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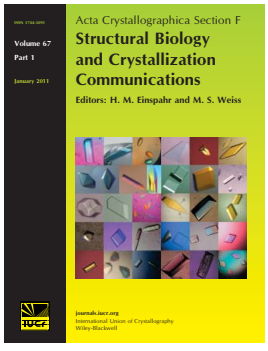

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Cloning, expression, crystallization and preliminary X-ray studies of the ferredoxin–NAD(P)$^+$ reductase from the thermophilic cyanobacterium *Thermosynechococcus elongatus* BP-1

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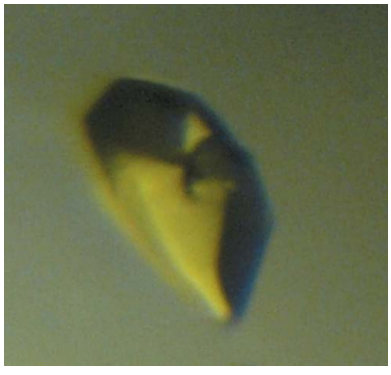
Ferredoxin–NADP$^+$ reductase (FNR) is a flavoenzyme that catalyses the reduction of NADP$^+$ in the final step of the photosynthetic electron-transport chain. FNR from the thermophilic cyanobacterium *Thermosynechococcus elongatus* BP-1 (*Te*FNR) contains an additional 9 kDa domain at its N-terminus 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 *Te*FNR and assigning a structural role to the small additional domain, the gene encoding *Te*FNR with and without an additional domain was engineered for heterologous expression and the recombinant proteins were purified and crystallized. Crystals of *Te*FNR without the additional domain belonged to space group $P2_1$, with unit-cell parameters $a = 55.05$, $b = 71.66$, $c = 89.73$ Å, $\alpha = 90$, $\beta = 98.21$, $\gamma = 90^\circ$.

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



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 (*Te*FNR) 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 *Te*FNR 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 *Te*FNR was amplified with the pair of primers The5 (5′-GC**CATATG**TACAA-TACAATGCGACGAATTCTCGC-3′) and The3 (5′-**GGATCC**TT-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 *Te*FNR, LB medium containing kanamycin at a concentration of 50 µg 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 µ*M* IPTG. Cells were harvested after a further 3 h growth at 310 K. For overexpression of the truncated *Te*FNR, LB medium containing ampicillin at a concentration of 50 µg 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 µg l^{-1} anhydrotetracycline. Cells were harvested after a further 5 h growth at 310 K. Following centrifugation, the resultant pellet was resuspended in 50 m*M* Tris–HCl pH 7.5 supplemented with 200 m*M* NaCl, 1 m*M* EDTA, 1 m*M* $MgCl_2$, 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 *Te*FNR, 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 m*M* Tris–HCl pH 7.5 supplemented with 500 m*M* NaCl) containing 30 m*M* imidazole, bound proteins were eluted using a step gradient of 50, 100 and 200 m*M* imidazole in buffer *A*. Most His-tagged proteins were recovered in the 200 m*M* imidazole fraction, which was dialyzed three times against 30 volumes of buffer *B* (50 m*M* Tris–HCl pH 7.5 supplemented with 150 m*M* NaCl) to remove the imidazole. The homogeneity of the purified proteins was confirmed by SDS–PAGE. The purified full-length *Te*FNR was concentrated to ∼10.5 mg ml^{-1} in buffer *B*. These samples were then used for all of the crystallization experiments. Truncated *Te*FNR 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 m*M* NaCl in 50 m*M* 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

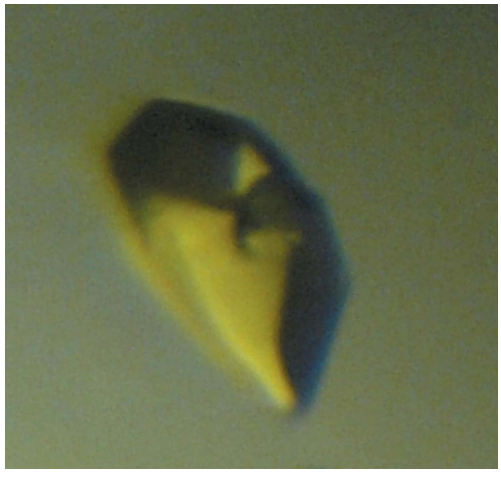


Figure 1
A typical crystal of *T. elongatus* FNR without the CpcD-like domain.

