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# Crystallization and preliminary X-ray studies of ferredoxin-NADP<sup>+</sup> oxidoreductase encoded by *Bacillus subtilis* *yumC*

Ferredoxin-NADP<sup>+</sup> oxidoreductase encoded by *Bacillus subtilis* *yumC* has been purified and successfully crystallized in complex with NADP<sup>+</sup> in two forms. Diffraction data from crystals of these two forms were collected at resolutions of 1.8 and 1.9 Å. The former belonged to space group  $P2_12_12$ , with unit-cell parameters  $a = 63.90$ ,  $b = 135.72$ ,  $c = 39.19$  Å, and the latter to space group  $C2$ , with unit-cell parameters  $a = 207.47$ ,  $b = 64.85$ ,  $c = 61.12$  Å,  $\beta = 105.82^\circ$ . The initial structure was determined by the molecular-replacement method using a thioredoxin reductase-like protein as a search model.

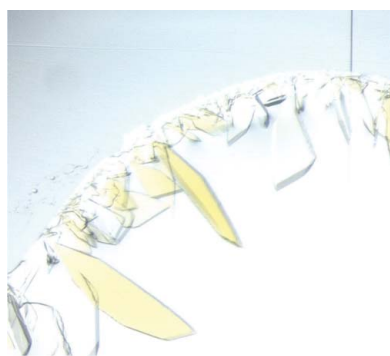
## 1. Introduction

Ferredoxin-NADP<sup>+</sup> oxidoreductases (FNRs) are ubiquitous flavo-enzymes that contain a noncovalently bound FAD. FNR mediates the redox reaction between NADP<sup>+</sup>/NADPH and ferredoxin, providing redox equivalents in cell-material synthesis (Knaff & Hirasawa, 1991; Ceccarelli *et al.*, 2004; Aliverti *et al.*, 2008). On the basis of phylogenetic and structural analyses, FNRs have been classified into two groups: plastid-type and glutathione reductase-type FNRs (Aliverti *et al.*, 2008). Both types exist as monomeric proteins composed of two nucleotide-binding domains, one for FAD binding and the other for NAD(P)H binding. Recently, it has been reported that a novel type of FNR has been obtained from several bacteria (Seo & Sakurai, 2002; Seo *et al.*, 2004; Mandai *et al.*, 2009). These FNRs occur as homodimers and share a higher level of amino-acid sequence homology with thioredoxin reductases (Waksman *et al.*, 1994) than with the two groups of FNRs mentioned above. BLAST searches (Altschul *et al.*, 1997) showed that a number of genes homologous to this novel FNR are present in some Gram-positive bacteria and archaea that have no gene coding for a protein with a high sequence identity to the known FNRs. This novel FNR constitutes a new group of FNRs in addition to the previously established plastid-type and glutathione reductase-type FNRs. In order to obtain the structure of this novel FNR from *Bacillus subtilis* (BsFNR), we performed its crystallization and preliminary X-ray analysis.

## 2. Materials and methods

### 2.1. Protein purification and crystallization

Expression and purification of BsFNR were carried out according to previously described methods (Seo *et al.*, 2009). Purified BsFNR was concentrated to 10 mg ml<sup>-1</sup> in 50 mM Tris-HCl pH 8.0 buffer containing 200 mM NaCl. The concentration of BsFNR was determined using a molar absorption coefficient of 12 300 M<sup>-1</sup> cm<sup>-1</sup> at 457 nm. For crystallization of the BsFNR-NADP<sup>+</sup> complex, 2.5 mM NADP<sup>+</sup> was added to the BsFNR solution. Crystallization screening was carried out using Crystal Screens I and II and PEG/Ion Screen from Hampton Research and the Cryo I and II sparse-matrix crystallization screens from Emerald BioSystems with the sitting-drop vapour-diffusion method at 293 K. Protein droplets prepared by mixing 1 µl protein solution and 1 µl reservoir solution were equilibrated against 100 µl reservoir solution at 293 K.

