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Crystallization and preliminary X-ray studies of ferredoxin-NAD(P)$^+$ reductase from *Chlorobium tepidum*

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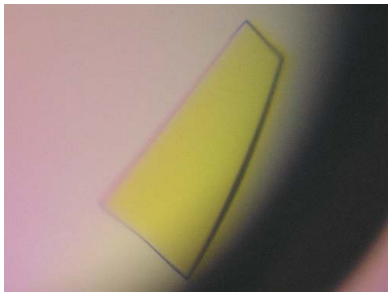
Ferredoxin-NAD(P)$^+$ reductase (FNR) is a key enzyme that catalyzes the photoreduction of NAD(P)$^+$ to generate NAD(P)H during the final step of the photosynthetic electron-transport chain. FNR from the green sulfur bacterium *Chlorobium tepidum* is a homodimeric enzyme with a molecular weight of 90 kDa; it shares a high level of amino-acid sequence identity to thioredoxin reductase rather than to conventional plant-type FNRs. In order to understand the structural basis of the ferredoxin-dependency of this unique photosynthetic FNR, *C. tepidum* FNR has been heterologously expressed, purified and crystallized in two forms. Form I crystals belong to space group $C222_1$ and contain one dimer in the asymmetric unit, while form II crystals belong to space group $P4_122$ or $P4_322$. Diffraction data were collected from a form I crystal to 2.4 Å resolution on the synchrotron-radiation beamline NW12 at the Photon Factory.

1. Introduction

Ferredoxin (Fd), a small acidic non-haem iron protein, plays a central role as an electron-transfer mediator in numerous metabolic pathways in various organisms, including bacteria, plants, algae and animals. Importantly, Fd can only transfer one electron at a time. The electron-transfer processes between Fd and redox-partner enzymes, called Fd-dependent enzymes, are accomplished by the formation of an electron-transfer complex (Knaff, 1996). Fd-dependent enzymes are diverse in terms of their molecular weight, prosthetic group requirements and physiological function. However, each Fd-dependent enzyme forms a productive 1:1 electron-transfer complex with Fd.

Fd-NADP$^+$ reductase (FNR; EC 1.18.1.2) is a ubiquitous Fd-dependent enzyme containing a flavin adenine dinucleotide (FAD) as a prosthetic group; it catalyzes the reversible redox reaction between Fd and NAD(P)$^+$/NAD(P)H (Karplus & Faber, 2004; Seo *et al.*, 2004). Based on their physiological function and amino-acid sequences, FNRs have been classified into three types: chloroplast-type, bacterial-type and mitochondrial-type FNRs. Chloroplast-type FNRs are distributed in both plant chloroplasts and cyanobacteria and are responsible for the production of NADPH during oxygenic photosynthesis. This type of FNR transfers reducing equivalents from the photo-reduced [2Fe–2S] Fd to NADP$^+$, providing the NADPH needed for CO_2 assimilation and other metabolic pathways (Karplus & Faber, 2004). Bacterial-type FNRs utilize NAD(P)H in order to reduce Fd and/or flavodoxin (Isas & Burgess, 1994; Liochev *et al.*, 1994). The physiological electron flow catalyzed by this type of FNR is the converse of that of chloroplast-type FNRs. *Azotobacter vinelandii* FNR and *Escherichia coli* FNR have been reported to be involved in the response to oxidative stress. The third class of FNRs are mitochondrial-type FNRs called adrenodoxin reductases (NADPH:adrenal ferredoxin oxidoreductase; AR), which constitute the first component in the mitochondrial cytochrome P450 electron-transfer cascade (Bernhardt, 1996). AR participates in the reduction

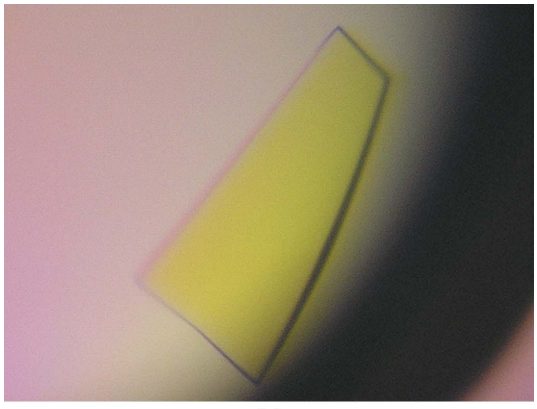


of adrenal Fd using NADPH as a source of reducing equivalents for all mitochondrial cytochrome P450-mediated reactions, including the conversion of cholesterol to pregnenolone and other steroid hormones as well as the hydroxylation reactions involved in the biosynthesis of bile acids and vitamin D.

