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Purification, crystallization and preliminary X-ray analysis of the membrane-bound [NiFe] hydrogenase from *Allochromatium vinosum*

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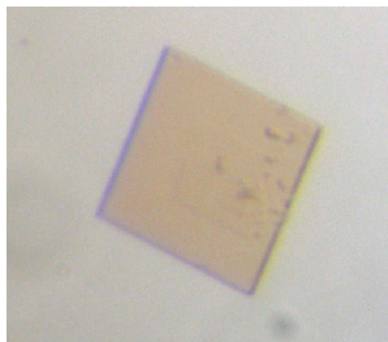
The membrane-bound [NiFe] hydrogenase is a unique metalloprotein that is able to catalyze the reversible oxidation of hydrogen to protons and electrons during a complex reaction cycle. The [NiFe] hydrogenase was isolated from the photosynthetic purple sulfur bacterium *Allochromatium vinosum* and its crystallization and preliminary X-ray analysis are reported. It was crystallized by the hanging-drop vapour-diffusion method using sodium citrate and imidazole as crystallization agents. The crystals belong to space group $P2_12_12$, with unit-cell parameters a = 205.00, b = 217.42, c = 120.44 Å. X-ray diffraction data have been collected to 2.5 Å resolution.



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1. Introduction

Hydrogenases (oxidoreductases) have been identified in different groups of phylogenetically independent organisms, including archaea, bacteria and eukaryotes, which consume hydrogen as an energy source or use it as an electron sink in their elementary metabolism (for a review, see Vignais & Billoud, 2007). These enzymes are of special interest since they are, in principle, capable of producing molecular hydrogen, a sustainable environmentally friendly energy carrier. Knowledge of the basic features of hydrogen conversion in nature is thus of major importance not only for basic research but also for possible applications in biotechnological processes (Esswein & Nocera, 2007; Cammack *et al.*, 2001). Furthermore, this would provide the foundation for the design of biomimetic or bio-inspired artificial hydrogenase catalysts for large-scale hydrogen production in the future (Kubas, 2007).

Three different classes of hydrogenases are generally known. These are the [NiFe] hydrogenases (Lubitz, van Gastel *et al.*, 2007), including the subgroup of [NiFeSe] hydrogenases, the [FeFe] hydrogenases (Nicolet *et al.*, 2002; Lubitz, Reijerse *et al.*, 2007) and the iron–sulfur cluster-free [Fe] hydrogenases, which are also known as hydrogen-forming methylenetetrahydromethanopterin dehydrogenases (Hmds; Shima & Thauer, 2007). All three hydrogenase classes have an unusual structural feature in common. Their active site contains an Fe atom that possesses both cyanide and CO or only CO as natural ligands. This has been interpreted as a possible sign of convergent evolution.

The most studied class of hydrogenases are the heterodimeric [NiFe] hydrogenases, which contain Ni and Fe atoms in the active site of the large subunit and three FeS clusters in the small subunit. The Fe atom in the active site carries two CN^- ions and one CO molecule as nonprotein ligands as determined from spectroscopic studies (Pierik *et al.*, 1999; Fichtner *et al.*, 2006; DeLacey *et al.*, 1997). The redox-active Ni atom is coordinated by four cysteine residues. Two of them bridge the Ni and Fe atoms.

Most of the investigated hydrogenases have been isolated from bacterial organisms, *e.g.* sulfate-reducing bacteria of species *Desulfovibrio*. The [NiFe] hydrogenase investigated in this study was isolated from the photosynthetic purple sulfur bacterium *Allochromatium vinosum*. This heterodimeric enzyme consists of an α-subunit (62 kDa

± 0.4%) and a β-subunit (29 kDa ± 0.4%). The molecular weights of both subunits were determined using MALDI–TOF MS (Fig. 1) and are in agreement with the known values for other standard hydrogenases.

