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Purification, crystallization and preliminary crystallographic analysis of the [NiFeSe] hydrogenase from *Desulfovibrio vulgaris* Hildenborough

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The [NiFeSe] hydrogenases belong to a subgroup of the [NiFe] proteins in which a selenocysteine is a ligand of the Ni. These enzymes demonstrate interesting catalytic properties, showing a very high H_2-producing activity that is sustained in the presence of low O_2 concentrations. The purification, crystallization and preliminary X-ray diffraction analysis of the [NiFeSe] hydrogenase isolated from *Desulfovibrio vulgaris* Hildenborough are reported. Crystals of the soluble form of this hydrogenase were obtained using 20% PEG 1500 as a precipitant and belonged to the monoclinic space group $P2_1$, with unit-cell parameters $a = 60.57$, $b = 91.05$, $c = 66.85$ Å, $\beta = 101.46^\circ$. Using an in-house X-ray diffraction system, they were observed to diffract X-rays to 2.4 Å resolution.

1. Introduction

As an energy carrier, hydrogen is a promising alternative to fossil fuels. It is considered to be a clean fuel since it does not contain carbon, sulfur or nitrogen, which cause pollution during combustion (Turner, 2004; Hallenbeck & Ghosh, 2009). However, no environmentally sustainable processes for hydrogen production have yet been developed (Turner, 2004). In nature, hydrogen is metabolized by very active enzymes named hydrogenases (Hases), the majority of which contain a binuclear Ni–Fe or Fe–Fe catalytic site that is more efficient than currently used Pt-based catalysts. The [NiFe] Hases are the most studied group. They are heterodimeric enzymes that are generally composed of a small subunit that contains up to three iron–sulfur clusters and a large subunit that contains the active site (Volbeda *et al.*, 1995; Vignais & Billoud, 2007). The [NiFeSe] hydrogenases are a subgroup of the [NiFe] proteins in which a selenocysteine is a ligand of the Ni (Matias *et al.*, 2002, 2005; Garcin *et al.*, 1999; De Lacey *et al.*, 2007). These hydrogenases demonstrate very high H_2-producing activities, become active immediately upon reduction and show less product inhibition than standard [NiFe] proteins (Valente *et al.*, 2005; De Lacey *et al.*, 2008; Parkin *et al.*, 2008). In addition, their H_2-producing activities are sustained in the presence of low molecular-oxygen concentrations (Parkin *et al.*, 2008), making these Hases excellent candidates for the development of oxygen-stable engineered proteins. Recently, a study was published in which an [NiFeSe] Hase from *Desulfomicrobium baculatum* was able to produce hydrogen in the presence of oxygen on a TiO_2 nanoparticle system for visible-light-driven H_2 production (Reisner *et al.*, 2009).

The [NiFeSe] hydrogenase from the sulfate reducer *Desulfovibrio vulgaris* Hildenborough (DvH) is associated with the cytoplasmatic membrane (Valente *et al.*, 2005). It is the major Hase synthesized by DvH when selenium is available (Valente *et al.*, 2006). This enzyme is a bacterial lipoprotein that is post-translationally modified to include a hydrophobic group bound to the N-terminus which, when cleaved, produces a soluble form (Valente *et al.*, 2005, 2007). The structural and functional characterization of [NiFe] Hases in their different redox states is well advanced, but comparatively scant information is available for the [NiFeSe] Hases. Only one three-dimensional crystal structure has been reported: the reduced form of the [NiFeSe] Hase

from *Desulfomicrobium baculatum* (Garcin *et al.*, 1999). Recently, a FTIR study of the [NiFeSe] Hase from DvH identified two different EPR-silent forms for the oxidized state (De Lacey *et al.*, 2008). A central issue is how the structural differences between the standard [NiFe] Hases and the [NiFeSe] Hases may be relevant to the higher activity and greater resistance to oxygen displayed by the [NiFeSe] Hases. Here, we report the purification, crystallization and preliminary X-ray diffraction analysis of the [NiFeSe] Hase from DvH.

