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Cloning, expression, purification, crystallization and preliminary X-ray diffraction analysis of a ferredoxin/ferredoxin-NADP(H) oxidoreductase (Bc0385) from *Bacillus cereus*

Ferredoxin/ferredoxin-NADP(H) oxidoreductases (FNRs) are key enzymes involved in catalysing electron transfer between ferredoxins/ferredoxins and NAD(P)H/NAD(P)⁺. In *Bacillus cereus* there are three genes that may encode FNRs, and the Bc0385 FNR has been cloned, overexpressed, purified and successfully crystallized in its NADPH/NADP⁺-free form. Diffraction data have been collected to 2.5 Å resolution from crystals belonging to the orthorhombic space group *P*₂₁₂₁₂, with unit-cell parameters *a* = 57.2, *b* = 164.3, *c* = 95.0 Å, containing two FNR molecules in the asymmetric unit. The structure of the Bc0385 FNR has been solved by molecular replacement, and is a member of the homodimeric thioredoxin reductase-like class of FNRs.

1. Introduction

Ferredoxin/ferredoxin-NADP(H) oxidoreductases (FNRs; EC 1.18.1.2) catalyse the reversible redox reaction between ferredoxins (Fds)/ferredoxins (Flds) and NAD(P)⁺/NAD(P)H. In oxygenic photosynthetic organisms FNRs catalyse the reduction of NADP⁺ by photosynthetically reduced Fd, while in many heterotrophs FNRs catalyse the NADPH-dependent reduction of Fd/Fld to subsequently provide reducing power to various Fd/Fld-dependent enzyme systems, *e.g.* cytochrome P450, ribonucleotide reductase and methionine synthase (Bianchi *et al.*, 1995; Seo *et al.*, 2009; Wan & Jarrett, 2002). All FNRs have a common two-nucleotide-binding architecture with a C-terminal FAD-binding domain and an N-terminal NADPH-binding domain. FNRs are found in a variety of organisms and are typically divided into four classes according to molecular phylogenetic analysis (Seo *et al.*, 2009; Aliverti *et al.*, 2008): (i) plastid-type FNRs, (ii) proteobacteria-type FNRs, (iii) mitochondria-type FNRs and, more recently, (iv) thioredoxin reductase-like (TrxR-type) FNRs.

The TrxR-type FNRs are widely distributed among bacteria and have catalytic properties similar to the other types of FNR. However, they are more homologous to bacterial NADPH-TrxRs than the other types of FNR (Komori *et al.*, 2010b). The plastid, proteobacteria and mitochondria types are monomers, while the TrxR-type FNRs are homodimers similar to the low-molecular-weight TrxRs (Muraki *et al.*, 2010). Structurally, these TrxR-like FNRs differ from the TrxRs by lacking the CXXC motif essential for disulfide isomerization and by the presence of an extended C-terminus. The extended C-terminus from each monomer covers the *re* face of the isoalloxazine ring of the other monomer, and can make a hydrogen bond between the C-terminal Thr/Ser of the first monomer and the isoalloxazine N5 of the FAD group of the second monomer. The structures of the TrxR-like FNRs from *Bacillus subtilis* and *Chlorobaculum tepidum* have been crystallized with and without NADP⁺, respectively, indicating flexibility and large movement of the NADPH domain relative to the FAD domain through the hinge region (Muraki *et al.*, 2010; Komori *et al.*, 2010b).

In *B. cereus*, there are three annotated FNRs and one TrxR. The *B. cereus* Bc0385 FNR has been crystallized in its NADP⁺/NADPH-free form and belongs to the TrxR-type FNRs. The Bc0385 FNR has a sequence identity of 41% with respect to the TrxR-like FNR from *B. subtilis*, which is the most similar homologue in the Protein Data Bank (Komori *et al.*, 2010b). To better understand the structure-

Table 1

Macromolecule-production information.