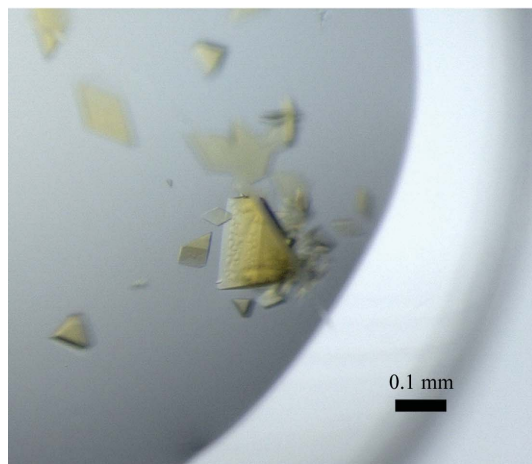


## 2.2. Dynamic light scattering

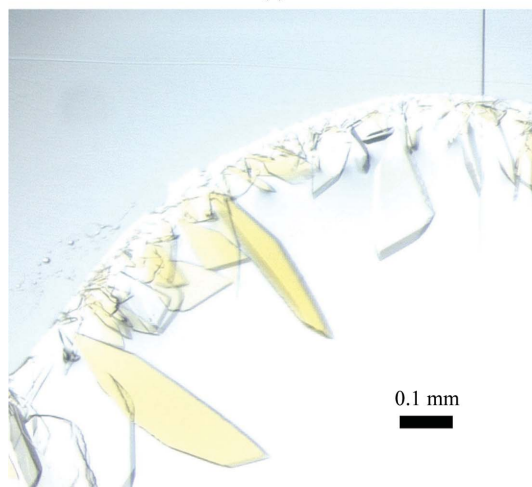
Solutions of 10 mg ml<sup>-1</sup> BsFNR and 2.5 mM NADP<sup>+</sup> were utilized for dynamic light-scattering (DLS) investigations. Debris in the protein solution was removed by centrifugation (20 000g, 10 min) before measurements. Automated measurements were carried out with a Zetasizer  $\mu$ V instrument (Malvern Instruments Ltd). A Peltier



(a)



(b)



(c)

**Figure 1**  
Crystals of *B. subtilis* FNR. (a) NADP<sup>+</sup>-free crystals. (b) Form I crystals of the NADP<sup>+</sup> complex. (c) Form II crystals of the NADP<sup>+</sup> complex.

**Table 1**

Summary of crystallographic data.

	Form I	Form II
Space group	<i>P</i> 2 <sub>1</sub> 2 <sub>1</sub> 2	<i>C</i> 2
Unit-cell parameters (Å, °)	<i>a</i> = 63.90, <i>b</i> = 135.72, <i>c</i> = 39.19	<i>a</i> = 207.47, <i>b</i> = 64.85, <i>c</i> = 61.12, $\beta$ = 105.82
Resolution (Å)	50.00–1.80 (1.86–1.80)	50.00–1.90 (1.97–1.90)
No. of unique reflections	32363	59771
Multiplicity	7.0 (6.1)	3.6 (3.0)
Completeness (%)	99.9 (99.5)	96.9 (80.4)
$\langle I/\sigma(I) \rangle$	23.0 (2.8)	22.6 (3.1)
<i>R</i> <sub>merge</sub> (%)	7.1 (38.8)	5.1 (22.0)

thermostatting system was used to maintain the temperature at 298 K. Size distributions were acquired at regular time intervals for BsFNR in 50 mM Tris–HCl buffer pH 8.0 containing 200 mM NaCl.

## 2.3. Data collection and crystallographic analysis

X-ray diffraction data sets were collected at 100 K on beamline BL38B1 ( $\lambda$  = 1.0000 Å) at SPring-8 using a Rigaku Jupiter210 CCD detector. The crystal-to-detector distance was maintained at 150 mm with an oscillation range per image of 1.0°, covering a total oscillation range of 180°. Determination of the unit-cell parameters and integration of reflections were performed using the *HKL*-2000 program package (Otwinowski & Minor, 1997). Molecular replacement was carried out using the program *Phaser* (McCoy *et al.*, 2007). Further refinement and model building are in progress using the programs *REFMAC* (Murshudov *et al.*, 1997) and *Coot* (Emsley & Cowtan, 2004).