2. Experimental procedures and results

2.1. Cell growth

Desulfovibrio vulgaris Hildenborough was grown anaerobically at 310 K in lactate/sulfate medium as described previously (Legall *et al.*, 1994). The preparation of the membrane extract was performed as described in Valente *et al.* (2001).

2.2. Protein purification

All purification procedures were carried out at pH 7.6 and 277 K in the presence of 0.2%(*w*/*v*) Zwittergent 3-12 (Sigma–Aldrich). The [NiFeSe] Hase was isolated using fast purification protocols adapted from Valente *et al.* (2001). The purification was performed aerobically using different ion-exchange chromatography columns. The detergent extract was loaded onto a Q-Sepharose fast-flow column equilibrated with 20 m*M* Tris–HCl pH 7.6 buffer. A stepwise gradient of increasing NaCl concentration was performed. Two bands were separated, eluting at 300 and 350 m*M* NaCl, respectively, for the soluble and membrane forms of the [NiFeSe] Hase. The membrane form of the [NiFeSe] Hase ($[NiFeSe]_m$) was purified as described in Valente *et al.* (2005). The fraction containing the soluble form of the [NiFeSe] Hase ($[NiFeSe]_s$) was further purified on a Pharmacia Q-Sepharose HP column (HiLoad 26/10). The fraction eluting at 200 m*M* NaCl was concentrated and the $[NiFeSe]_s$ Hase was finally purified on a ceramic HTP column equilibrated with 5 m*M* phosphate pH 7.6 buffer, applying a stepwise gradient to 1 *M* phosphate. The fraction that eluted at 225 m*M* phosphate was concentrated. This step yielded pure $[NiFeSe]_s$ Hase as judged by SDS–PAGE and activity-stained native gel (illustrated in Fig. 1). The sample was washed and concentrated to 10 mg ml^{-1} in 10 m*M* Tris–HCl pH 7.6.

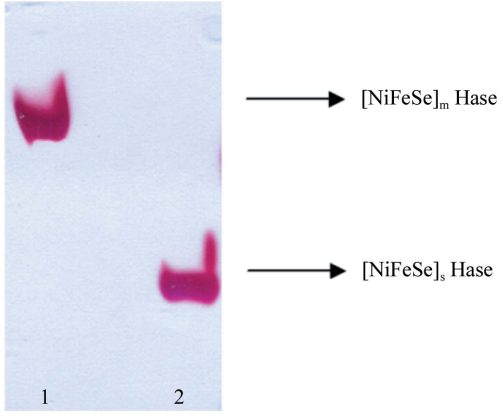

Figure 1
Activity-stained native PAGE. Lane 1, DvH $[NiFeSe]_m$ Hase; lane 2, DvH $[NiFeSe]_s$ Hase.

Table 1
Crystallographic data collection and processing.

Values in parentheses are for the highest resolution shell.

Wavelength (Å)	1.5418
Space group	$P2_1$
Unit-cell parameters (Å, °)	a = 60.57, b = 91.05, c = 66.85, β = 101.46
Resolution range (Å)	32.76–2.40 (2.50–2.40)
Scan type	ω-scan
Total angular range† (°)	619.5
Total No. of frames†	2478
Exposure time per frame (s)	90
No. of observations	271135
Unique reflections	27886 (3181)
$\langle I/\sigma(I)\rangle$	17.2 (3.8)
R_{merge}‡ (%)	9.50 (34.8)
$R_{p.i.m.}$§ (%)	3.1 (16.0)
Completeness (%)	99.8 (97.6)
Multiplicity	9.7 (6.0)
Wilson B factor (Å^2)	43.0
No. of molecules in ASU	1
V_M (Å^3 Da^{-1})	2.13
Estimated solvent content (%)	42.4

† In ten different crystal settings. ‡ $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$. § $R_{p.i.m.} = \sum_{hkl}[1/(N-1)]^{1/2}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the observed intensity, $\langle I(hkl)\rangle$ is the average intensity of multiple observations from symmetry-related reflections and N is their redundancy.