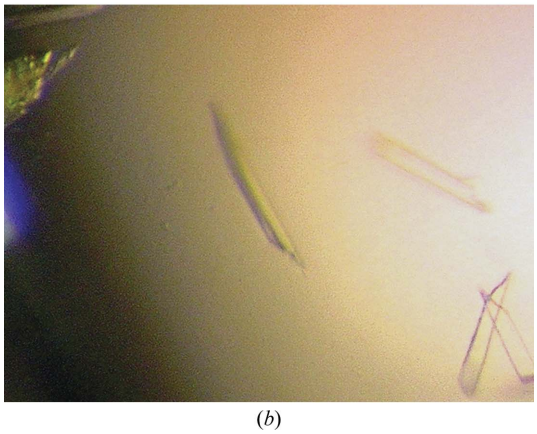
The three types of FNR have been extensively studied and their catalytic mechanisms are biochemically and structurally well characterized. The X-ray crystal structure of the chloroplast-type FNR from spinach was solved in 1991 (Karplus *et al.*, 1991). The enzyme is monomeric and is composed of FAD- and $NADP^+$-binding domains, with a single non-covalently bound FAD molecule located between the two functional domains. The structure of the complexes formed between the pea FNR variant and $NADP^+$, and between maize FNR and Fd have been independently reported (Deng *et al.*, 1999; Kurisu *et al.*, 2001). Based on these structures, a detailed mechanism of electron transfer between Fd and FNR has been proposed. It has also been reported that the polypeptide folds of *E. coli* and *A. vinelandii* FNRs are homologous to those of chloroplast-type FNRs, even though the direction of physiological electron transfer is converse (Ingelman *et al.*, 1997; Sridhar Prasad *et al.*, 1998). In contrast to the structural similarity between plant and bacterial FNRs, the X-ray structure of bovine AR revealed that there was no chain-fold similarity to other functionally related FNRs (Ziegler *et al.*, 1999). A striking feature of bovine AR is the asymmetric charge distribution that controls the approach of adrenodoxin.

Recently, a novel FNR was found in the green sulfur bacterium *Chlorobium tepidum* (Seo & Sakurai, 2002), a phototrophic anaerobic bacterium. The bacterium possesses an iron–sulfur-type reaction centre which is evolutionarily related to photosystem I in chloroplasts and is able to reduce Fd photosynthetically. It was anticipated that *C. tepidum* might possess a homologue of the plant-type FNR because the electron-transfer reactions involving Fd are quite similar to those that occur in the chloroplast. However, no homologue of the plant-type FNRs was found in the genome sequence of *C. tepidum*. Instead, the novel FNR found in the cell extract of *C. tepidum* is distinct from previously characterized FNRs in its oligomerization state and amino-acid sequence. Unlike conventional FNRs, which are monomeric proteins, FNR from *C. tepidum* is a homodimeric enzyme with a non-covalently bound FAD molecule. The *C. tepidum* FNR shares a higher level of amino-acid sequence identity to NAD(P)H-thioredoxin reductases (TR) rather than to the functionally related plant-type FNR. TR is homodimeric and catalyzes the reduction of thioredoxin (Trx) using NADPH (Biterova *et al.*, 2005). Trx is also an electron-transfer protein with a similar molecular size to Fd; it maintains the intracellular redox status by modulating its intramolecular disulfide bond.

Intriguingly, *C. tepidum* FNR is exclusively dependent on Fd and independent of Trx, despite its apparent structural similarity to TR based on amino-acid sequence alignments. In order to understand the structural basis of the Fd-dependency of this unique FNR, we have overexpressed *C. tepidum* FNR and crystallized the protein for further structural studies. Here, we report the purification, crystallization and preliminary X-ray crystallographic analysis of *C. tepidum* FNR.



(*a*)



(*b*)

Figure 1
Crystals of *C. tepidum* FNR. (*a*) Plate-shaped form I crystal. (*b*) Rod-shaped form II crystal.

