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Crystallization and preliminary X-ray analysis of NADH:rubredoxin oxidoreductase from *Clostridium acetobutylicum*

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NADH:rubredoxin oxidoreductase (NROR), an O_2-inducible protein, is a versatile electron donor for scavengers of O_2 and reactive oxygen species (ROS) in *Clostridium acetobutylicum*. Recombinant NROR was overexpressed in *Escherichia coli* and purified to homogeneity; it was subsequently crystallized using the sitting-drop vapour-diffusion method at 293 K. Preliminary crystallographic analysis revealed that the crystals belonged to space group $P4_122$ or $P4_322$, with unit-cell parameters $a = b = 98.6$, $c = 88.3$ Å, and diffracted to 2.1 Å resolution. Assuming that the crystals contained one molecule per asymmetric unit, the Matthews coefficient was calculated to be 2.7 Å^3 Da^{-1} and the solvent content to be 54.1%.

1. Introduction

Clostridia, which are typical obligate anaerobes, have been studied from the viewpoint of their application in biodegradation and bioenergy fermentation processes and are known to be formidable pathogens. Although it has long been accepted that the growth of *Clostridium* species is severely impaired in aerobic environments (Holdeman *et al.*, 1977), previous studies have shown that some species can survive in aerobic environments with the aid of O_2/reactive oxygen species (ROS) detoxification systems. *C. acetobutylicum* is a representative of such species and it has been proposed that NAD(P)H-oxidation systems react with O_2, resulting in an imbalance in the redox potential and the eventual cessation of cell growth instead of cell death (O'Brien & Morris, 1971). In a subsequent study, NADH-dependent O_2-consuming activity was detected in crude extracts of *Clostridium* strains (Kawasaki *et al.*, 1998); furthermore, a H_2O-forming NADH oxidase from *C. aminovalericum* has recently been purified and found to be an O_2 scavenger (Kawasaki, Ishikura, Chiba *et al.*, 2004). Although analysis of the genome of *C. acetobutylicum* revealed that the gene encoding the H_2O-forming NADH oxidase is absent, a homologue of this gene with 17% identity, formerly characterized as NADH:rubredoxin oxidoreductase (NROR), has been identified (Nolling *et al.*, 2001; Kawasaki *et al.*, 2005; Guedon & Petitdemange, 2001). The NROR enzyme does not harbour a cysteine residue at the active site, suggesting the absence of NADH-dependent O_2-reductase activity. A previous study showed that the O_2-responsive enzymes of the *nror* operon, including flavoprotein A2 and desulfoferrodoxin, and the previously characterized O_2-responsive protein rubperoxin (Kawasaki, Ishikura, Watamura *et al.*, 2004; Kawasaki *et al.*, 2007) have O_2- or ROS-scavenging activity (Kawasaki *et al.*, 2009; Riebe *et al.*, 2009) and the kinetic data led to the proposal that *C. acetobutylicum* contains a multi-enzyme complex that can efficiently function to eliminate the toxicity of O_2 and ROS by using NROR as the chief electron donor (Kawasaki *et al.*, 2009).

In order to reveal the detailed mechanism of the O_2-scavenging system in *C. acetobutylicum* on the basis of its structure at atomic resolution, we initiated crystal structure analysis of the components of the system. In this paper, we report the results of crystallization and preliminary X-ray analysis of NROR from *C. acetobutylicum*.

2. Materials and methods

2.1. Cloning, expression and purification of NROR from *C. acetobutylicum*

The experiment was performed as described previously (Kawasaki *et al.*, 2009). In brief, the gene encoding NROR [The Institute for Genomic Research (TIGR) No. CAC2448] was amplified from the genomic DNA of *C. acetobutylicum* by PCR using the oligonucleotide primer pair 5′-ATG**CATCATCATCATCATCAT**AAAAGCACA-AAAATTTTAATC and 5′-CTATAAATTATTTAATATTGC; the sequence in bold encodes a six-histidine tag used for purification. No additional residues (*e.g.* a proteolytic cleavage site and linker) were included in the open reading frame apart from the six-histidine tag. The purified PCR products were ligated into the pET7Blue vector, resulting in the generation of a pNROR plasmid. *Escherichia coli* strain Tuner (DE3) (Novagen) was transformed with pNROR and cultured at 310 K in Luria–Bertani broth containing 50 µg ml^{-1} ampicillin. When the optical density of the culture reached 0.7 at 660 nm, protein synthesis was induced by the addition of 1 m*M* isopropyl β-D-1-thiogalactopyranoside and the culture was incubated for a further 3 h at 310 K. The induced cells were harvested from 10 l culture, washed once with buffer *A* (50 m*M* potassium phosphate buffer pH 7.0 containing 0.1 m*M* dithiothreitol) and then resuspended in 60 ml of the same buffer.

All purification procedures were performed at 277 K or on ice. Cell-free extracts were prepared by disruption of the cells in a French press (140 MPa) followed by centrifugation at 15 000*g* for 15 min. The supernatant was then applied onto Talon Metal Affinity Resin (Clontech) and subsequently washed with buffer *A* containing 50 m*M* imidazole; NROR was eluted with buffer *A* containing 150 m*M* imidazole. The buffer was changed to 50 m*M* potassium phosphate buffer pH 7.0 by repeating 100-fold concentration and dilution two times using an Amicon Ultra 3000 kDa cutoff concentrator (Millipore).

