Crystallization and preliminary X-ray analysis of the
NAD⁺-reducing [NiFe] hydrogenase from
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NAD⁺-reducing [NiFe] hydrogenases catalyze the oxidoreduction of dihydrogen concomitant with the interconversion of NAD⁺ and NADH. Here, the isolation, purification and crystallization of the NAD⁺-reducing [NiFe] hydrogenase from *Hydrogenophilus thermoluteolus* TH-1 are reported. Crystals of the NAD⁺-reducing [NiFe] hydrogenase were obtained within one week from a solution containing polyethylene glycol using the sitting-drop vapour-diffusion method and micro-seeding. The crystal diffracted to 2.58 Å resolution and belonged to space group C2, with unit-cell parameters $a = 131.43$, $b = 189.71$, $c = 124.59$ Å, $\beta = 109.42^\circ$. Assuming the presence of two NAD⁺-reducing [NiFe] hydrogenase molecules in the asymmetric unit, V_M was calculated to be $2.2 \text{ Å}^3 \text{ Da}^{-1}$, which corresponds to a solvent content of 43%. Initial phases were determined by the single-wavelength anomalous dispersion method using the anomalous signal from the Fe atoms.

1. Introduction

Hydrogenases catalyze the reversible oxidation of dihydrogen into two protons and electrons. They play a key role in the H₂ metabolism of various microorganisms, providing reducing power for chemical reactions in the cells and also contributing to the disposal of surplus electrons as dihydrogen. Hydrogenases are metalloenzymes and are classified into three phylogenetically unrelated types, [Fe], [FeFe] and [NiFe] hydrogenases, according to the metal content of the active site. [NiFe] hydrogenases can be divided into four or five groups, and some of the groups have been further sorted into subgroups (Vignais & Billoud, 2007). Two structures of the group 1 standard [NiFe] hydrogenase from sulfate-reducing bacteria were determined using X-ray crystallography in the 1990s (Volbeda *et al.*, 1995; Higuchi *et al.*, 1997). The physicochemical properties and catalytic mechanism of the standard [NiFe] hydrogenases have been discussed in some recent reviews (Shomura & Higuchi, 2013; Yagi *et al.*, 2014; Lubitz *et al.*, 2014).

Cytoplasmic heteromultimeric NAD⁺-reducing [NiFe] hydrogenase was first found in *Ralstonia eutropha* H16 (Schneider & Schlegel, 1976). It is a group 3 (subgroup d) [NiFe] hydrogenase, and has been found in several bacteria. It catalyzes bidirectional oxidoreduction of hydrogen concomitant with interconversion between NAD⁺ and NADH. The NAD⁺-reducing [NiFe] hydrogenase from *Hydrogenophilus thermoluteolus* TH-1 (HtTH-1), a thermophilic H₂-oxidizing bacterium (Goto *et al.*, 1977), is composed of two heterodimeric catalytic subcomplexes, HoxHY (hydrogenase unit) and HoxFU (diaphorase unit), and is known to be a minimum essential protein complex of this type of enzyme. HoxH and HoxY correspond to the large and small subunits of the group 1 standard [NiFe] hydrogenases, respectively. HoxH has four conserved cysteine residues that provide thiol ligands to the Ni–Fe active site, while the C-terminal region found in the small subunit of the standard [NiFe] hydrogenases is truncated in HoxY, suggesting that there is only one Fe–S cluster, which is most proximal to the Ni–Fe active site in the large subunit. Furthermore, in addition to a flavin mononucleotide (FMN) firmly attached to the diaphorase (HoxFU) subcomplex, another FMN, which is loosely bound to HoxY and essential for

NAD⁺ reduction by H₂, has been detected in the NAD⁺-reducing [NiFe] hydrogenase from *R. eutropha* H16 (Van der Linden *et al.*, 2004).

A recent spectroscopic study showed that the structure of the Ni-Fe active site of the NAD⁺-reducing [NiFe] hydrogenases is similar to that of the standard [NiFe] hydrogenases (Horch *et al.*, 2010). That is, Ni is coordinated by four S atoms of cysteine residues, with two of them also coordinated to Fe, thus making a bridge between the two metals. Fe has three additional diatomic ligands: two CN ligands and one CO ligand. Some [NiFe] hydrogenases, including the NAD⁺-reducing [NiFe] hydrogenases, have been reported to be oxygen-tolerant (Burgdorf *et al.*, 2005; Vincent *et al.*, 2005; Lukey *et al.*, 2010; Pandelia *et al.*, 2010). It has recently been proposed that the O₂ tolerance of the group 1 membrane-bound [NiFe] hydrogenases is owing to the flexible [4Fe-3S]-6Cys-type cluster most proximal to the Ni-Fe active site (Shomura *et al.*, 2011; Fritsch *et al.*, 2011). Although the possible O₂ tolerance of the NAD⁺-reducing [NiFe] hydrogenase from *R. eutropha* H16 has been proposed to be owing to a slow rate of association of O₂ with the Ni-Fe active site (Lauterbach *et al.*, 2011), the real mechanism has been the subject of debate.