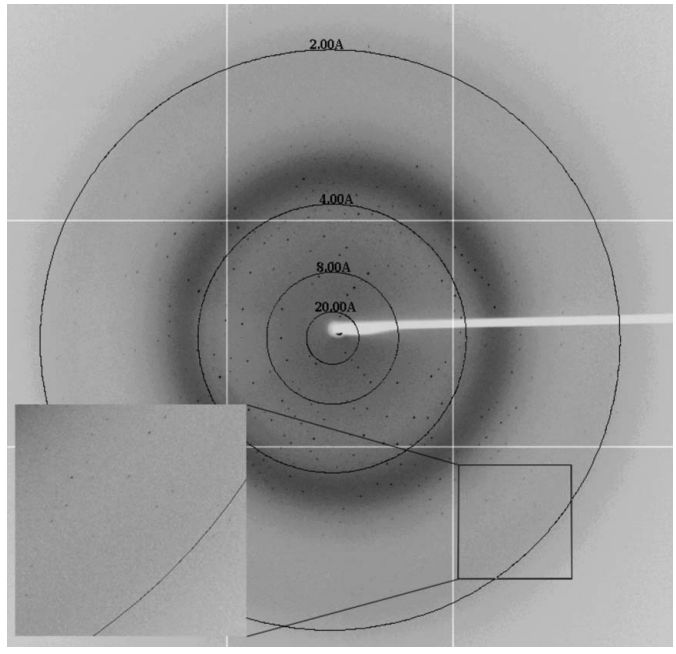


Figure 2
A diffraction image from truncated *Te*FNR recorded at the Photon Factory.

the final sample was assessed by SDS–PAGE and UV–Vis spectroscopy at 280 and 457 nm. The purified *Te*FNR lacking the CpcD-like domain was concentrated to ∼10.0 mg ml^{-1} and was used for all the crystallization experiments. The yield of the full-length and truncated forms of *Te*FNR 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 µl protein solution with 1 µl reservoir solution and were equilibrated against 150 µl 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 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 m*M* PMSF to prevent proteolysis of the susceptible hinge region. Crystals of full-length FNR were grown in 1.0 M NaH_2PO_4/K_2HPO_4 pH 6.9.

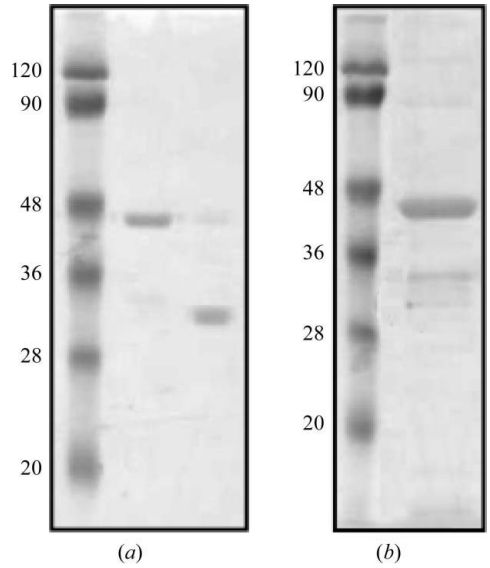


Figure 3
SDS–PAGE analysis of full-length FNR. (*a*) Freshly purified FNR was applied in the middle lane. Dissolved crystals of *Te*FNR without any protein inhibitor were applied in the right lane. (*b*) Dissolved crystals prepared in the presence of 10 m*M* PMSF were applied to the right gel. The left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

Table 1
Diffraction data statistics for truncated *Te*FNR.

Values in parentheses are for the highest resolution bin.

Space group	$P2_1$
Unit-cell parameters	
a (Å)	55.05
b (Å)	71.66
c (Å)	89.73
β (°)	98.21
Completeness (%)	99.9 (100.0)
R_{merge} (%)	10.4 (54.3)
Resolution (Å)	2.1 (2.14–2.10)
Measured reflections	150514
Unique reflections	40292

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 m*M* PMSF clearly inhibited

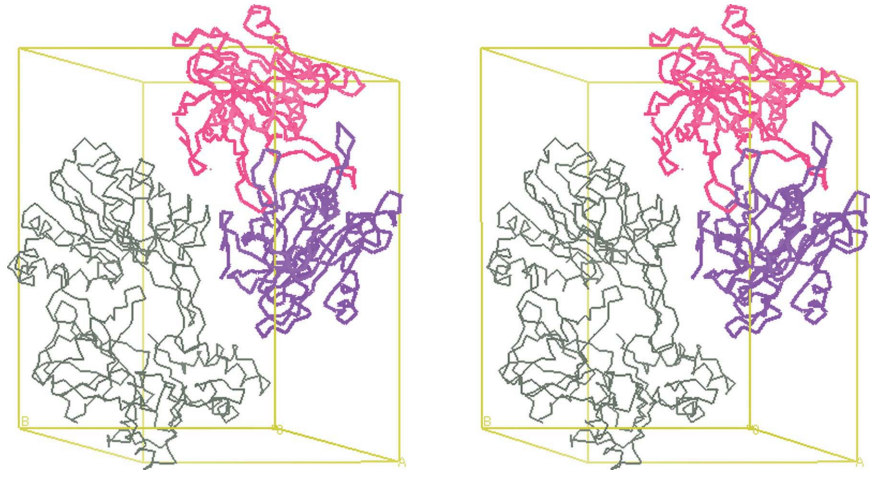


Figure 4
Molecular packing drawn as a stereo pair projected along the *c* axis. The asymmetric unit contains two molecules.

cleavage even after one week of incubation at room temperature. A second round of crystallization screening in the presence of 10 m*M* 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 *Te*FNR 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 *Synechecoccus* 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 *Te*FNR 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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