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Cloning, expression, crystallization and preliminary X-ray studies of the ferredoxin–NAD(P)⁺ reductase from the thermophilic cyanobacterium *Thermosynechococcus elongatus* BP-1

Ferredoxin–NADP⁺ reductase (FNR) is a flavoenzyme that catalyses the reduction of NADP⁺ in the final step of the photosynthetic electron-transport chain. FNR from the thermophilic cyanobacterium *Thermosynechococcus elongatus* BP-1 (*TeFNR*) contains an additional 9 kDa domain at its N-terminus relative to chloroplastic FNRs and is more thermostable than those from mesophilic cyanobacteria. With the aim of understanding the structural basis of the thermostability of *TeFNR* and assigning a structural role to the small additional domain, the gene encoding *TeFNR* with and without an additional domain was engineered for heterologous expression and the recombinant proteins were purified and crystallized. Crystals of *TeFNR* without the additional domain belonged to space group *P*2₁, with unit-cell parameters *a* = 55.05, *b* = 71.66, *c* = 89.73 Å, α = 90, β = 98.21, γ = 90°.

1. Introduction

Ferredoxin–NADP⁺ reductase (FNR; EC 1.18.1.2) is a ubiquitous ferredoxin-dependent enzyme that contains flavin adenine dinucleotide (FAD) as a prosthetic group. Based on phylogenetic and structural information, FNRs are classified into two distinct families (Aliverti *et al.*, 2008): glutathione reductase-type (GR-type) and plant-type FNRs (Schulz *et al.*, 1978; Karplus *et al.*, 1991; Serre *et al.*, 1996). GR-type FNRs mediate a wide range of physiological functions, providing electrons for various metabolic processes as diverse as steroid hydroxylation in mitochondria and reductive activation of biosynthetic enzymes. In contrast, plant-type FNRs are mainly distributed in plant chloroplasts and cyanobacteria. Plant-type FNRs utilize electrons received from two ferredoxins to reduce NADP⁺, which is required for the biosynthesis of carbohydrates in the dark reaction of photosynthesis.

Since their discovery in pea thylakoids, plant-type FNRs have been investigated by numerous biochemical studies (Avron & Jagendorf, 1956; Hurley *et al.*, 2002; Hanke *et al.*, 2004). Early reports suggested that the molecular mass of a typical cyanobacterial FNR was similar to that of those from higher plants (~35 kDa; Rowell *et al.*, 1981). High-resolution X-ray crystal structural analyses have shown that higher plant and cyanobacterial FNRs share a common three-dimensional arrangement, with two domains that bind FAD and NADPH, respectively (Serre *et al.*, 1996; Hurley *et al.*, 2002; Hanke *et al.*, 2004). In contrast, the FNR encoded by a cyanobacterial gene (*petH*) was reported to have a molecular mass of 45 kDa (van Thor *et al.*, 1998). The larger than anticipated molecular mass of this protein arises from the presence of a small domain that is attached to the N-terminus of the FAD domain *via* a hinge region. The estimated molecular mass of this small domain, which displays significant sequence similarity to the phycobilisome (PBS) binding protein CpcD, is 9 kDa (de Lorimier *et al.*, 1990; Schluchter & Bryant, 1991). It was proposed that the full-length cyanobacterial FNR is located in close proximity to the thylakoid membrane. Three forms of FNR can be purified from the thermophilic cyanobacterium *Thermosynechococcus elongatus* BP-1 (Nakajima *et al.*, 2002): (i) a 34 kDa

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form lacking the CpcD-like domain (probably owing to proteolytic cleavage at the hinge region), (ii) a 45 kDa form with an intact CpcD-like domain and (iii) a 78 kDa form in complex with phycocyanin, which is a major pigment-protein complex of PBS. Although the three-dimensional arrangement of the CpcD-like domain relative to the other domains remains unclear, the hinge region is assumed to be flexible and therefore susceptible to proteolytic cleavage (Nakajima *et al.*, 2002; van Thor *et al.*, 1999; Gómez-Lojero *et al.*, 2003).

We are interested in studying the structural and biochemical characteristics of recombinant FNRs with and without the CpcD-like domain. We anticipated that the structure of *T. elongatus* FNR (*TeFNR*) would provide an important insight into the mechanism of the protein–protein interaction not only with ferredoxin but also with phycocyanin. Additionally, given that *T. elongatus* is a thermophilic cyanobacterium, it could also be interesting to determine how the protein achieves thermostability despite displaying a high sequence similarity to mesophilic cyanobacterial FNRs. Here, we report the crystallization of *TeFNR* and the preliminary results of the X-ray crystal structure analysis.