The iron–sulfur clusters are responsible for electron transport from or to the active site. Channels for proton and gas transport through the protein matrix of the large subunit from the surface to the active site have been described (Montet *et al.*, 1997; Teixeira *et al.*, 2008). This information basically relies on analysis of the crystal structures and sequence alignments of nonphotosynthetic hydrogenases (Volbeda *et al.*, 1995; Ogata *et al.*, 2005; Fontecilla-Camps *et al.*, 2007). Alignments of the amino-acid sequences (*ClustalW* 1.83; Thompson *et al.*, 1994) of the large subunits of the hydrogenases from *D. vulgaris* Miyazaki F, *D. gigas* and *D. desulfuricans* with the *A. vinosum* hydrogenase sequence show 45, 48 and 45% identity, respectively. Alignments of the equivalent small subunit show 36, 28 and 40% identity. The close relation to the membrane-bound [NiFe] hydrogenase from *Thiocapsa roseopersicina*, another photosynthetic bacterial organism that belongs to the same taxonomic order (Chromatiales), is reflected in the values of 83% identity for the large subunit and 81% identity for the small subunit.

To date, no [NiFe] hydrogenases from species other than *Desulfovibrio* have been crystallized. The lack of structural information about hydrogenases of different origins impedes a detailed structural comparison and prevents us from developing a picture of the essential features of the hydrogen-conversion mechanism. The differences that might appear during comparison of the results from a photosynthetic and a nonphotosynthetic organism as previously indicated by the amino-acid alignment (see above) are possibly of crucial importance. To address this, we purified the [NiFe] hydrogenase from the physiologically different photosynthetic bacterium *A. vinosum* and crystallized it in its 'as isolated' (aerobic) state. In this paper, the results of the first preliminary X-ray analysis of the crystals obtained of this membrane-bound catalytic [NiFe] hydrogenase are presented.

2. Experimental procedures and results

2.1. Purification

The cells were cultivated in a 1100 l glass fermenter and purified according to the procedure described previously (Coremans *et al.*, 1992). The method was further modified and optimized for crystallization. All purification steps were performed at 277 K under aerobic conditions.

The cells were washed automatically in a newly developed cooled combined distillation and Soxhlet-extraction apparatus using acetone containing 5%(*v*/*v*) water to remove the photosynthetic pigments and to break the cells. The protein was extracted from the cell powder with a buffer containing 2%(*w*/*v*) Triton X-100 *via* stirring at 277 K. After high-speed centrifugation (2 h at 18 600*g*), the extract was applied onto an initial anion-exchange column (Toyopearl DEAE 650C, Tosoh, Japan) equilibrated with 50 m*M* Tris–HCl buffer pH 7.4 using an ÄKTA basic system (GE Healthcare, Uppsala, Sweden). The protein was eluted with the same buffer and a gradient of increasing NaCl concentration (0–0.6 *M* NaCl).

The hydrogenase-containing fractions were identified after each step of the chromatographic purification procedure by a hydrogenase-activity test using an inverse-polarized polarographic electrode (Yellow Springs, Ohio, USA) and benzylviologen (1,1′-dibenzyl-4,4′-bipyridinium dichloride; Sigma–Aldrich, Seelze, Germany). The hydrogen-uptake ability of the hydrogenase was detected. Fractions that showed significant activity were pooled and further purified.

The second chromatography step was based on the hydrophobic interaction (HIC) principle. The sample was charged with 0.2 *M* ammonium sulfate and applied onto an HIC column (Phenyl Sepharose CL 4B, GE Healthcare, Uppsala, Sweden) previously equilibrated with 150 m*M* ammonium sulfate in 50 m*M* Tris–HCl pH 8.0. The protein was eluted during a decreasing salt gradient to ammonium sulfate-free buffer (5 m*M* Tris–HCl pH 8.0).

The chromatographic procedure was supplemented by chymotrypsin treatment. The protein solution was incubated twice for 30 min at 312 K with 0.1 mg α-chymotrypsin from bovine pancreas (Sigma–Aldrich, Seelze, Germany) per milligram of protein. The solution was washed twice with 50 m*M* Tris–HCl buffer containing 100 m*M* NaCl pH 8.0. The sample was finally concentrated to 15 ml to apply it onto the third column.

