Improved purification, crystallization and crystallographic study of Hyd-2-type [NiFe]-hydrogenase from *Citrobacter* sp. S-77

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The purification procedure of Hyd-2-type [NiFe]-hydrogenase from *Citrobacter* sp. S-77 was improved by applying treatment with trypsin before chromatography. Purified protein samples both with and without trypsin treatment were successfully crystallized using the sitting-drop vapour-diffusion method with polyethylene glycol as a precipitant. Both crystals belonged to space group *P*2₁, with unit-cell parameters $a = 63.90$, $b = 118.89$, $c = 96.70$ Å, $\beta = 100.61^\circ$ for the protein subjected to trypsin treatment and $a = 65.38$, $b = 121.45$, $c = 98.63$ Å, $\beta = 102.29^\circ$ for the sample not treated with trypsin. The crystal obtained from the trypsin-treated protein diffracted to 1.60 Å resolution, which is considerably better than the 2.00 Å resolution obtained without trypsin treatment. The [NiFe]-hydrogenase from *Citrobacter* sp. S-77 retained catalytic activity with some amount of O₂, indicating that it has clear O₂ tolerance.

1. Introduction

Hydrogenases play an important role in the energy metabolism of the cells of a broad range of microorganisms. Recently, hydrogenases have received much attention owing to their potential use in enzyme-based hydrogen fuel cells, as dihydrogen is a strong candidate for next-generation clean energy sources. Hydrogenases catalyze not only the reversible oxidation of H₂ but also hydrogen–deuterium exchange and spin–isomer conversion reactions (Vignais & Colbeau, 2004; Vignais & Billoud, 2007). Hydrogenases are classified into three phylogenetically unrelated types according to the metal-ion content of their active site: nickel–iron hydrogenases ([NiFe]-hydrogenases), di-iron hydrogenases ([FeFe]-hydrogenases) and mono-iron hydrogenases ([Fe]-hydrogenases). Of these three types, [NiFe]-hydrogenases are the most abundant and are often more active in H₂ oxidation (Lubitz *et al.*, 2014). [NiFe]-Hydrogenases are further classified into at least four groups (groups 1–4) and some subgroups (Vignais & Billoud, 2007). One crucial shortcoming in the applications of hydrogenases is their sensitivity to O₂. The ‘standard’ [NiFe]-hydrogenases in group 1, which are generally composed of two subunits with an Ni–Fe active site in the large subunit and three iron–sulfur clusters in the small subunit, are reversibly inactivated by O₂ oxidation. They do not display catalytic activity in the presence of even a trace amount of O₂ (Lubitz

Table 1

Purification of Hyd-2-type [NiFe]-hydrogenase from *Citrobacter* sp. S-77.

The starting bacterial cell pellet was 65.0 g for purification procedures both with and without trypsin treatment.

Purification step	Activity (U)		Protein (mg)		Specific activity (U mg ⁻¹)		Recovery (%)		Purity (fold)	
	+	–	+	–	+	–	+	–	+	–
Trypsin treatment (0.1 mg ml ⁻¹)										
Membrane preparation	6420	7254	22630	48109	0.28	0.15	100	100	1	1
Solubilization	6040	4903	678	2583	8.91	1.90	94.1	67.6	31.8	12.7
DEAE FF	5612	3259	59.9	384	93.7	8.49	87.4	44.9	335	56.6
HiTrap Q HP	3403	2195	21.8	90.0	156	24.4	53.0	30.3	557	163
HiTrap Phenyl HP	3114	915	5.37	3.92	580	233	48.5	12.6	2071	1553

et al., 2014). Some membrane-bound [NiFe]-hydrogenases (MBHs), however, have been found to be tolerant to O₂ and exhibit catalytic activity at ambient O₂ concentrations (Cracknell *et al.*, 2009; Pandelia *et al.*, 2011). Crystal structures of O₂-tolerant MBHs revealed that they do not have a normal [4Fe–4S]–4Cys cluster but have a unique [4Fe–3S]–6Cys cluster at the proximal position to the active site (Shomura *et al.*, 2011; Fritsch *et al.*, 2011). In contrast to the standard [NiFe]-hydrogenases, two extra cysteine residues in the small subunit enable it to coordinate the unique [4Fe–3S] cluster. Based on electrochemical and spectroscopic data (Goris *et al.*, 2011; Pandelia *et al.*, 2011), it has been proposed that the mechanism underlying the O₂ tolerance of MBH involves the ability of the [4Fe–3S]–6Cys cluster to donate additional electrons to the active site, which is synchronized with a change in its conformation, when the enzyme is inactivated by O₂ (Shomura *et al.*, 2011).

