å. X-ray data-collection statistics are summarized in Table 1. Analysis of the intensities after scaling displayed reflections for Miller indices $00l$ when $l = 4n$ and $h00$ when $h = 2n$, indicating space group $P4_12_12$ or $P4_32_12$ as the most probable. Specific volume calculations indicated the presence of one molecule in the asymmetric unit and a solvent content of 64% ($V_M = 3.4$ $Å^3$ Da^{-1}; Matthews, 1968).

Structural resolution was carried out using the molecular-replacement method with the flavodoxin from *Anabaena* PCC7120 (PDB code 1flv), which shares 41% sequence identity and 60% sequence homology, as a model. The molecular-replacement method was performed using the program *MOLREP* (Vagin & Teplyakov, 1997) with reflections in the resolution range 25.6–3.0 Å. A single and unambiguous solution for the rotation and translation functions was obtained, which yielded a final correlation coefficient of 0.38 and an R factor of 0.53. The space group was confirmed to be $P4_12_12$ and the asymmetric unit contained a single protein monomer. Structural refinement of the NifF model is currently in progress.

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