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Crystallization and preliminary X-ray diffraction analysis of membrane-bound respiratory [NiFe] hydrogenase from *Hydrogenovibrio marinus*

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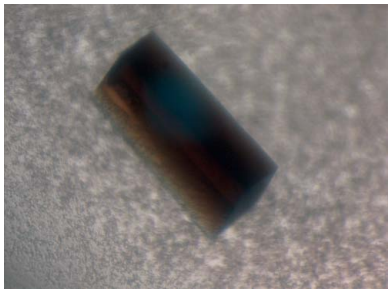

Membrane-bound respiratory [NiFe] hydrogenase is an H_2-uptake enzyme found in the periplasmic space of bacteria that plays a crucial role in energy-conservation processes. The heterodimeric unit of the enzyme from *Hydrogenovibrio marinus* was purified to homogeneity using chromatographic procedures. Crystals were grown using the sitting-drop vapour-diffusion method at room temperature. Preliminary crystallographic analysis revealed that the crystals belonged to space group $P2_1$, with unit-cell parameters a = 75.72, b = 116.59, c = 113.40 Å, β = 91.3°, indicating that two heterodimers were present in the asymmetric unit.

1. Introduction

Hydrogenases are essential enzymes in hydrogen metabolism in many prokaryotes and some eukaryotes, catalyzing the formation and decomposition of H_2 with the aid of redox partners (Vignais & Billoud, 2007). The enzymes are classified into three types based on the metal composition of the catalytic centre: [NiFe], [FeFe] and [Fe] hydrogenases. The [NiFe] hydrogenases are phylogenetically and functionally further divided into several groups. The representative member of the largest group (termed group 1) is 'standard' [NiFe] hydrogenase, which is mainly isolated from obligate anaerobes such as sulfate-reducing bacteria and is known to be an O_2-sensitive enzyme. The physiological function of group 1 [NiFe] hydrogenases is mostly related to H_2 uptake in the periplasmic space. The basic unit of the enzyme is composed of a large and a small subunit. The former accommodates an Ni–Fe cluster using four cysteinyl thiolates, two of which bridge Ni and Fe atoms and the other two of which coordinate the Ni atom as terminal ligands. In addition, a third bridging ligand, which is liberated upon reduction (Higuchi *et al.*, 1999; Higuchi & Yagi, 1999), is observed between Ni and Fe in the oxidized state (Higuchi *et al.*, 1997; Ogata *et al.*, 2009). Fe is also coordinated by two cyano ligands and one carbonyl ligand (Happe *et al.*, 1997). The small subunits of group 1 [NiFe] hydrogenases usually contain three Fe–S clusters that function as an electron-transfer unit (Fontecilla-Camps *et al.*, 2007). The Fe–S clusters are termed the proximal, medial and distal clusters, referring to their distances from the catalytic centre. Standard [NiFe] hydrogenases have [4Fe–4S]-type proximal and distal clusters and a [3Fe–4S]-type medial cluster. [NiFeSe] hydrogenases, which have a selenocysteinyl ligand for the Ni atom instead of a cysteine, also fall into the same group as the standard enzymes based on their functional and structural characteristics. In contrast to standard [NiFe] hydrogenases, all three Fe–S clusters of [NiFeSe] enzymes are of the [4Fe–4S] type. To date, seven crystal structures of [NiFe] hydrogenases in various redox states have been reported, all of which are members of group 1: four standard [NiFe] hydrogenases from sulfate-reducing *Desulfovibrio* species (Volbeda *et al.*, 1995; Higuchi *et al.*, 1997; Montet *et al.*, 1997; Matias *et al.*, 2001; Ogata *et al.*, 2002, 2005), two [NiFeSe] hydrogenases from sulfate reducers (Garcin *et al.*, 1999; Marques *et al.*, 2010) and the recently reported [NiFe] hydrogenase from the photosynthetic purple sulfur bacterium *Allochromatium vinosum* (Ogata *et al.*, 2010). Membrane-bound respiratory hydrogenases (MBHs) are members of the group 1 [NiFe]

hydrogenases. The heterodimeric catalytic unit associates with an intrinsic membrane protein, a *b*-type cytochrome subunit (Cyt*b*), and the inner membrane through a C-terminal transmembrane helix in the small subunit which is not found in other members of this group. The MBH–Cyt*b* complex couples the oxidation of H_2 in the periplasmic space with the reduction of quinone species in the inner membrane, taking up protons from the cytoplasmic side and resulting in the formation of a proton motive force across the membrane (Gross *et al.*, 2004). MBH is known to be an O_2-tolerant enzyme, although the determinant for O_2 tolerance is largely unknown (Vincent *et al.*, 2005; Luo *et al.*, 2009). An obligately lithoautotrophic and aerobic hydrogen-oxidizing bacterium (knallgas bacterium), *Hydrogenovibrio marinus*, has been isolated from the aerobic surface region of seawater (Nishihara *et al.*, 1989, 2005). Many knallgas bacteria are known to be microaerophiles, but this strain can grow rapidly without a lag period even in an atmosphere of 40% oxygen (Nishihara *et al.*, 1991) and the respiratory MBH is remarkably resistant to oxidative inactivation (Nishihara *et al.*, 1997; Yoon *et al.*, 2011). In order to provide an X-ray crystallographic insight into the O_2-tolerant enzymatic activity of MBH, the enzyme was purified from *H. marinus* and crystallized.