Sequence analyses showed that NAD⁺-reducing [NiFe] hydrogenases are very similar to some of the peripheral subunits of respiratory complex I (NADH:quinone oxidoreductase; Böhm *et al.*, 1990). HoxF (N-terminal portion), HoxF (C-terminal portion), HoxU, HoxY and HoxH correspond to Nqo2, Nqo1, Nqo3, Nqo4 and Nqo6, respectively, of complex I from *Thermus thermophilus* HB8 (Albracht, 1993; Sazanov & Hinchliffe, 2006). Recently, three-dimensional structures of complex I from *T. thermophilus* HB-8 have been determined (Sazanov & Hinchliffe, 2006; Berrisford & Sazanov, 2009; Baradaran *et al.*, 2013). Structural comparison of the subunit folding and the spatial arrangement of the cofactors (*i.e.* Fe-S clusters, NAD⁺ and FMNs) between the NAD⁺-reducing [NiFe] hydrogenase and complex I is of great interest to elucidate the evolution of the catalytic building blocks of the energy metabolic machineries in the cells.

In this paper, we report the purification, crystallization and preliminary X-ray structure analysis of the NAD⁺-reducing [NiFe] hydrogenase from HtTH-1.

2. Materials and methods

2.1. Cultivation of HtTH-1 and purification of NAD⁺-reducing [NiFe] hydrogenase

HtTH-1 has been reported to be a facultatively chemolitho-autotrophic hydrogen-oxidizing bacterium in the β -subclass of the Proteobacteria (Goto *et al.*, 1977; Hayashi *et al.*, 1999). Its NAD⁺-reducing [NiFe] hydrogenase was thought to be expressed when it is cultivated autotrophically with hydrogen, carbon dioxide and oxygen. We found, however, that the efficiency of the expression of the NAD⁺-reducing [NiFe] hydrogenase in heterotrophically grown cells is similar to that in autotrophically cultivated cells by screening the cultivation conditions.

HtTH-1 was grown under aerobic conditions in 2×YT medium at pH 6.8 at 323 K for 20 h in a 30 l jar fermentor (MSJ-U3; B. E. Marubishi Co. Ltd). The obtained cells (200 g) were suspended in 25 mM Tris-HCl buffer pH 7.4 and then disrupted by ultrasonication (SUW150D; Suzuki Co. Ltd). The lysate was ultracentrifuged (150 000g) for 60 min at 277 K. The hydrogenase in the supernatant fraction was separated by 30% (w/v) ammonium sulfate fractionation and precipitated by centrifugation at 20 000g for 30 min at 277 K. The pellet was dissolved in 25 mM Tris-HCl buffer pH 7.4 and then

dialyzed against the same buffer overnight. The dialyzed fraction was loaded onto TOYOPEARL DEAE-650S (50 mm × 10 cm, TOSOH) equilibrated with 25 mM Tris-HCl buffer pH 7.4. The hydrogenase fraction was eluted using a linear gradient of 0–400 mM NaCl (six column volumes) and a flow rate of 4.0 ml min⁻¹. The obtained fraction was diluted with 20 mM bis-tris buffer pH 6.5 and then loaded onto a HiTrap Q HP column (2 × 5 ml; GE Healthcare) equilibrated with the same buffer. The hydrogenase was eluted with a linear gradient of 20–200 mM bis-tris buffer pH 5.0 (15 column volumes). The fractions with NAD⁺-reducing [NiFe] hydrogenase activity were pooled and concentrated using an Amicon Ultra-15 50000 NMWL (Merck Millipore) and then further purified by gel-filtration chromatography on a HiLoad Superdex 200 pg 16/60 column (GE Healthcare) equilibrated with 25 mM Tris-HCl buffer pH 7.4 containing 150 mM NaCl. The normal H₂-uptake activity was assayed by measuring the reducing rate of 3 mM benzyl viologen with H₂ in each purification step, and the NAD⁺-reducing activity was estimated by using a spectrophotometer at 323 K to measure the increase in the absorption at 340 nm of the final purified sample owing to the reduction of NAD⁺ to NADH with H₂. One unit of NAD⁺-reducing activity of the [NiFe] hydrogenase is defined as the amount of the enzyme needed to catalyze the reduction of 1 μ mol NAD⁺ per minute. Protein concentration was determined by the method of Bradford (Bio-Rad) and its purity was checked by native PAGE and SDS-PAGE.