Gene	Bc0385 (NP_830224.1)
Source organism	<i>Bacillus cereus</i>
Forward primer	GGAATTCATATGATCGAGAAGTAATGTTTGTATGAACCG
Reverse primer	AGGAAGCTTATTTTCATCATCTGCTTAATCAGTTCGG
Expression vector	pET-22b
Expression host	<i>Escherichia coli</i> BL21(DE3)
Complete amino-acid sequence of the construct produced	MNREELFDVTIVIGGGPAGLYSAFYSGLRMRTKIIIEFPHLG-GKIHVYPEKMIWDVGGLLPVTGDKLIEQLVQGLTFKPEV-VLDTKVESIIRNQDGTFTLKTSTGEEHFSKTVIVATGSGI-LKPQKLSIEGAERFEVSNLMTYVSKLRFKGTVIISGGG-NSAVDWANELEPIAKKVYVYTYRKEELSGHEAQVKQLMNSS-AECFFNTSITKLIAGDNHEAIEYVELTNHETGEVSHLPID-EVIINHGYERDITLLENSELDVAIIDNYYIAGNANSESSV-DGLYAAGDILKHEGKLHLIAGAFQDAGNAVKNKAKQFIQPD-ASEYGMVSSHNEVFKKRNRELKQMMK

function relation of the TrxR-type FNRs, further studies of this type of FNR are needed to reveal the flexibility of the NADPH domain during NADPH oxidation and electron transfer to Fds or Flds. Here, we report the cloning, purification, crystallization and preliminary X-ray analysis of the Bc0385 FNR from *B. cereus*.

2. Materials and methods

2.1. Macromolecule production

2.1.1. Cloning. The *bc0385* (*B. cereus* ATCC 14579) gene was amplified by PCR using genomic DNA of *B. cereus* and the gene-specific forward and reverse primers listed in Table 1. The *bc0385* PCR product and pET-22b expression vector (Novagen) were separately double-digested with FastDigest *Nde*I and *Hind*III restriction enzymes (Fermentas) and mixed for ligation. The ligation product was first transformed into *Escherichia coli* XL10-Gold Ultra-competent cells (Stratagene) and in a second step was transformed into *E. coli* BL21(DE3) Competent cells (New England BioLabs); the latter were used for protein expression. The resulting pET-22b-Bc0385 plasmid does not contain the *pelB* leader sequence owing to the use of the *Nde*I and *Hind*III restriction enzymes, and the Bc0385 FNR is expressed without any tags.

2.1.2. Expression. *E. coli* BL21(DE3) cells containing pET-22b-Bc0385 were first grown from freeze stocks on agar plates, before a single colony was used for overnight culture growth. Luria–Bertani (LB) medium containing 100 µg ml⁻¹ ampicillin at 310 K was used for the plates and the cultures. Protein expression was carried out in 1 l Terrific Broth (TB) medium containing 100 µg ml⁻¹ ampicillin inoculated with 10 ml of the overnight culture, and was vigorously agitated at 310 K until the OD₆₀₀ reached 0.9 before cooling on ice until the temperature had dropped to 293 K. Overexpression of Bc0385 was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and the cultures were incubated at 293 K for 14 h with vigorous agitation before harvesting by centrifugation at 5000g for 15 min at 277 K.

2.1.3. Purification. The harvested bacteria (~30 g) were cooled with liquid N₂ and thoroughly crushed before cell lysis with an AB X-press system (Magnusson & Edebo, 1976) pre-cooled to 253 K. The lysed cells were dissolved in purification buffer (100 mM Tris–HCl pH 7.5, 1 mM EDTA, 2 mM DTT) supplemented with one cComplete Protease Inhibitor Cocktail Tablet (Roche). DNA was precipitated by adding streptomycin sulfate to 2.5% (w/v) and removed by centrifugation at 39 000g before the protein was precipitated with ammonium sulfate (0.43 mg ml⁻¹) and collected by centrifugation. Precipitated protein was dissolved and desalted using a XK26 column containing 70 ml Sephadex G-25 Fine material (GE Healthcare) run on an

AKTApurifier FPLC system (GE Healthcare). The desalted protein was applied onto an XK26 column containing 70 ml High Performance Q anion-exchange material (GE Healthcare) and eluted with a linear gradient of KCl (18–40% of 1 M KCl in purification buffer) run over 20 column volumes. Because the FNR absorbs light at 450 nm,

Figure 1

Purification of Bc0385 FNR. (a) Anion-exchange chromatogram. (b) Size-exclusion chromatogram. Both chromatograms show absorbance at 280 nm (red line), absorbance at 450 nm (blue line) and conductivity (grey line). (c) SDS-PAGE. Lane M, protein marker (labelled in kDa); lane 1, FNR peak after anion-exchange chromatography; lane 2, FNR peak after size-exclusion chromatography.