2. Materials and methods

2.1. Bacterial cultivation and purification of MBH

H. marinus MH-110 was grown lithoautotrophically under a continuous flow of a gas mixture (7:2:1 H_2:O_2:CO_2, 4 l min^{-1}, 750 rev min^{-1}) using a 10 l jar fermenter (Nishihara *et al.*, 1991). Purification of MBH was performed as described previously (Yoon *et al.*, 2011). In brief, cells were suspended in 20 m*M* potassium phosphate buffer pH 7.0 (buffer *A*) to give a concentration of 1 g wet cells per 5 ml. The cell suspension was disrupted twice (Sonifier 250; Branson Ultrasonics Corp., Connecticut, USA) at 50 W for 10 min, centrifuged at 7000*g* for 30 min at 277 K and the supernatant was further ultracentrifuged at 100 000*g* for 1 h at 277 K. The precipitated membrane was washed with buffer *A* containing 1 *M* ammonium sulfate, solubilized in the presence of 1% Triton X-100 for 1 h at 277 K under argon gas and then incubated at 333 K for 20 min. The solubilized membrane proteins were subjected to a Q-Sepharose high-performance column (2.6 × 10 cm; GE Healthcare UK Ltd, Buckinghamshire, England) that had been pre-equilibrated with 50 m*M* Bis-Tris buffer pH 7.0 containing 0.025% Triton X-100 and eluted using a linear gradient of 0–300 m*M* NaCl. The hydrogenase fraction was applied onto a hydroxyapatite column (1.6 × 10 cm; Bio-Rad Laboratories Inc.) pre-equilibrated with 1 m*M* potassium phosphate buffer pH 7.0 containing 0.025% Triton X-100, which was subsequently eluted with a linear gradient of 1–400 m*M* potassium phosphate in the same buffer. The obtained active fraction was concentrated (Amicon Ultra-15, 30 000 NMWL; Millipore Corp., Billerica, Massachusetts, USA) and subjected to Superdex 200 column chromatography (1.6 × 60 cm; GE Healthcare UK Ltd) using 10 m*M* potassium phosphate buffer pH 7.0 containing 0.1 *M* NaCl and 0.025% Triton X-100. Column chromatography was performed anaerobically at room temperature. H_2-oxidation activity was measured spectrophotometrically at 600 nm at 333 K using benzyl viologen as the electron acceptor.

2.2. Crystallization

Initial screening of crystallization conditions was carried out at 293 K by the sitting-drop vapour-diffusion method using 96-well CrystalClear Strips (Douglas Instruments, UK) and JBScreen, Wizard I and II (Jena Bioscience GmbH, Germany). Crystallization using the optimized condition was performed by the sitting-drop vapour-diffusion method at room temperature in 24-well Cryschem plates (Hampton Research, USA); the drop was prepared by mixing 2 µl protein solution consisting of 27.5 mg ml^{-1} MBH, 10 m*M* Tris–HCl pH 7.4 and 2 m*M* DTT with 2 µl reservoir solution consisting of 100 m*M* PIPES pH 6.5, 15% PEG 3350, 300 m*M* Li_2SO_4 and 5 m*M* DTT.

2.3. Data collection and processing

The fresh crystals were cryopreserved in a solution consisting of 100 m*M* PIPES pH 6.5, 20% PEG 3350, 300 m*M* Li_2SO_4, 20% glycerol and 5 m*M* DTT. The crystals were mounted on LithoLoops (Molecular Dimensions, USA) and subsequently cooled using liquid nitrogen. X-ray diffraction data were collected on beamlines BL41XU and BL44XU at SPring-8 (Hyogo, Japan) using *Beamline Schedule Software* (*BSS*; Ueno *et al.*, 2005). The crystals were maintained at

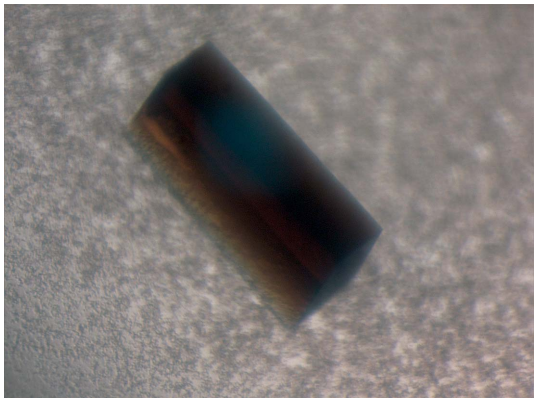

Figure 1
A dark brown crystal of MBH with dimensions of 0.1 × 0.1 × 0.3 mm.