The [NiFe]-hydrogenase isolated from *Citrobacter* sp. S-77 (CS-77) has been reported to be a heterodimeric hydrogenase (Eguchi *et al.*, 2012). As pointed out by Matsumoto *et al.* (2014), pairwise sequence alignments of the heterodimeric part of Hyd-1 (MBH-col) and Hyd-2 (HYD2-col) from *Escherichia coli*, MBH from *Hydrogenovibrio marinus* (MBH-mar) and the standard [NiFe]-hydrogenase from *Desulfovibrio vulgaris* Miyazaki F (STD-dvm) with the enzyme from CS-77 showed 40, 96, 41 and 43% sequence identity, respectively, indicating that the heterodimeric hydrogenase from CS-77 in this report is the hydrogenase unit of an Hyd-2-type [NiFe]-hydrogenase (referred to in the following as HYD2-s77). This analysis suggests that purified HYD2-s77 has lost the two membrane-bound subunits, namely the *b*-type cytochrome and the iron–sulfur protein, during the purification procedure, as has often been reported for MBHs. Hyd-2-type enzymes have been reported to be sensitive to O₂ and can be reactivated with H₂ after inactivation by O₂ like standard enzymes (Lukey *et al.*, 2010). Despite high sequence similarity to other Hyd-2-type enzymes, biochemical studies of HYD2-s77 showed high oxygen stability and a strong specific activity of 661 U mg⁻¹ (Eguchi *et al.*, 2012) for H₂ oxidation at pH 7, which is comparable to the value of 566 U mg⁻¹ for STD-dvm (Yagi *et al.*, 1978). Furthermore, HYD2-s77 has a 637-fold higher mass activity than Pt at 50 mV in a hydrogen half-cell (Matsumoto *et al.*, 2014). These data show that HYD2-s77 offers many advantages as an efficient H₂-activating biocatalyst for future hydrogenase technologies.

Here, we report an improved purification of the heterodimeric hydrogenase unit (HYD2-s77) of the Hyd-2-type [NiFe]-hydrogenase from CS-77 using trypsin treatment, as well as its crystallization and preliminary X-ray diffraction analysis and new enzymatic data that show its possible O₂ tolerance. Since no structural information on Hyd-2-type [NiFe]-hydrogenases has yet been reported, the crystal structure of HYD2-s77 will provide a more detailed structural comparison with the other types of [NiFe]-hydrogenases and structural evidence for its high specific activity and possible O₂ tolerance, even though it is an Hyd-2-type enzyme.

2. Materials and methods

2.1. Purification and assay of oxygen tolerance of the enzyme

Cultivation and disruption of the bacterial cells used, and extraction of HYD2-s77 from the pellet, were carried out according to a previously described protocol (Eguchi *et al.*, 2012). The series of purification procedures was improved in order to achieve high yields of the enzyme with a high specific activity. For each cycle of the purification experiment, 65.0 g of harvested bacterial wet cells were used. The extracted membrane-protein fractions with 1.0% Triton X-100 in the ultracentrifuged supernatant were treated with trypsin (final concentration 0.1 mg ml⁻¹) for 1 h at 310 K and then loaded onto the first purification column. The protein sample prepared without trypsin treatment was also purified and crystallized and was compared with that obtained with trypsin treatment.

All purification procedures described below were carried out at room temperature under strictly anaerobic conditions in an anaerobic chamber (Model B, Coy Laboratory Products, Grass Lake, Michigan, USA; gas phase 98% N₂ and 2% H₂). All buffer solutions for chromatographic procedures were degassed before chromatography, and all chromatography columns were equilibrated with a degassed buffer solution prior to use. Liquid chromatography was performed using an ÄKTAprius Plus (GE Healthcare, Little Chalfont, England).

Solubilized membrane proteins were loaded onto an initial DEAE Sepharose FF anion-exchange column (2.6 × 12 cm in an XK26/20 column, GE Healthcare) equilibrated with 10 mM MOPS pH 7.0, 0.03% Triton X-100, 1.0 mM DTT. The hydrogenase was eluted at approximately 250 mM NaCl from a linear concentration gradient of NaCl (0–0.5 M in four