2.3. Crystallization and cryoprotection

Initial crystallization screenings were performed for the membrane and soluble forms of the [NiFeSe] Hase in 96-well plates at the nanolitre scale using a Cartesian Dispensing Systems robot (Genomic Solutions). No crystals appeared for the membrane form of the protein. Crystalline forms appeared in the EasyXtal Classics Suite and PEG Suite screens (Qiagen) for $[NiFeSe]_s$ Hase. Additional searches were performed around the initial conditions in order to obtain good-quality crystals. Crystallization trials were carried out aerobically using the sitting-drop vapour-diffusion method at 293 K. After several crystal-optimization experiments, crystals were grown after 2 d by mixing 1.5 µl reservoir solution containing 20% polyethylene glycol (PEG) 1500, 0.1 *M* Tris–HCl pH 8.5 and an equal volume of a solution composed of 10 mg ml^{-1} protein in 10 m*M* Tris–HCl buffer pH 7.6 (Fig. 2). Crystals were cryoprotected using the reservoir solution supplemented with 10% glycerol and flash-cooled at 100 K in a nitrogen-gas stream (Oxford Cryosystems 700).

2.4. X-ray data collection and preliminary crystallographic analysis

A 2.4 Å diffraction data set was measured in-house using a Bruker AXS Proteum Pt135 CCD detector system coupled to a Bruker AXS

Figure 2
[NiFeSe] Hase crystals grown in 20% PEG 1500, 0.1 *M* Tris–HCl pH 8.5.

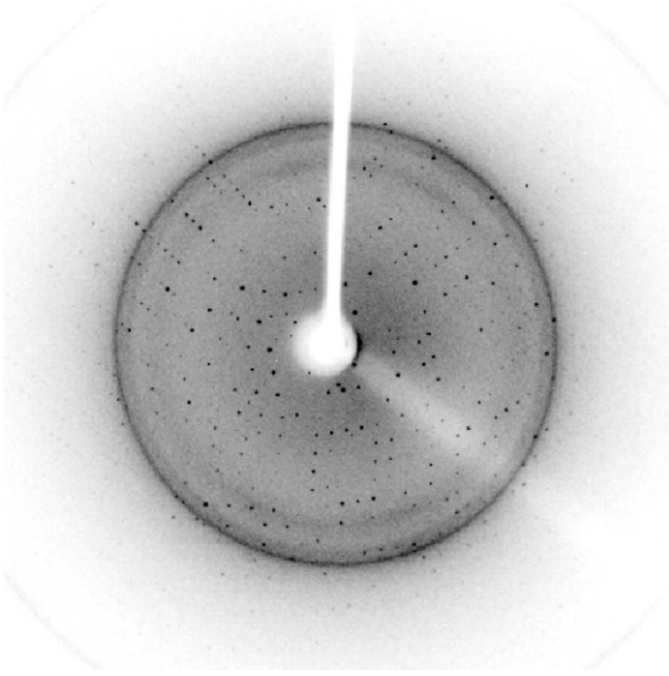

Figure 3
Diffraction image of an $[NiFeSe]_s$ Hase crystal collected with in-house equipment.

Microstar-I rotating-anode X-ray generator with Montel mirrors (see Fig. 3). The data were integrated with *SAINT* and scaled with *SADABS* as part of the Bruker AXS *Proteum* software suite. Diffraction data statistics were obtained with *XPREP* (Bruker AXS). The data were then converted and further processed for use with the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994). A summary of the data-collection and processing statistics is given in Table 1.

A molecular-replacement structure solution was readily obtained with *Phaser* (Storoni *et al.*, 2004) using the homologous structure from *Desulfomicrobium baculatum* (Garcin *et al.*, 1999) as a search model (each subunit was considered separately). However, there were some regions in the DvH structure which were difficult to rebuild because of poor electron density. Therefore, a MAD experiment at the Fe *K* absorption edge will be carried out at the earliest opportunity in order to obtain experimental and unbiased phase information.

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