2. Materials and methods

2.1. Protein expression and purification

The thermophilic cyanobacterium *T. elongatus* strain BP-1 was originally isolated from a hot spring in Beppu, a city in the southern island of Japan, as described previously (Yamaoka *et al.*, 1978) and the genomic DNA of *T. elongatus* was extracted as described previously (Nakamura *et al.*, 2002). The gene encoding *TeFNR* was amplified with the pair of primers The5 (5'-GCCATATGTACAA-TACAATGCGACGAATTCTCGC-3') and The3 (5'-GGATCCTT-TTAGTAGGTTTCCACGTGCCA-3') and the PCR product was cloned into vector pET28a for overexpression in *Escherichia coli* BL21 (DE3) cells. The truncated FNR construct lacking the CpcD-like domain was engineered as follows: a DNA fragment encoding part of the FAD- and NADP-binding domains was amplified by PCR using the FNR gene cloned from the *T. elongatus* genomic DNA as a template. The amplified product was then cloned into pASK-IBA7 lacking the affinity tag.

E. coli cells transformed with each expression plasmid were cultivated in LB medium at 310 K. For overexpression of full-length *TeFNR*, LB medium containing kanamycin at a concentration of

50 $\mu\text{g ml}^{-1}$ was used. The culture was allowed to reach an OD_{600} of 0.4 before inducing heterologous gene expression by the addition of 100 μM IPTG. Cells were harvested after a further 3 h growth at 310 K. For overexpression of the truncated *TeFNR*, LB medium containing ampicillin at a concentration of 50 $\mu\text{g ml}^{-1}$ was used. Once the cell culture reached an OD_{600} of 0.5, heterologous gene expression was induced by the addition of 100 $\mu\text{g l}^{-1}$ anhydrotetracycline. Cells were harvested after a further 5 h growth at 310 K. Following centrifugation, the resultant pellet was resuspended in 50 mM Tris–HCl pH 7.5 supplemented with 200 mM NaCl, 1 mM EDTA, 1 mM MgCl_2 , 0.1% β -mercaptoethanol and PMSF. Cells of each recombinant strain were disrupted by sonication and the cell debris was removed by centrifugation. For the full-length *TeFNR*, the supernatant containing the His-tagged protein was applied onto a nickel-affinity column (Ni-NTA, Qiagen, Hilden, Germany). After washing the column with ten volumes of buffer A (50 mM Tris–HCl pH 7.5 supplemented with 500 mM NaCl) containing 30 mM imidazole, bound proteins were eluted using a step gradient of 50, 100 and 200 mM imidazole in buffer A. Most His-tagged proteins were recovered in the 200 mM imidazole fraction, which was dialyzed three times against 30 volumes of buffer B (50 mM Tris–HCl pH 7.5 supplemented with 150 mM NaCl) to remove the imidazole. The homogeneity of the purified proteins was confirmed by SDS–PAGE. The purified full-length *TeFNR* was concentrated to $\sim 10.5 \text{ mg ml}^{-1}$ in buffer B. These samples were then used for all of the crystallization experiments. Truncated *TeFNR* lacking the affinity tag was purified as follows: nucleic acids and negatively charged proteins were first removed by anion-exchange chromatography using a DE52 column equilibrated in buffer B. Yellow fractions containing FNR were precipitated between 40 and 70% ammonium sulfate and the pellets were then dialyzed against NaCl-free buffer. The yellow solution was applied onto a Resource Q column and the bound protein was subsequently eluted with a linear gradient from 0 to 500 mM NaCl in 50 mM Tris–HCl pH 7.5. The pooled fractions were further purified by Phenyl-Sepharose chromatography with a gradient of 40–0% ammonium sulfate, which was dialyzed against buffer B. The purity of

Figure 1
A typical crystal of *T. elongatus* FNR without the CpcD-like domain.

Figure 2
A diffraction image from truncated *TeFNR* recorded at the Photon Factory.

the final sample was assessed by SDS–PAGE and UV–Vis spectroscopy at 280 and 457 nm. The purified *TeFNR* lacking the CpcD-like domain was concentrated to $\sim 10.0 \text{ mg ml}^{-1}$ and was used for all the crystallization experiments. The yield of the full-length and truncated forms of *TeFNR* from *E. coli* was 4 and 20 mg l^{-1} , respectively.