## 3. Results and discussion

The recombinant protein was purified to apparent homogeneity on SDS–PAGE. Its molecular mass was approximately 40 kDa on SDS–PAGE. The UV–Vis absorption spectra of the air-oxidized FNR suggested that almost all protein molecules bound a flavin. DLS was used to assess the influence of NADP<sup>+</sup> on the oligomerization of BsFNR. The deduced diameters of BsFNR without and with NADP<sup>+</sup> were 3.94 ± 0.66 and 3.90 ± 1.45 nm, respectively. The hydrodynamic radius of BsFNR was not significantly affected by the addition of NADP<sup>+</sup>. The estimated molecular weight of about 80 000 indicated dimer formation. We first tried to crystallize BsFNR without NADP<sup>+</sup> by extensive screening; needle-like crystals were obtained using polyethylene glycol 3350 (PEG 3350) as precipitant (Fig. 1). However, the crystals diffracted to a resolution of approximately 10 Å and were not suitable for further crystallographic analysis. Therefore, we attempted the crystallization of BsFNR in complex with NADP<sup>+</sup> and obtained crystals with different shapes (forms I and II). The crystallization conditions of the BsFNR–NADP<sup>+</sup> complex were refined and two different crystal forms were obtained using reservoir solutions consisting of 0.1 M HEPES buffer pH 7.5, 30% 1,2-propanediol, 20% PEG 400 (form I) and 20% PEG 3350, 0.2 M sodium fluoride and 5% trehalose (form II) (Fig. 1). For data collection, a form I crystal was transferred directly from the mother liquor to a nitrogen cold stream at 100 K. A form II crystal was soaked in a cryoprotectant solution (25% PEG 3350, 0.2 M sodium fluoride and 15% trehalose) for a few minutes prior to freezing in the nitrogen cold stream. The diffraction of the form I and II crystals of the NADP<sup>+</sup> complex extended to 1.8 and 1.9 Å resolution, respectively. The form I crystal belonged to space group *P*2<sub>1</sub>2<sub>1</sub>2, with unit-cell parameters *a* = 63.90, *b* = 135.72, *c* = 39.19 Å. The form II crystal belonged to space group *C*2, with unit-cell parameters *a* = 207.47, *b* = 64.85, *c* = 61.12 Å,

$\beta = 105.82^\circ$ . The calculated Matthews coefficients ( $V_M$ ) of 2.12 and  $2.47 \text{ \AA}^3 \text{ Da}^{-1}$  for forms I and II correspond to solvent contents of 42.1 and 50.3% assuming the presence of one and two protomers in the asymmetric unit, respectively, and a protein molecular weight of 40 000 (Matthews, 1968). The data-collection statistics are summarized in Table 1. Structure determination was performed by the molecular-replacement method using the structure of the thioredoxin reductase-like protein from *Thermus thermophilus* HB8 (PDB code 2zbw; A. Ebihara, M. Manzoku, Y. Fujimoto, Y. Kitamura, S. Yokoyama & S. Kuramitsu, unpublished work) as a search model (45% sequence identity). The X-ray diffraction data used to solve the BsFNR structure were collected from a form I crystal. Thioredoxin reductase consists of two globular domains (the FAD-binding and NADPH-binding domains) connected by a two-stranded  $\beta$ -sheet (Waksman *et al.*, 1994). Thioredoxin reductase has been proposed to undergo a conformational change on binding NADPH, leading to a rotation of the two domains by around  $60^\circ$  relative to each other (Lennon *et al.*, 2000). BsFNR should also consist of two domains connected by a flexible linker. In order to allow conformational flexibility of the two domains, the search model was divided into two parts (the FAD-binding and NADPH-binding domains). Molecular replacement and consequent structure refinement are now in progress.

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