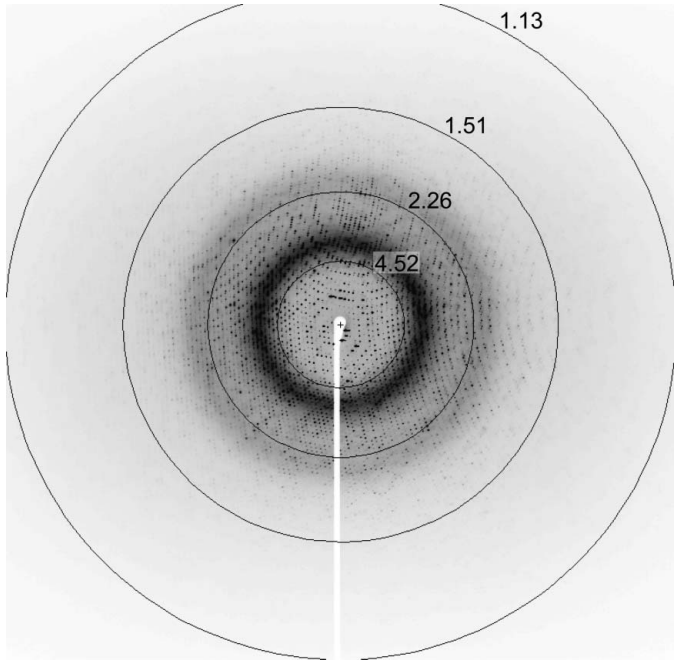

Figure 2
Diffraction image of the MBH crystal collected on beamline BL44XU at SPring-8. The resolution limits of the circles are labelled in Å.

Table 1
Diffraction data-processing statistics.

Values in parentheses are for the highest resolution shell.

Wavelength (Å)	0.9000
Space group	$P2_1$
Unit-cell parameters (Å, °)	a = 75.72, b = 116.59, c = 113.40, β = 91.3
Mosaicity (°)	0.38–0.58
Resolution (Å)	20.00–1.25 (1.27–1.25)
Total No. of reflections	2034450
No. of unique reflections	538698 (26924)
Multiplicity	3.8 (3.3)
Completeness (%)	99.8 (99.9)
$R_{\rm merge}$† (%)	7.2 (49.0)
Mean $I/\sigma(I)$	14.0 (3.2)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the scaled observed intensity of the ith symmetry-related observation for reflection hkl and $\langle I(hkl)\rangle$ is the average intensity.

90 K using a gaseous nitrogen stream during data collection. The diffraction data were integrated and scaled with the *HKL*-2000 program suite (Otwinowski & Minor, 1997).

3. Results and discussion

The specific activity of the purified protein used for crystallization was 265 U mg^{-1}. A fraction of the small subunit of the purified protein lacked the C-terminal transmembrane region and it is speculated that this region was cleaved off after dissociation from Cyt*b* or that the cleavage of this region caused the dissociation of Cyt*b*. Elimination of Triton X-100 from the protein solution prior to crystallization setup significantly improved the size and quality of the crystals. SDS–PAGE analyses revealed that the small subunits in the crystals did not contain the C-terminal region (data not shown). For X-ray diffraction experiments, dark brown crystals of suitable size (approximate dimensions 0.1 × 0.1 × 0.3 mm) were obtained from the optimized condition within 2 d (Fig. 1). X-ray diffraction data for MBH were successfully collected to 1.25 Å resolution (Fig. 2). The crystals belonged to space group $P2_1$, with unit-cell parameters a = 75.72, b = 116.59, c = 113.40 Å, β = 91.3°. Under the assumption that the asymmetric unit contained two heterodimers, the Matthews coefficient was calculated to be 2.6 Å^3 Da^{-1} with a solvent content of 52.0%. The statistics of data collection are shown in Table 1. As expected, molecular replacement using the *MOLREP* program (Murshudov *et al.*, 2011) with the atomic coordinates of the enzyme from *D. vulgaris* MF (PDB entry 1wuj; Ogata *et al.*, 2005) as a search model yielded two unique solutions. Model building and refinement is now in progress.

The synchrotron-radiation experiments were performed on BL41XU (proposal No. 2010A1223) and BL44XU (Proposal No. 2010A/B6520) with the approval of JASRI. The MX225-HE CCD detector (Rayonix) at BL44XU was financially supported by Academia Sinica and the National Synchrotron Radiation Research Center (Taiwan). This work was supported by Grant-in-Aids for Scientific Research from MEXT (20051022 and 22770111 to YS, 18GS0207, 22370061 and 22657031 to YH), a Grant-in-Aid for Scientific Research from JSPS (20580094 to HN), a Grant-in-Aid for Research and Education from the University of Hyogo (YS) and a Grant-in-Aid for Young Scientists from Hyogo Science and Technology Association (YS). This work was also partially supported by the GCOE Program (YH), the Japanese Aerospace Exploration Agency Project (YH) and the Basic Research Programs CREST-type 'Development of the Foundation for Nano-Interface Technology' from JST, Japan (YH).

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