2.2. Crystallization

The preliminary step of crystallization involved transfer of the purified NROR protein to 20 m*M* Tris–HCl buffer pH 7.0, followed by concentration to 14 mg ml^{-1} using a Vivaspin 500 10 000 MWCO PES concentrator (GE Healthcare). This concentration was confirmed using the Bradford absorption method at 595 nm (Bradford, 1976). Crystallization trials were performed using the sitting-drop vapour-diffusion method at 293 K and initial screening of crystallization conditions was carried out using Wizard I and II (Emerald BioSystems) in CrystalClear D 96-well plates (Douglas Instruments). The protein and reservoir solutions were manually mixed in 1 µl drops and the drops were equilibrated against 100 µl reservoir solution. The optimized reservoir solution was composed of 35%(*v*/*v*) PEG 400, 0.1 *M* Tris–HCl pH 8.5 and 0.15 *M* $MgCl_2$.

2.3. Data collection and processing

X-ray diffraction experiments were performed using the BL26B1 and BL41XU beamlines at SPring-8 with the Beamline Scheduling Software (administration software for automatic operation; Ueno *et al.*, 2005, 2006). Data extending to 2.1 Å resolution were collected on BL41XU using an ADSC Quantum 315 detector with a crystal-to-detector distance of 260 mm, a wavelength of 1.0000 Å and 1° oscillation steps over a range of 180°.

Because the concentration of PEG 400 in the crystallization drops was sufficiently high for cryoprotection, the crystals were directly transferred to liquid N_2 for vitrification and mounted in a stream of N_2 gas at 90 K. All data were integrated and merged using the *HKL*-2000 program package (Otwinowski & Minor, 1997) and subsequent data processing was performed using the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994). The *Phaser* (Storoni *et al.*, 2004) and *MOLREP* (Murshudov *et al.*, 1997) programs were used for molecular replacement.

Table 1
Diffraction data-processing statistics.

Values in parentheses are for the highest resolution shell.

Wavelength (Å)	1.0000
Space group	$P4_122$ or $P4_322$
Unit-cell parameters (Å)	$a = b = 98.6$, $c = 88.3$
Resolution (Å)	49.29–2.10 (2.21–2.10)
Total No. of reflections	198423 (29020)
No. of unique reflections	25940 (3693)
Multiplicity	7.6 (7.9)
Completeness (%)	99.8 (100.0)
$R_{\rm merge}$† (%)	6.2 (39.8)
Mean $I/\sigma(I)$	21.4 (5.2)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$.

3. Results and discussion

Single crystals were obtained using Wizard I condition No. 25 [30%(*v*/*v*) PEG 400, 0.1 *M* Tris–HCl pH 8.5 and 0.2 *M* $MgCl_2$]. Crystals of suitable size for X-ray diffraction experiments, with dimensions of approximately 0.2 × 0.2 × 0.15 mm, were obtained using a micro/macro-seeding technique (Fig. 1). The yellowish colour of the crystals can be attributed to the presence of FAD in NROR, which has been identified in a previous study (Petitdemange *et al.*, 1979).

X-ray diffraction data for NROR were successfully collected to 2.1 Å resolution. The crystals were found to belong to space group $P4_122$ or $P4_322$, with unit-cell parameters $a = b = 98.6$, $c = 88.3$ Å. Under the assumption that the asymmetric unit contains one molecule, the Matthews coefficient was calculated to be 2.7 Å^3 Da^{-1} and the solvent content to be 54.1%. The statistics of data collection are shown in Table 1. Molecular replacement using the *Phaser* (Storoni *et al.*, 2004) and *MOLREP* (Vagin & Teplyakov, 1997) programs with Protein Data Bank entries 1q1r (putidaredoxin reductase from *Pseudomonas putida*; 20% sequence identity to NROR), 2v3a [NAD(P)H:rubredoxin reductase from *P. aeruginosa*] and 1xhc (NADH oxidase from *Pyrococcus furiosus*; 20% sequence identity to

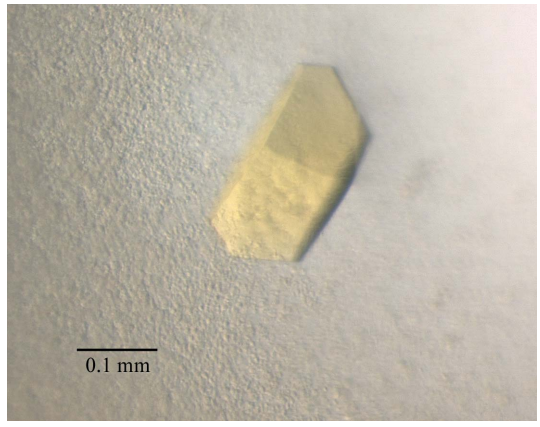

Figure 1
A yellow crystal of recombinant NROR with dimensions of 0.2 × 0.2 × 0.15 mm.

NROR) as search models yielded no solutions. The preparation of heavy-metal derivative crystals is currently under way.

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