The protein solution was applied onto a size-exclusion chromatography column (Ultrogel AcA 44, Biosepra, Dreieich, Germany) equilibrated with 50 m*M* Tris–HCl buffer containing 100 m*M* NaCl pH 8.0. The Q-Sepharose anion-exchange column (HiLoad 16/10 Q-Sepharose HP, GE Healthcare, Uppsala, Sweden) was equilibrated with 50 m*M* Tris–HCl buffer pH 8.0. The protein was eluted using an increasing salt gradient (0–1 *M* NaCl) in the same buffer. It was concentrated and applied onto the last Superdex 200 size-exclusion column (HiLoad 16/60 Superdex 200 prep grade, GE Healthcare, Uppsala, Sweden) and eluted with an isocratic gradient with 50 m*M* Tris–HCl and 100 m*M* NaCl pH 8.0. The preparation of 2 kg of cells yielded ∼100 mg protein.

To achieve highly purified protein for crystallization, the last two columns were repeated. The isolated enzyme was rebuffered in 50 m*M* Tris–HCl pH 8.0 and immediately used for crystallization attempts. The purity was confirmed using SDS–PAGE and MALDI–TOF mass spectrometry (Fig. 1). The enzyme was isolated in oxidized redox states (no strict anaerobic conditions) and showed a mixture of Ni-B (∼70%) and Ni-A (∼30%) as determined from FT-IR and EPR spectroscopy.

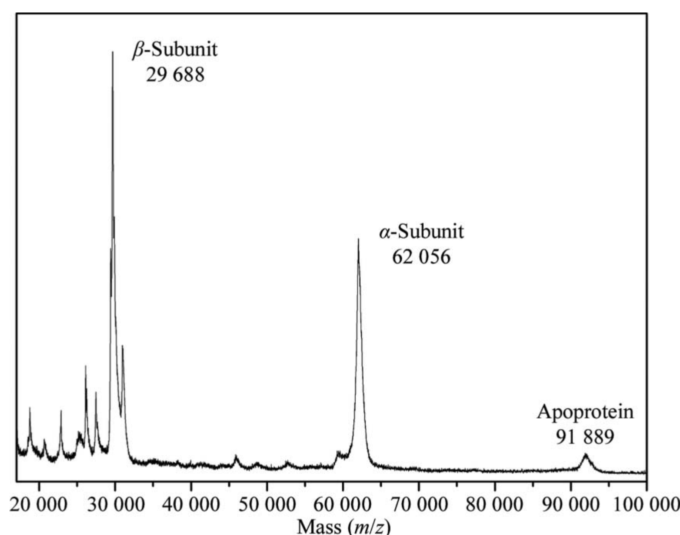

Figure 1
MALDI–TOF MS spectrum of [NiFe] hydrogenase isolated from *A. vinosum*. The spectrum was recorded with a Voyager-DE PRO Workstation (Applied Biosystems, Darmstadt); a sinapic acid matrix was used (error < 0.4%).

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

	Native	Peak	Inflection
Wavelength (Å)	0.91841	1.74014	1.74213
Space group	$P2_12_12$	$P2_12_12$	$P2_12_12$
Unit-cell parameters (Å)			
a	205.00	207.71	208.31
b	217.42	216.73	217.19
c	120.44	119.88	120.94
Resolution range (Å)	68.36–2.50 (2.64–2.50)	103.70–5.00 (5.27–5.00)	104.26–5.00 (5.27–5.00)
Observed reflections	721710	335628	339404
Unique reflections	166469	24116	24451
Completeness (%)	99.8 (100.0)	100.0 (100.0)	100.0 (100.0)
R_{merge}† (%)	9.0 (44.2)	16.4 (25.3)	22.7 (41.4)
$\langle I/\sigma(I)\rangle$	6.8 (1.7)	4.4 (2.9)	3.2 (1.8)
FOM		0.34	
No. of molecules in ASU	4		