2.2. Crystallization

The purified NAD⁺-reducing [NiFe] hydrogenase was concentrated to 15 mg ml⁻¹ by centrifugation using an Amicon Ultra-4 50000 NMWL (Merck Millipore). Initial screening for crystallization conditions was carried out at 277 K by the sitting-drop vapour-diffusion method using 2 μ l drops (1:1 protein:well solution ratio) with Crystal Screen, Crystal Screen 2, PEG/Ion, PEG/Ion 2 and PEGRx1 from Hampton Research and Cryo I, Cryo II, Wizard I, Wizard II, Wizard III and Wizard IV from Rigaku. Tiny crystals, with a size of about 5 μ m, were obtained from a solution containing polyethylene glycol, magnesium chloride and Tris-HCl after four months. To improve the quality of the crystals, the concentration of polyethylene glycol, the pH range, the additives and the number of the seeds introduced into the crystallization droplets were optimized.

2.3. Data collection and processing

Crystals of the NAD⁺-reducing [NiFe] hydrogenase were soaked in a cryoprotectant solution consisting of 0.1 M Tris-HCl buffer pH 8.5, 35% PEG 3350, 0.2 M magnesium chloride, 20 mM NAD⁺, flash-cooled directly in liquid nitrogen and stored until use, when they were transferred into a nitrogen cryostream (100 K) for data collection. A single-wavelength anomalous dispersion (SAD) data set was collected near the absorption edge of Fe ($\lambda = 1.7300$ Å) on the BL44XU beamline at SPring-8, Japan using an MX225-HE detector (Rayonix). The crystal-to-detector distance, oscillation angle, exposure time for each film and total number of frames were 160 mm, 1.0°, 20 s and 180, respectively. A diffraction data set at an energy level remote from the absorption edge ($\lambda = 0.9000$ Å) was collected on the BL44XU beamline at SPring-8 using an MX225-HE detector (Rayonix). The crystal-to-detector distance, oscillation angle, exposure time for each film and total number of frames were 250 mm, 1.0°, 6.0 s and 162, respectively. The diffraction data sets were processed using XDS (Kabsch, 2010) and SCALA (Evans, 2006). The data-collection statistics are listed in Table 1.

3. Results and discussion

A total of 2 mg of NAD⁺-reducing [NiFe] hydrogenase with a specific activity of 200 U mg⁻¹ was purified from 200 g of wet cells. Light brown crystals (0.1 × 0.05 × 0.02 mm in size) of the NAD⁺-reducing [NiFe] hydrogenase were obtained after one week in 0.1 M Tris–HCl buffer pH 8.5 containing 10% PEG 3350 and 0.2 M magnesium chloride with 20 mM NAD⁺ as an additive using micro-seeding at 277 K (Fig. 1). The crystal diffracted to 2.58 Å resolution and belonged to space group C2, with unit-cell parameters $a = 131.43$, $b = 189.71$, $c = 124.59$ Å, $\beta = 109.42^\circ$ (Fig. 2 and Table 1). By assuming the presence of two molecules in the asymmetric unit, the Matthews coefficient was calculated to be 2.2 Å³ Da⁻¹. Initial phases were determined by the SAD method using the anomalous signal from the

Figure 1
Crystal of the NAD⁺-reducing [NiFe] hydrogenase from *H. thermoluteolus* TH-1 (scale bar: 0.1 mm).

Figure 2
Diffraction image of an NAD⁺-reducing [NiFe] hydrogenase crystal obtained on BL44XU at SPring-8. The 2.58 Å resolution limit is shown by a circle. The crystal-to-detector distance, oscillation angle and exposure time were 250 mm, 1.0° and 6.0 s, respectively.

Table 1

Data collection and processing.

Values in parentheses are for the outer shell.

Data set	Fe absorption edge	Remote
Diffraction source	BL44XU	BL44XU
Wavelength (Å)	0.9000	1.7300
Temperature (K)	100	100
Detector	MX225-HE	MX225-HE
Crystal-to-detector distance (mm)	250	160
Rotation range per image (°)	1.0	1.0
Total rotation range (°)	162	180
Exposure time per image (s)	6	20
Space group	C2	C2
a, b, c (Å)	131.43, 189.71, 124.59	131.65, 190.22, 124.83
α, β, γ (°)	90.00, 109.42, 90.00	90.00, 109.48, 90.00
Mosaicity (°)	0.19	0.19
Resolution range (Å)	19.83–2.58 (2.72–2.58)	20.00–3.20 (3.37–3.20)
Total No. of reflections	311380 (45957)	180324 (26119)
No. of unique reflections	89715 (13139)	47442 (6934)
Completeness (%)	99.4 (99.9)	99.5 (99.9)
Multiplicity	3.5 (3.5)	3.8 (3.8)
$\langle I/\sigma(I) \rangle$	12.8 (2.3)	13.5 (2.5)
R_{meas}	0.099 (0.664)	0.119 (0.614)
Overall B factor from Wilson plot (Å ²)	44.0	58.0

Fe atoms. One NAD⁺-reducing [NiFe] hydrogenase molecule contains four [4Fe–4S] clusters, one [2Fe–2S] cluster and one Ni–Fe active site. 29 of 38 Fe sites in an asymmetric unit were successfully assigned and these peaks were refined using *PHENIX* (Adams *et al.*, 2010). Model building and structure refinement of the NAD⁺-reducing [NiFe] hydrogenase is now in progress.