2.2. Crystallization

Crystallization screens were carried out with Crystal Screen, Crystal Screen 2 and the PEG/Ion Screen (Hampton Research, Aliso Viejo, California, USA) using the hanging-drop vapour-diffusion method at 277 and 293 K. Crystallization droplets were prepared on siliconized cover slips by mixing $1 \mu\text{l}$ protein solution with $1 \mu\text{l}$ reservoir solution and were equilibrated against $150 \mu\text{l}$ reservoir solution. A crystallization robot (Hanging Drop Maker FK1, Furukawa Co. Ltd, Tokyo, Japan) was used to set up the crystallization plates. Crystals of FNR without the CpcD-like domain were obtained

Figure 3 SDS–PAGE analysis of full-length FNR. (a) Freshly purified FNR was applied in the middle lane. Dissolved crystals of *TeFNR* without any protein inhibitor were applied in the right lane. (b) Dissolved crystals prepared in the presence of 10 mM PMSF were applied to the right gel. The left lanes contain molecular-mass markers (labelled in kDa).

Figure 4 Molecular packing drawn as a stereo pair projected along the *c* axis. The asymmetric unit contains two molecules.

Table 1

Diffraction data statistics for truncated *TeFNR*.

Values in parentheses are for the highest resolution bin.

Space group	<i>P</i> 2 ₁
Unit-cell parameters	
<i>a</i> (Å)	55.05
<i>b</i> (Å)	71.66
<i>c</i> (Å)	89.73
β (°)	98.21
Completeness (%)	99.9 (100.0)
<i>R</i> _{merge} (%)	10.4 (54.3)
Resolution (Å)	2.1 (2.14–2.10)
Measured reflections	150514
Unique reflections	40292

at 277 K. After optimizing the crystallization conditions, rounded rectangular crystals were grown in 0.1 M HEPES pH 7.5, 20% polyethylene glycol 10 000 as a precipitant within a few days (Fig. 1). Crystallization screening of full-length FNR was carried out after adding 10 mM PMSF to prevent proteolysis of the susceptible hinge region. Crystals of full-length FNR were grown in 1.0 M $\text{NaH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ pH 6.9.

2.3. X-ray data collection

X-ray diffraction data were collected from a single crystal of FNR lacking the CpcD-like domain using synchrotron radiation of wavelength 1 Å on beamline BL-17A at Photon Factory, Tsukuba, Japan (Fig. 2). For data collection under cryogenic conditions, crystals of FNR were soaked in artificial mother liquor containing different concentrations of ethylene glycol (0 – 20%). A full set of X-ray images was collected at 2.1 Å resolution and was processed with *HKL-2000* (Otwinowski & Minor, 1997). Data-collection statistics are summarized in Table 1.

3. Results and discussion

During initial crystallization screening of full-length FNR, significant proteolysis was observed by SDS–PAGE analysis (Fig. 3). The N-terminal amino-acid sequence of truncated FNR was subsequently determined to be GAAPVK (data not shown), which corresponds to the hinge region. We thought that a specific protease from *E. coli* might be responsible for cleavage at the hinge region. Thus, we screened a range of different protease inhibitors in order to prevent proteolytic cleavage. We found that 10 mM PMSF clearly inhibited

cleavage even after one week of incubation at room temperature. A second round of crystallization screening in the presence of 10 mM PMSF was then performed. Even after optimization of crystallization conditions, the yellow needle crystals were too thin to be used for X-ray data collection. However, the existence of full-length FNR with the CpcD-like domain in the crystals was confirmed by SDS–PAGE analysis (Fig. 3).

Crystals of *TeFNR* lacking the CpcD-like domain belonged to the monoclinic space group $P2_1$, with unit-cell parameters $a = 55.05$, $b = 71.66$, $c = 89.73$ Å, $\beta = 98.21^\circ$. Molecular-replacement calculations were performed with *BALBES* (Long *et al.*, 2008). The FNR from the mesophilic cyanobacterium *Synechococcus* sp. PCC7002 (PDB entry 2b5o; M. R. Sawaya, C. A. Kerfeld, C. Gómez-Lojero, D. Krogmann, D. A. Bryant & T. O. Yeates, unpublished work) shares 76.1% similarity with *TeFNR* and was used as the molecular-replacement search model. The calculations provided a unique solution with two FNR molecules in the asymmetric unit (Fig. 4). Structure determination including model rebuilding and refinement is in progress.

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