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

2.2. Crystallization

Crystallization trials for the membrane-bound [NiFe] hydrogenase from *A. vinosum* were conducted using the sitting-drop and hanging-drop vapour-diffusion methods. A wide range of conditions were tested using kits from Hampton Research (Crystal Screen, Crystal Screen 2, Crystal Screen Cryo, Grid Screen Ammonium Sulfate, Grid Screen MPD and Grid Screen PEG 6000) and Emerald Biosystems (Cryo I, Cryo II, Wizard I and Wizard II). The protein concentration was kept at 30 mg ml^{-1} and the drops were prepared by mixing equal volumes (1 µl) of protein solution in 50 m*M* Tris–HCl buffer pH 8.0 and crystallization solution. The screens were set up at 277 K using Crystal Clear strips or Cryschem plates (sitting drops) or VDX plates (hanging drops) from Hampton Research. 100–500 µl reservoir solution was placed in each well. Crystals suitable for diffraction experiments were obtained using the hanging-drop vapour-diffusion method with the following conditions: 1.0 *M* sodium citrate and 100 m*M* imidazole pH 8.0. Thin crystal plates of triangular or square shape appeared after approximately 14 d. The crystal dimensions were typically 0.2 × 0.2 × 0.05 mm (Fig. 2).

2.3. Data collection

For data collection, a crystal was separated, dipped into mineral oil (light oil; Sigma Aldrich, Seelze, Germany) and subsequently frozen in a stream of nitrogen gas at 100 K. A complete native data set was collected to 2.5 Å resolution. In addition, two-wavelength Fe-MAD data sets were collected to 5.0 Å resolution. All diffraction data were collected at 100 K using beamline BL14.1 at BESSY II (Berlin, Germany). The detector was a fast-scanning 225 mm CCD-mosaic detector (MAR Research, Norderstedt, Germany). For the native data set, 135 images of 10 s exposure time and 1.0° oscillation were collected. The distance between the crystal and the detector was maintained at 330 mm. For the Fe-MAD data set, 360 images of 10 s exposure time each and 1.0° oscillation were collected. The distance between the crystal and the detector was maintained at 180 mm. Diffraction images were indexed using the program *MOSFLM* (Leslie, 1992) and processed using the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994). The conditions used for data collection and the results obtained are summarized in Table 1.

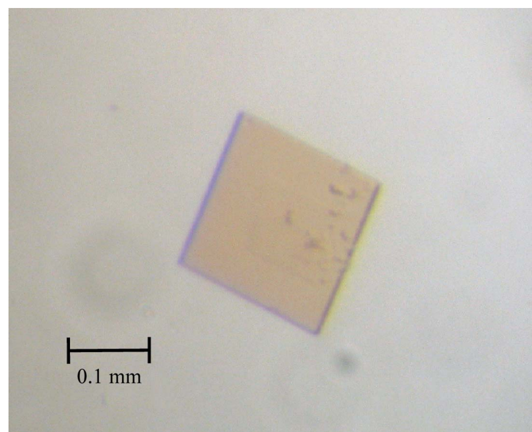

Figure 2
Crystal of [NiFe] hydrogenase from *A. vinosum*.

3. Summary and conclusion

The membrane-bound [NiFe] hydrogenase was highly purified from the purple sulfur bacterium *A. vinosum*. It could be crystallized using the sitting-drop and hanging-drop vapour-diffusion methods. The X-ray diffraction data showed that the crystals belonged to the orthorhombic space group $P2_12_12$, with unit-cell parameters a = 205.00, b = 217.42, c = 120.44 Å. The calculated Matthews coefficient (V_M) of 3.65 $Å^3\,Da^{-1}$ with a solvent content of 66.3% indicated the presence of four molecules in the asymmetric unit. The crystals diffracted to 2.5 Å resolution. Initial phases were calculated from the Fe-MAD data set using the *CCP*4 program suite. The phase calculation confirmed the presence of four molecules in the asymmetric unit. Subsequently, the molecular-replacement method was applied using the program *CNS* (Brünger *et al.*, 1998). The structure of the [NiFe] hydrogenase from *D. vulgaris* Miyazaki F was used as a search model (PDB code 1h2r). Model building is now in progress.

This study reports the first successful crystallization and X-ray diffraction analysis of a membrane-bound [NiFe] hydrogenase isolated from a photosynthetic bacterium. The structure of this enzyme supplements previous results from other standard non-photosynthetic hydrogenases and will help to extract further information about the general mechanism of hydrogen conversion. The data derived from the X-ray structure provide the basis for a detailed interpretation of the results from the spectroscopic characterization of the redox states carried out by EPR, FT-IR and electrochemical methods (Albracht, 1994; Gessner *et al.*, 1999; Bleijlevens *et al.*, 2004; Vincent *et al.*, 2007).

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