2. Materials and methods

2.1. Protein expression and purification

The gene encoding *C. tepidum* FNR (CT1512; Eisen *et al.*, 2002) was engineered for overexpression by subcloning into the pETBlue-1 vector (Novagen, Madison, Wisconsin, USA). The resultant expression plasmid was then transformed into *E. coli* Tuner(DE3)pLacI cells. A single colony of transformed cells was picked and cultured in LB medium supplemented with 100 µg ml^{-1} ampicillin and 34 µg ml^{-1} chloramphenicol at 310 K to an OD_{600} of ∼1.5. Heterologous gene expression was then induced by the addition of 0.5 m*M* isopropyl *β*-D-1-thiogalactopyranoside (IPTG) and growth was continued at 300 K overnight before harvesting the cells by centrifugation. The cell pellet was resuspended in 50 m*M* Tris–HCl buffer pH 7.8 containing 200 m*M* NaCl, 1 m*M* EDTA and 1 m*M* PMSF. Cells were disrupted by sonication and the crude lysate was clarified by centrifugation at 10 000*g* for 20 min. Ammonium sulfate was added to the supernatant to 40% saturation. The resultant precipitate was suspended in 0.1 *M* potassium phosphate buffer pH 6.0 (buffer *A*) and dialyzed three times against 5 l buffer *A* for 2 h each. The sample was applied onto a Matrex Red A column (Amicon, Beverly, Massachusetts, USA) pre-equilibrated with buffer *A*. The column was washed with sufficient volumes of buffer *A* containing 50 m*M* NaCl and the sample was eluted with 0.1 *M* glycine–NaOH buffer pH 10.5 containing 1.5 *M* KCl. After dialysis against 50 m*M* Tris–HCl buffer pH 7.8, the sample was loaded onto a DEAE-5PW column (Tosoh, Tokyo, Japan). The fractions were eluted with a linear gradient of NaCl (0–200 m*M*) in the same buffer. The FNR fractions, which had a yellow colour, were dialyzed against 20 m*M* MOPS buffer pH 6.0 and applied onto a hydroxyapatite column (Bio-Rad Laboratories Inc., Hercules, California, USA). FNR was eluted with a linear gradient of 20 m*M* MOPS buffer pH 6.0 to 0.2 *M* potassium phosphate buffer pH 7.0. Finally, FNR was loaded onto a Superdex-200 gel-permeation column (GE Healthcare Biosciences, Piscataway, New Jersey, USA) equilibrated with 50 m*M* Tris–HCl buffer pH 7.8 containing 200 m*M*

NaCl. The purified enzyme was identical to the native protein without any additional tag and gave a single band on SDS–PAGE. The protein concentration was determined using the Protein Assay Rapid kit (Wako Pure Chemical Industries, Osaka, Japan). The purified *C. tepidum* FNR was concentrated to approximately 5.5 mg ml^{-1} in the last buffer and the sample was used at this concentration in all crystallization experiments

2.2. Crystallization

Crystallization screening was carried out with Crystal Screens I and II and PEG/Ion Screen (Hampton Research, Aliso Viejo, California, USA) using the hanging-drop vapour-diffusion method at 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 180 µl of the same reservoir solution. The *C. tepidum* FNR was crystallized in two forms, I and II, from screening conditions consisting of 20%(*w*/*v*) PEG 3350 containing 200 m*M* ammonium sulfate or diammonium tartrate as precipitant. The two crystal forms co-exist in the same drops. Initially, form I crystals were plate-shaped and tended to cluster together. The crystals from the cluster had a thickness of 0.01 mm and were too thin for X-ray experiments. Form II crystals were rod-shaped, but their reproducibility was very low even using identical crystallization conditions. After refinement of the crystallization conditions, the best crystals of the two forms were obtained using 0.1 *M* MES buffer pH 5.8 containing 16%(*w*/*v*) PEG 6K and 0.1 *M* ammonium sulfate as precipitant (Fig. 1).

2.3. Data collection

For data collection under cryogenic conditions, crystals were briefly soaked in reservoir solution containing 15%(*v*/*v*) glycerol. Crystals were mounted in a nylon loop and flash-cooled in a stream of gaseous nitrogen at 100 K. Native diffraction data sets were collected on beamline NW-12 at the Photon Factory, Tsukuba, Japan.

