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Crystallization and preliminary X-ray crystallographic study of [NiFe]-hydrogenase maturation factor HypE from *Thermococcus kodakaraensis* KOD1

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The hydrogenase maturation protein HypE is involved in the biosynthesis of the CN ligands of the active-site iron of [NiFe] hydrogenases using carbamoyl-phosphate as a substrate. Here, the crystallization and preliminary crystallographic analysis of HypE from *Thermococcus kodakaraensis* KOD1 are reported. Crystals of HypE (338 amino acids, 35.9 kDa) have been obtained by the sitting-drop vapour-diffusion method using 2-methyl-2,4-pentanediol (MPD) as a precipitant. The crystals belong to space group $P2_12_12$, with unit-cell parameters a = 88.3, b = 45.8, c = 75.1 Å. There is one HypE molecule in the asymmetric unit. A complete native X-ray diffraction data set was collected to a maximum resolution of 1.55 Å at 100 K.

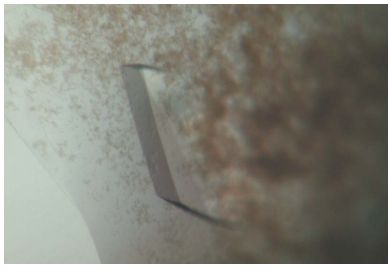
1. Introduction

[NiFe] hydrogenases catalyze the reversible formation of hydrogen gas from protons and electrons. These enzymes, which are found in a variety of bacteria and archaea, play an important role in energy metabolism in microorganisms (Vignais *et al.*, 2001). Studies of hydrogenases have attracted considerable attention because understanding of their catalytic mechanism may contribute to biotechnological applications in the production of hydrogen as a future energy source (Cammack *et al.*, 2001; Evans & Pickett, 2003; Volbeda & Fontecilla-Camps, 2003). [NiFe] hydrogenases carry a metal centre composed of Fe and Ni atoms at the active site. The Fe atom is further coordinated by two CN ligands and one CO ligand (Happe *et al.*, 1997; Pierik *et al.*, 1999). Biosynthesis of the complex metal centre of [NiFe] hydrogenases requires specific accessory proteins (Böck *et al.*, 2006; Forzi & Sawers, 2007; Leach & Zamble, 2007). In *Escherichia coli*, the *hyp* genes (*hypABCDEF*) are required for the synthesis and incorporation of the metal centre of [NiFe] hydrogenases (Lutz *et al.*, 1991; Jacobi *et al.*, 1992). Similar sets of genes are also found in many bacteria and archaea (Vignais *et al.*, 2001).

HypA and HypB are involved in nickel insertion into the precursor large subunit of [NiFe] hydrogenases (Waugh & Boxer, 1986; Olson *et al.*, 2001). They form a heterodimer and require GTP hydrolysis for the nickel insertion. The crystal structure of HypB from *Methanocaldococcus jannaschii* suggests that a GTP-dependent conformational change of HypB is presumably coupled with nickel delivery (Gasper *et al.*, 2006). An additional auxiliary protein, SlyD, has also been identified as being involved in this step (Zhang *et al.*, 2005).

HypC and HypD are assumed to be involved in the insertion of the Fe atom with diatomic ligands into the precursor of the large subunit (Blokesch, Böck *et al.*, 2002). HypC interacts with the precursor large subunit and maintains a conformation capable of metal incorporation (Drapal & Böck, 1998; Magalon & Böck, 2000*a*,*b*). HypD is the only maturation protein that has a redox cofactor, the [4Fe–4S] cluster, which is assumed to be required in the maturation process (Blokesch, Albracht *et al.*, 2004; Blokesch & Böck, 2006).