column volumes) in the same buffer solution (flow rate of 5.0 ml min^{-1}). The fractions that showed significant activity were collected and pooled for the next step. The pooled fractions were further purified by using a second application of anion-exchange chromatography with a HiTrap Q HP column (5 ml; GE Healthcare) equilibrated with 10 mM MOPS pH 7.0, 1.0 mM DTT without detergent. The hydrogenase eluted at approximately 200 mM NaCl using a linear concentration gradient of NaCl (0–0.4 M in 20 column volumes) in the same buffer solution (flow rate of 4.0 ml min^{-1}). The final step used here was hydrophobic chromatography. The pooled hydrogenase fractions were merged with the same volume of 10 mM MOPS pH 7.0, 1.0 mM DTT containing 0.4 M ammonium sulfate and applied onto a HiTrap Phenyl HP column (5 ml; GE Healthcare). The column was pre-equilibrated with 0.2 M ammonium sulfate in 10 mM MOPS pH 7.0, 1.0 mM DTT. The protein eluted at approximately 80 mM ammonium sulfate using a linear concentration gradient of ammonium sulfate (0.2–0 M in ten column volumes). Fractions containing the target hydrogenase were combined and dialyzed against

10 mM MOPS pH 7.0, 0.2 M NaCl. Dialyzed protein solution was concentrated to 30 mg ml^{-1} using a Vivaspinn concentrator (5000 Da molecular-weight cutoff; GE Healthcare). The protein samples were prepared according to the methods reported previously for MBH-mar (Yoon *et al.*, 2011) and STD-dvm (Higuchi *et al.*, 1987). The results of the purification steps are summarized in Table 1.

After running native PAGE, the polyacrylamide gel (8%) was soaked in 50 ml 20 mM MOPS pH 7.0 containing 1 mM benzyl viologen (1,1'-dibenzyl-4,4'-bipyridinium dichloride; Sigma–Aldrich, Seelze, Germany), 1 mM 2,3,5-triphenyl-2H-tetrazolium chloride (TTC; BD, Sparks, Maryland, USA) and incubated at 303 K with H_2 bubbling (50 ml min^{-1} , 0.1 MPa) for 10 min. The migrated active hydrogenase in the gel displayed a red band caused by the reduction of TTC to 1,3,5-triphenylformazan (TPF) owing to the H_2 -dependent catalytic reaction of the enzyme. The hydrogenase activity in each fraction of the chromatography was checked by native PAGE activity staining and active fractions were pooled and applied onto the next column. The quantitative enzyme assay for each

Figure 1
The effect of O_2 on the H_2 -consumption activity of the purified enzymes from (a) *H. marinus* (MBH-mar), (b) *D. vulgaris* Miyazaki F (STD-dvm) and (c) *Citrobacter* sp. S-77 (HYD2-s77). The inset in each figure shows a magnification of the absorbance data with various amounts of O_2 . The O_2 tolerance of all hydrogenases was estimated by measuring the time taken for revival of the activity in buffer solutions with triphenyltetrazolium chloride and a certain amount of H_2 and O_2 . The O_2 -tolerance indexes in the presence of 4.2, 8.4, 12.6, 31.5 and 63.0 nmol O_2 were calculated to be 120.0, 98.4, 93.8, 14.4 and 8.30 for MBH-mar and 46.9, 20.5, 17.1, 2.44 and 1.34 for HYD2-s77, respectively.

Table 2
Crystallization information.

Protein sample	HYD2-s77 with trypsin treatment	HYD2-s77 without trypsin treatment
Method	Sitting-drop vapour diffusion	Sitting-drop vapour diffusion
Plate type	MRC 2-well (Hampton Research)	MRC 2-well (Hampton Research)
Temperature (K)	283	283
Protein concentration (mg ml ⁻¹)	15	11
Buffer composition of protein solution	10 mM MOPS pH 7.0, 0.2 M NaCl	10 mM MOPS pH 7.0, 0.2 M NaCl
Composition of reservoir solution	19% (w/v) PEG 10 000, 0.1 M NaCl, 0.1 M Tris-HCl pH 8.5, 20% (w/v) glycerol	18% (w/v) PEG 10 000, 0.1 M NaCl, 0.1 M Tris-HCl pH 8.5, 20% (w/v) glycerol
Volume and ratio of drop (μl)	0.5:0.5	0.5:0.5
Volume of reservoir (μl)	70	70

Table 3
Data collection and processing.

Values in parentheses are for the outer shell.