Table 2

Crystallization.

Method	Sitting drop
Plate type	Swissci MRC2
Temperature (K)	277 for 1 d then 293
Protein concentration (mg ml ⁻¹)	9
Buffer composition of protein solution	100 mM Tris-HCl pH 7.5, 1 mM EDTA, 2 mM DTT
Composition of reservoir solution	20 mM sodium formate, 20 mM ammonium acetate, 20 mM sodium citrate, 20 mM sodium potassium tartrate, 20 mM sodium oxamate, 50 mM HEPES, 50 mM MOPS pH 7.5, 12.5% (v/v) MPD, 12.5% (w/v) PEG 1K, 12.5% (w/v) PEG 3350
Volume and ratio of drop	1 µl (2:3 ratio of protein:reservoir)
Volume of reservoir (µl)	100

the purification was monitored at both 450 and 280 nm on the ÄKTApurifier. The FNR peak is clearly shown in the chromatogram (Fig. 1a). Additionally, the purity was monitored by SDS-PAGE under reducing and denaturing conditions (Fig. 1c). After concentration, the Bc0385 FNR was further purified by size-exclusion chromatography on an XK16 column containing 120 ml Superdex 200 material (GE Healthcare) (Fig. 1b), giving a final yield of ~5 mg pure Bc0385 FNR per litre of culture. The highly purified Bc0385 FNR

Table 3

Data collection and processing.

Values in parentheses are for the outer shell.

Diffraction source	ID29, ESRF
Wavelength (Å)	0.9763
Temperature (K)	100
Detector	Pilatus 6M
Crystal-to-detector distance (mm)	533
Rotation range per image (°)	0.15
Total rotation range (°)	119.1
Exposure time per image (s)	0.037
Flux (photons s ⁻¹)	6.4 × 10 ¹¹
Beam size (µm)	30 × 50
Crystal size (µm)	50 × 50 × 150
Absorbed X-ray dose (MGy)	4.5
Space group	<i>P</i> 2 ₁ 2 ₁ 2
Unit-cell parameters (Å)	<i>a</i> = 57.2, <i>b</i> = 164.3, <i>c</i> = 95.0
Mosaicity (°)	0.14
Resolution range (Å)	54.8–2.5 (2.6–2.5)
Total No. of reflections	137105
No. of unique reflections	31641
Completeness (%)	99.5 (98.7)
Multiplicity	4.3 (4.1)
$\langle I/\sigma(I) \rangle$	8.8 (2.6)
$R_{\text{meas}}^{\dagger}$	0.14 (0.597)
Overall <i>B</i> factor from Wilson plot (Å ²)	24.1

$\dagger R_{\text{meas}} = \sum_{hkl} \{N(hkl)/[N(hkl) - 1]\}^{1/2} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $N(hkl)$ is the multiplicity, $I_i(hkl)$ is the observed intensity and $\langle I(hkl) \rangle$ is the average intensity for multiple measurements.

sample from size-exclusion chromatography (Figs. 1b and 1c) was used for crystallization.

2.2. Crystallization

The purified Bc0385 FNR was concentrated to ~30 mg ml⁻¹ in 100 mM Tris-HCl pH 7.5 buffer containing 1 mM EDTA and 2 mM DTT. The concentration of Bc0385 FNR was determined using the molar extinction coefficient of *B. subtilis* FNR of 12 300 M⁻¹ cm⁻¹ at 457 nm (Komori *et al.*, 2010a). Crystallization screening was carried out with an Oryx6 crystallization robot (Douglas Instruments) using the commercial crystallization screens JCSG+ (Molecular Dimensions), Morpheus (Molecular Dimensions; Gorrec, 2009) and Index (Hampton Research) with the sitting-drop vapour-diffusion method. Yellow (oxidized) crystals were observed using two conditions: Index H12 [0.15 M potassium bromide, 30% (w/v) PEG MME 2000] and Morpheus G8 [20 mM sodium formate, 20 mM ammonium acetate, 20 mM sodium citrate, 20 mM sodium potassium tartrate, 20 mM sodium oxamate, 50 mM HEPES, 50 mM MOPS pH 7.5, 12.5% (v/v) MPD, 12.5% (w/v) PEG 1K, 12.5% (w/v) PEG 3350]. Manual gradient screening by changing the protein and precipitant concentrations was performed to further optimize the two initial crystallization conditions. The Index H12 condition gave cubic and needle-shaped crystals (Fig. 2a), while the Morpheus G8 condition gave rod-shaped crystals (Fig. 2b). Only the crystals from the Morpheus G8 condition gave diffraction-quality crystals that were structurally solvable (Table 2).