HypE and HypF catalyze the synthesis of the CN ligand of the active-site iron of the [NiFe] hydrogenases using carbamoyl phosphate and ATP as substrates (Paschos *et al.*, 2002; Reissmann *et al.*, 2003; Blokesch, Paschos *et al.*, 2004). HypF first forms the postulated carbamoyladenylate by utilizing carbamoyl phosphate and ATP as substrates and transfers the carbamoyl moiety of carbamoyladenylate



to the conserved C-terminal cysteine residue of HypE to yield HypE-thiocarbamate. Subsequently, HypE dehydrates the *S*-carbamoyl moiety by an ATP-dependent reaction to produce HypE-thiocyanate (Reissmann *et al.*, 2003). Finally, this cyano group appears to be transferred to the Fe atom. It was demonstrated that the cyano group of HypE-thiocyanate is transferred to a complex formed by HypC and HypD *in vitro*, provided that the complex is prepared under anaerobic conditions (Blokesch, Albracht *et al.*, 2004).

To elucidate the mechanism of the cyanation reaction, we have purified, crystallized and performed preliminary X-ray diffraction analysis of HypE from *Thermococcus kodakaraensis* KOD1.

2. Cloning, expression and purification

T. kodakaraensis KOD1 (Atomi *et al.*, 2004; Fukui *et al.*, 2005) was the source of the genomic DNA. *Escherichia coli* DH5α and plasmid pUC18 were used for gene cloning, sequencing and DNA manipulation. *E. coli* BL21-CodonPlus(DE3)-RIL (Stratagene, La Jolla, CA, USA) and pET21a(+) (Novagen, Madison, WI, USA) were used for gene expression. *E. coli* strains were cultivated in Luria–Bertani medium with 100 µg ml^{-1} ampicillin at 310 K. The HypE gene was amplified from the genomic DNA of *T. kodakaraensis* with the primer set HypE5 (5′-ATT**CATATG**GGTGAAAAGATAAAGCT-3′; the *Nde*I site is shown in bold) and HypE3 (5′-CGG**GAATTC**-AATGGCGACCTTCTGGG-3′; the *Eco*RI site is shown in bold). After confirming the sequences of the DNA fragments, they were inserted into the *Nde*I/*Eco*RI sites of pET21a(+). After introduction into *E. coli* BL21-CodonPlus(DE3)-RIL cells, gene expression was induced with 0.1 m*M* isopropyl β-D-1-thiogalactopyranoside at the mid-exponential growth phase, with further incubation for 6 h at 310 K. After induction of gene expression, cells were washed with 50 m*M* Tris–HCl pH 8.0, 150 m*M* NaCl and resuspended in the same buffer. Cells were sonicated on ice and the supernatant after centrifugation (20 000*g*, 30 min at 277 K) was subjected to heat treatment at 358 K for 15 min. After centrifugation (20 000*g*, 30 min at 277 K), the supernatant was applied onto an anion-exchange column (HiTrapQ, GE Healthcare, Little Chalfont, UK) equilibrated with 50 m*M* Tris–HCl pH 8.0, 150 m*M* NaCl. Proteins were eluted with a linear gradient of NaCl from 0.15 to 0.5 *M*. After desalting with a HiPrep26/10 column (GE Healthcare), 100 m*M* DTT was added to the sample and it was kept at 277 K for 1 h. The sample was then applied onto a gel-filtration column (Superdex75 HR 10/30, GE Healthcare) equilibrated with 50 m*M* Tris–HCl pH 8.0, 150 m*M* NaCl.

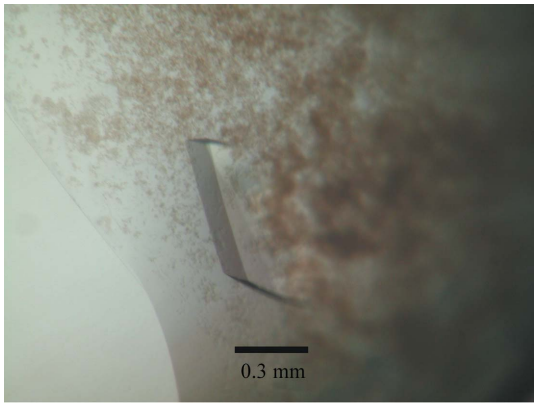


Figure 1
Crystals of HypE from *T. kodakaraensis* KOD1 with dimensions of 0.6 × 0.3 × 0.1 mm belonging to space group $P2_12_12$.