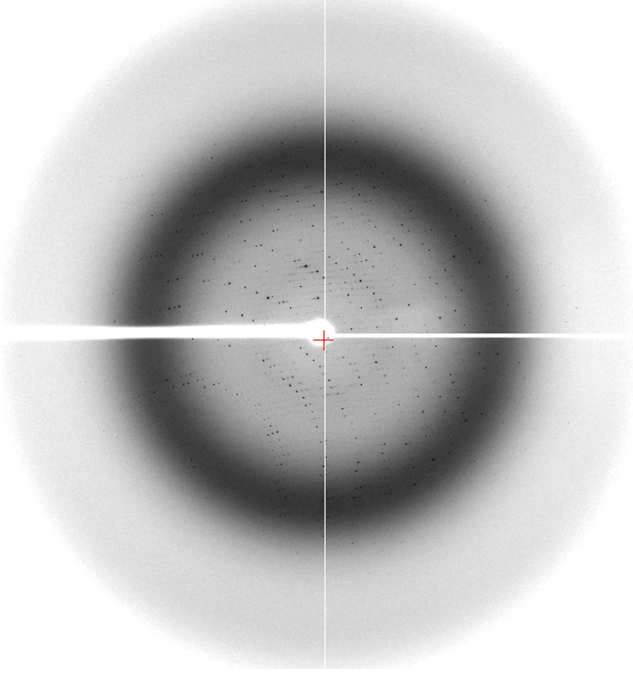


Figure 2
X-ray diffraction image from a form I crystal of *C. tepidum* FNR.

Table 1
Diffraction data statistics for crystals of FNR from *C. tepidum*.

Values in parentheses are for the highest resolution shell.

	Form I	Form II
Space group	$C222_1$	$P4_122$ or $P4_322$
Unit-cell parameters		
a (Å)	100.5	82.0
b (Å)	128.0	82.0
c (Å)	128.4	162.7
Temperature (K)	100	100
Wavelength (Å)	0.9831	1.0000
Resolution range (Å)	50.00–2.40 (2.49–2.40)	50.00–3.30 (3.42–3.30)
Total reflections	242503	75173
Unique reflections	32978	8168
Completeness (%)	100.0 (100.0)	99.8 (100)
$R_{\rm merge}(I)$† (%)	8.0 (48.5)	11.7 (43.1)
$I/\sigma(I)$	12.0 (3.92)	8.9 (5.44)

† $R_{\rm merge}(I) = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the value of the *i*th measurement of the intensity of a reflection, $\langle I(hkl)\rangle$ is the mean value of the intensity of that reflection and the summation is over all measurements.

Diffraction images were collected at 100 K using an ADSC Quantum210 CCD detector and Rigaku GN_2 cryosystem (Fig. 2). A total of 180 images for each data set were recorded with an oscillation range of 1.0° and an exposure time of 5 s per image. All diffraction data were processed and scaled with the *HKL*-2000 program package (Otwinowski & Minor, 1997). The data-collection statistics are summarized in Table 1.

3. Results and discussion

C. tepidum FNR comprises 360 amino acids and has a predicted molecular weight of about 40 kDa. However, gel-filtration chromatographic analysis showed an apparent molecular weight of about 90 kDa, indicating that the enzyme is homodimeric in solution. Form I crystals belong to the orthorhombic space group $C222_1$, with unit-cell parameters a = 100.5, b = 128.0, c = 128.4 Å. Assuming the presence of one dimer in the asymmetric unit, the Matthews coefficient ($V_{\rm M}$) is 2.6 Å^3 Da^{-1}, corresponding to a solvent content of 53.2%. Self-rotation functions calculated using the diffraction data from the form I crystal show one obvious peak, ω = 44.1, φ = 90.0, κ = 180°, corresponding to a noncrystallographic twofold axis (Collaborative Computational Project, Number 4, 1994). These calculations imply that the asymmetric unit of the form I crystals contains a dimer. Form II crystals belong to the tetragonal space group $P4_122$ or $P4_322$, with unit-cell parameters a = 82.0, c = 162.7 Å. The $V_{\rm M}$ value of 3.5 Å^3 Da^{-1} indicates that the form II crystal contains one protein molecule per asymmetric unit.

We attempted to carry out molecular replacement using the program *MOLREP* from the *CCP*4 program suite. The search models for the molecular-replacement calculation showed approximately 25% similarity to *C. tepidum* FNR and included TR from *M. tuberculosis* (PDB code 2a87; Akif *et al.*, 2005), alkyl hydroperoxide reductase from *S. typhimurium* (PDB code 1hyu; Wood *et al.*, 2001) and alkyl hydroperoxide reductase (PDB code 1fl2; Bieger & Essen, 2001) and TRs from *E. coli* (PDB codes 1tde and 1clo; Lennon *et al.*, 1999; Waksman *et al.*, 1994). Despite using monomeric, dimeric and partial models, we were unable to obtain good solutions for the molecular-replacement calculations. We therefore abandoned our attempt to obtain phase information using the molecular-replacement method.

Recently, we have succeeded in obtaining crystals of selenomethionine-substituted *C. tepidum* FNR, which has seven methio-

nines per monomer. Furthermore, selenium signals from the crystals were confirmed by an X-ray fluorescence scan. Efforts towards structure determination using the multiwavelength anomalous dispersion method are currently in progress.

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