2.3. Data collection and processing

X-ray diffraction experiments were performed on beamline ID29 at the ESRF. The cubic and needle-shaped crystals based on the Index H12 condition diffracted to only 8 and 3 Å resolution, respectively. For the latter a weak data set was collected to 3 Å resolution, but attempts to solve the structure by molecular replacement (MR) were unsuccessful (space group *C*2, unit-cell parameters *a* = 71.7, *b* = 132.9, *c* = 79.7 Å, β = 107.6°). The rod-shaped crystals grown from the Morpheus G8 condition (Table 2) were directly flash-cooled in CryoLoops (Hampton Research) in liquid N₂

(a)

(b)

Figure 2

Crystals of Bc0385 FNR from (a) Index condition H12 and (b) Morpheus condition G8.

since the crystallization condition also served as a cryoprotectant. Several data sets were collected to ~ 2.5 Å resolution from these crystals, which belonged to space group $P2_12_12$. The data sets were indexed and integrated with *XDS* (Kabsch, 2010) and scaled with *AIMLESS* (Evans & Murshudov, 2013). The statistics for the best data set are listed in Table 3, with the absorbed X-ray dose calculated by *RADDOSE* (Paithankar *et al.*, 2009; Murray *et al.*, 2004). The number of molecules in the asymmetric unit was estimated to be two, giving a Matthews coefficient of $2.9 \text{ Å}^3 \text{ Da}^{-1}$ and a solvent content of 58% (Matthews, 1968). The Bc0385 FNR structure was solved by MR using the *BALBES* program/server (Long *et al.*, 2008) by separately using the NADPH and FAD domains of *B. subtilis* FNR (PDB entry 3lzx; 41% sequence identity; Komori *et al.*, 2010b) as search models. The solution had an MR score of 11.7 and a *Q*-factor of 0.76, with an initial *R* and *R*_{free} of 41 and 44%, respectively, which fell to 34 and 39% in the first refinement cycle in *BALBES* (Murshudov *et al.*, 2011; Keegan *et al.*, 2011).

3. Results and discussion

The Bc0385 FNR (38.7 kDa) was successfully cloned, overexpressed and purified to high purity by anion-exchange and size-exclusion chromatography (Fig. 1). However, some slight contamination by two other proteins can be seen on the SDS-PAGE. The most prominent contaminants at 80–110 kDa could not be separated during size-exclusion chromatography and indicate a similar molecular weight to Bc0385 in their native state. This would correspond to FNR being purified as a dimer (77.4 kDa), which is consistent with the asymmetric unit having two copies of the protein and belonging to the TrxR-like FNRs. Two crystallization conditions were found for the NADPH/NADP⁺-free form of Bc0385 FNR. The crystals from the Morpheus G8 condition (Table 2) diffracted to 2.5 Å resolution and the structure was solvable by MR. The Bc0385 FNR has a sequence identity of 41% with respect to the TrxR-like FNR from *B. subtilis*, and the domains from the latter structure were used as starting models to solve the Bc0385 structure by MR (Komori *et al.*, 2010b). The previous structures of TrxR-like FNRs have shown that they are homodimers, have a C-terminal extension that covers the *re* face of the isoalloxazine ring of the FAD moiety of the other monomer and exhibit flexibility and movement of the NADPH domain (Komori *et al.*, 2010b; Muraki *et al.*, 2010). The Bc0385 FNR structure is currently being refined, and the structure as well as additional studies could provide further insight into the structure–function relationship of the electron-transfer mechanism of the TrxR-like FNRs and the

previously observed flexibility of the different domains. Additionally, Bc0385 FNR is an important piece in understanding the redox networks in *B. cereus* involving the Fds, Flds, FNRs and their redox partners.

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