Table 1
Data collection from the native crystals of HypE.

Values in parentheses are for the highest resolution shell.

Space group	$P2_12_12$
Unit-cell parameters	
a (Å)	88.34
b (Å)	45.83
c (Å)	75.05
Wavelength (Å)	1.0000
Resolution (Å)	50–1.55 (1.61–1.55)
Total reflections	302334
Unique reflections	44379
Completeness (%) ($I > 1\sigma$)	94.6 (78.2)
Redundancy	7.1
$I/\sigma(I)$	34.5 (4.2)
R_{sym}† (%)	5.0 (28.3)

† $R_{sym} = \sum|I_h - \langle I_h\rangle|/\sum\langle I_h\rangle$, where I_h is the observed intensity and $\langle I_h\rangle$ is the average intensity over symmetry-equivalent measurements.

3. Crystallization

Crystallization trials of HypE were initially performed by the sitting-drop vapour-diffusion method at 293 K using 96-well Intelli-plates (Hampton Research, Aliso Viejo, CA, USA) and a Hydra II Plus One system (Matrix Technologies Corporation, Hudson, NH, USA). The protein concentration was 20 mg ml^{-1} in 20 m*M* Tris–HCl pH 8.0 and 2 m*M* DTT. An initial search for crystallization conditions was performed using the following screening kits: Crystal Screens I and II, Crystal Screen Cryo, PEG/Ion Screen, Index, SaltRx, Natrix and MembFac (Hampton Research), Wizard I and II, and Cryo I and II (Emerald Biostructures, Bainbridge Island, WA, USA). Although small crystals of HypE were grown under various conditions, the most reproducible result was obtained using MembFac No. 1 [0.1 *M* sodium acetate pH 4.6, 0.1 *M* NaCl, 12%(*v*/*v*) MPD]. Subsequently, the crystallization conditions were optimized by varying the pH and the concentrations of NaCl and MPD. Drops were prepared by mixing 1.5 µl each of the protein solution and the reservoir solution and were equilibrated against 100 µl reservoir solution in Crystal-Clear Strips (Hampton Research). Finally, crystals suitable for data collection were obtained in 7 d using reservoir solution containing 0.1 *M* sodium acetate pH 5.0, 22%(*v*/*v*) MPD and 200 m*M* NaCl. The crystals grew to typical dimensions of 0.6 × 0.3 × 0.1 mm (Fig. 1)

4. X-ray diffraction study

X-ray diffraction experiments were performed on beamline BL41XU at SPring-8. Prior to data collection, cryoprotectant solution [0.1 *M* sodium acetate pH 5.0, 40%(*v*/*v*) MPD, 200 m*M* NaCl] was added to the drops several times. The crystals were then mounted in nylon loops (Hampton Research) and flash-cooled in a nitrogen stream at 100 K. Diffraction data were collected using an oscillation angle of 1.0°, an exposure time of 1 s per frame and a wavelength of 1.0000 Å using an ADSC Quantum 315 CCD detector (Area Detector Systems Corporation). The crystal-to-detector distance was set at 160 mm. The crystals diffracted to beyond 1.4 Å resolution. The data were processed using the programs *DENZO* and *SCALEPACK* from the *HKL*-2000 package (Otwinowski & Minor, 1997). The crystals belong to space group $P2_12_12$, with unit-cell parameters a = 88.3, b = 45.8, c = 75.1 Å. Assuming that the asymmetric unit contains one HypE molecule, the calculated Matthews coefficient (Matthews, 1968) was

2.11 Å^3 Da^{-1}, which corresponds to a solvent content of 41.7%. A complete native X-ray diffraction data set was successfully collected at 1.55 Å resolution at 100 K. Data-collection statistics are summarized in Table 1. The structure was successfully determined and is described elsewhere (Watanabe *et al.*, 2007).

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