Data set	HYD2-s77 with trypsin treatment	HYD2-s77 without trypsin treatment
Diffraction source	BL44XU, SPring-8	BL44XU, SPring-8
Wavelength (Å)	0.9000	0.9000
Temperature (K)	100	100
Detector	MX300HE	MX300HE
Crystal-to-detector distance (mm)	160	190
Rotation range per image (°)	0.5	1.0
Total rotation range (°)	180	180
Exposure time per image (s)	0.5	1.0
Space group	<i>P</i> 2 ₁	<i>P</i> 2 ₁
<i>a</i> , <i>b</i> , <i>c</i> (Å)	63.90, 118.89, 96.70	65.38, 121.45, 98.63
α , β , γ (°)	90.00, 100.61, 90.00	90.00, 102.29, 90.00
Mosaicity (°)	0.59	0.53
Resolution range (Å)	28.75–1.60 (1.69–1.60)	33.68–2.00 (2.11–2.00)
Total No. of reflections	93446	51136
No. of unique reflections	26992	14294
Completeness (%)	99.9 (99.4)	98.7 (97.0)
Multiplicity	3.7 (3.5)	3.7 (3.6)
$\langle I/\sigma(I) \rangle$	8.5 (3.3)	10.8 (3.0)
<i>R</i> _{int}	0.093 (0.478)	0.121 (0.414)
Overall <i>B</i> factor from Wilson plot (Å ²)	7.4	21.1

purification step was performed according to the protocols described in Eguchi *et al.* (2012). The concentration of the protein sample from each purification step was determined using the DC Protein Assay (Bio-Rad, California, USA).

A solution of 25 mM potassium phosphate (KP) buffer pH 7.0 with 5% (w/v) TTC was pre-saturated with argon bubbling and then saturated with H₂. A solution of hydrogenases (HYD2-s77, MBH-mar and STD-dvm) in 25 mM KP buffer was pre-activated by H₂ bubbling for 30 min at a flow rate of 50 ml min⁻¹. Monitoring of the enzymatic reaction was initiated by the addition of 300 μl TTC buffer solution to 2700 μl of a reaction mixture with 3.3 × 10⁻³ μM of each enzyme in a glass cuvette with a rubber stopper and aluminium cap (path length 10 mm) at 310 K. In order to determine the O₂ tolerance of the H₂-consumption activity, TTC with O₂ and TTC saturated with H₂ buffer solutions were mixed in various ratios and introduced into the reaction cuvette. The TTC buffer solution containing O₂ was prepared under aerobic conditions. This solution is air-equilibrated and is thought to contain 210 μM O₂ at ambient temperature and pressure (Hocking, 1998). H₂-consumption activity was monitored and estimated

by the increase in the absorption at 546 nm owing to the H₂-dependent reduction of TTC using a spectrophotometer. The index of O₂ tolerance was defined as 100 times the inverse of the reactivation time (in minutes) of the enzyme when the H₂-consumption catalytic reaction rate, the slope ($\Delta\text{Abs}/\Delta t$) of the tangent line of the H₂-consumption activity curves shown in Fig. 1, reached its maximum value with each amount of O₂.

2.2. Crystallization

Crystals of HYD2-s77 with and without trypsin treatment were prepared by the sitting-drop vapour-diffusion method using an MRC 2-well crystallization plate (Hampton Research, Aliso Viejo, California, USA). For the sample without trypsin treatment, a 0.5 μl drop of protein solution (11 mg ml⁻¹ HYD2-s77, 10 mM MOPS pH 7.0, 0.2 M NaCl) was mixed with a 0.5 μl drop of precipitant [18% (w/v) PEG 10 000, 0.1 M NaCl, 0.1 M Tris-HCl pH 8.5, 20% (w/v) glycerol] and equilibrated against a 70 μl reservoir consisting of precipitant solution. For the trypsin-treated sample, a 0.5 μl drop of protein solution (15 mg ml⁻¹ HYD2-s77, 10 mM MOPS pH 7.0, 0.2 M NaCl) was mixed with a 0.5 μl drop of precipitant [19% (w/v) PEG 10 000, 0.1 M NaCl, 0.1 M Tris-HCl pH 8.5, 20% (w/v) glycerol] (Table 2). Crystals appeared after 2–3 d at 283 K.

2.3. Data collection and processing

The X-ray diffraction experiment was carried out on the BL44XU beamline at SPring-8 using an MX300HE detector. The crystals were soaked in a cryoprotectant solution (reservoir solution), mounted in nylon loops (Hampton Research, Aliso Viejo, California, USA) and then flash-cooled in a nitrogen stream at 100 K. The X-ray wavelength employed for data collection was 0.9000 Å. The crystal-to-detector distance, rotation range, exposure time per frame and total number of frames for the sample not treated with trypsin were 190 mm, 1.0°, 1.0 s and 180, respectively, whereas those for the sample subjected to trypsin treatment were 160 mm, 0.5°, 0.5 s and 360, respectively. Details of data collection and processing are summarized in Table 3. Diffraction images were indexed using *iMosflm* from the *CCP4* suite (Winn *et al.*, 2011). Each data set was merged and scaled using *SCALA* (Evans, 2006).