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References

- Adams, P. D. *et al.* (2010). *Acta Cryst.* **D66**, 213–221.
- Albracht, S. P. J. (1993). *Biochim. Biophys. Acta*, **1144**, 221–224.
- Baradaran, R., Berrisford, J. M., Minhas, G. S. & Sazanov, L. A. (2013). *Nature (London)*, **494**, 443–448.
- Berrisford, J. M. & Sazanov, L. A. (2009). *J. Biol. Chem.* **284**, 29773–29783.
- Böhm, R., Sauter, M. & Böck, A. (1990). *Mol. Microbiol.* **4**, 231–243.
- Burgdorf, T., van der Linden, E., Bernhard, M., Yin, Q. Y., Back, J. W., Hartog, A. F., Muijsers, A. O., de Koster, C. G., Albracht, S. P. J. & Friedrich, B. (2005). *J. Bacteriol.* **187**, 3122–3132.
- Evans, P. (2006). *Acta Cryst.* **D62**, 72–82.
- Fritsch, J., Scheerer, P., Frielingsdorf, S., Kroschinsky, S., Friedrich, B., Lenz, O. & Spahn, C. M. (2011). *Nature (London)*, **479**, 249–252.
- Goto, E., Kodama, T. & Minoda, Y. (1977). *Agric. Biol. Chem.* **41**, 685–690.
- Hayashi, N. R., Ishida, T., Yokota, A., Kodama, T. & Igarashi, Y. (1999). *Int. J. Syst. Bacteriol.* **49**, 783–786.
- Higuchi, Y., Yagi, T. & Yasuoka, N. (1997). *Structure*, **5**, 1671–1680.
- Horch, M., Lauterbach, L., Saggi, M., Hildebrandt, P., Lendzian, F., Bittl, R., Lenz, O. & Zebger, I. (2010). *Angew. Chem. Int. Ed.* **49**, 8026–8029.
- Kabsch, W. (2010). *Acta Cryst.* **D66**, 125–132.
- Lauterbach, L., Liu, J., Horch, M., Hummel, P., Schwarze, A., Haumann, M., Vincent, K. A., Lenz, O. & Zebger, I. (2011). *Eur. J. Inorg. Chem.* **7**, 1067–1079.
- Linden, E. van der, Faber, B. W., Bleijlevens, B., Burgdorf, T., Bernhard, M., Friedrich, B. & Albracht, S. P. J. (2004). *Eur. J. Biochem.* **271**, 801–808.

- Lubitz, W., Ogata, H., Rüdiger, O. & Reijerse, E. (2014). *Chem. Rev.* **114**, 4081–4148.
- Lukey, M. J., Parkin, A., Roessler, M. M., Murphy, B. J., Harmer, J., Palmer, T., Sargent, F. & Armstrong, F. A. (2010). *J. Biol. Chem.* **285**, 3928–3938.
- Pandelia, M. E., Fourmond, V., Tron-Infossi, P., Lojou, E., Bertrand, P., Léger, C., Giudici-Orticoni, M. T. & Lubitz, W. (2010). *J. Am. Chem. Soc.* **132**, 6991–7004.
- Sazanov, L. A. & Hinchliffe, P. (2006). *Science*, **311**, 1430–1436.
- Schneider, K. & Schlegel, H. G. (1976). *Biochim. Biophys. Acta*, **452**, 66–80.
- Shomura, Y. & Higuchi, Y. (2013). *Rev. Inorg. Chem.* **33**, 173–192.
- Shomura, Y., Yoon, K.-S., Nishihara, H. & Higuchi, Y. (2011). *Nature (London)*, **479**, 253–256.
- Vignais, P. M. & Billoud, B. (2007). *Chem. Rev.* **107**, 4206–4272.
- Vincent, K. A., Cracknell, J. A., Lenz, O., Zebger, I., Friedrich, B. & Armstrong, F. A. (2005). *Proc. Natl Acad. Sci. USA*, **102**, 16951–16954.
- Volbeda, A., Charon, M. H., Piras, C., Hatchikian, E. C., Frey, M. & Fontecilla-Camps, J. C. (1995). *Nature (London)*, **373**, 580–587.
- Yagi, T., Ogo, S. & Higuchi, Y. (2014). *Int. J. Hydrogen Energy*, **39**, 18543–18550.