Figure 2

Crystals of the hydrogenase unit of the [NiFe]-hydrogenase from *Citrobacter* sp. S-77 (HYD2-s77). (a) A crystal obtained from the protein sample with trypsin treatment. (b) Crystals obtained from the protein sample without trypsin treatment (the scale bar is 0.05 mm in length).

3. Results and discussion

The results of the purification steps are summarized in Table 1. After the purification procedures both with and without trypsin treatment, 5.37 and 3.92 mg of HYD2-s77 were obtained from 65.0 g of bacterial cell pellets, respectively. The specific activity (580 U mg^{-1}) and recovery (49%) of the purified enzyme with trypsin treatment were better than those obtained using only Triton X-100 treatment without trypsin (233 U mg^{-1} and 13%, respectively) (Table 1). The specific activity obtained from the sample with trypsin treatment was lower than the value of 661 U mg^{-1} obtained by the original method of Eguchi *et al.* (2012), but the recovery (16.3%) was considerably improved. These results clearly show that trypsin treatment was effective in obtaining a sufficient amount of the protein sample with high purity for further crystallization screening (Supplementary Fig. S1).

By using TTC as the electron acceptor, the O_2 tolerances of three different enzymes were investigated and compared. The H_2 -consumption activity of MBH-mar promptly recovered even in the presence of a significant amount (63.0 nmol) of O_2 , indicating that the enzyme retained the activity and consumed O_2 owing to its catalytic reaction, which produces an H_2 -dependent reducing power (Fig. 1a). STD-dvm, the typical standard [NiFe]-hydrogenase, showed a strong H_2 -consumption activity without O_2 , but the activity could not be recovered in the presence of a small amount (4.2 nmol) of O_2 (Fig. 1b). HYD2-s77 showed a relatively prolonged lag time for the various O_2 concentrations compared with MBH-mar, but the activity was recovered in the presence of 63.0 nmol O_2 (Fig. 1c). The calculated O_2 -tolerance indexes in the presence of 4.2, 8.4, 12.6, 31.5 and 63.0 nmol O_2 were 120.0, 98.4, 93.8, 14.4 and 8.30 for MBH-mar and 46.9, 20.5, 17.1, 2.44 and 1.34 for HYD2-s77, respectively. The results presented here demonstrate that HYD2-s77 also retained catalytic activity of H_2 consumption in the presence of some amount of O_2 , suggesting that HYD2-s77, one of the Hyd-2-type enzymes, has an obvious O_2 tolerance.

HYD2-s77 purified both with and without trypsin treatment was successfully crystallized using the sitting-drop vapour-diffusion method. The crystallization conditions for both samples were almost the same except for the concentrations of the enzyme (15 or 11 mg ml^{-1}) and the precipitant (19 or 18% PEG 10 000). The crystals obtained from the trypsin-treated sample were observed to be plate-like and generally larger than the crystals obtained without trypsin treatment, which were observed to be pillar-shaped (Fig. 2).

The X-ray diffraction data showed that the HYD2-s77 crystals belonged to the monoclinic space group $P2_1$, with unit-cell parameters $a = 63.90$, $b = 118.89$, $c = 96.70 \text{ \AA}$, $\beta = 100.61^\circ$ for the crystals obtained with trypsin treatment and $a = 65.38$, $b = 121.45$, $c = 98.63 \text{ \AA}$, $\beta = 102.29^\circ$ for the crystals obtained without trypsin treatment (Table 3). The crystals from the samples with and without trypsin treatment diffracted to 1.60 and 2.00 $\text{\AA}$ resolution, respectively, which indicates that the diffraction quality of the crystal was considerably improved by treatment with trypsin. The values of other statistics, such as $R_{\text{f.i.m.}}$ and the overall B factor, were also improved by trypsin treatment. These improvements were probably owing to the high homogeneity of the trypsin-treated protein sample, in particular with regard to the evenly trimmed long C-terminal loop of the small subunit, which is often randomly eliminated by proteases during purification. Data-collection statistics are summarized in Table 3. Based on the presence of two heterodimeric molecules in the asymmetric unit of the crystals from samples purified with and without trypsin treatment, the Matthews coefficients were calculated to be 2.01 and $2.13 \text{ \AA}^3 \text{ Da}^{-1}$, with solvent contents of about 39 and 42%, respectively. Subsequently, the molecular-replacement method was applied using *MOLREP* from *CCP4*. Model building is now in progress.

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