Acta Crystallographica Section F
Structural Biology and Crystallization Communications
ISSN 1744-3091

Crystallization and preliminary X-ray crystallographic studies of the [NiFe] hydrogenase maturation proteins HypC and HypD

Satoshi Watanabe,[a] Rie Matsumi,[b] Haruyuki Atomi,[b] Tadayuki Imanaka[b] and Kunio Miki[a,c]*

[a]Department of Chemistry, Graduate School of Science, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan, [b]Department of Synthetic Chemistry and Biological Chemistry, Graduate School of Engineering, Kyoto University, Katsura, Nishikyo-ku, Kyoto 615-8510, Japan, and [c]RIKEN SPring-8 Center at Harima Institute, Koto 1-1-1, Sayo, Hyogo 679-5148, Japan

Correspondence e-mail: miki@kuchem.kyoto-u.ac.jp

Received 14 March 2007
Accepted 14 May 2007

HypC and HypD proteins are required for the insertion of the Fe atom with diatomic ligands into the large subunit of [NiFe] hydrogenases, an important step in the maturation process of this type of hydrogenase. The crystallization and preliminary crystallographic analysis of HypC and HypD from *Thermococcus kodakaraensis* KOD1 are reported. Crystals of HypC grew in two different forms. Monoclinic crystals of HypC in space group $C2$ with unit-cell parameters $a = 78.2$, $b = 59.1$, $c = 54.0$ Å, $\beta = 109.0^\circ$ were obtained using PEG 4000 and ammonium sulfate or sodium bromide as precipitants. They diffracted X-rays to 1.8 Å resolution and were suitable for structure determination. Crystals of HypD were also obtained in two different forms. The monoclinic crystals obtained using PEG 4000 and magnesium chloride diffracted X-rays to beyond 2.1 Å resolution, despite growing as clusters. They belong to space group $P2_1$, with unit-cell parameters $a = 42.3$, $b = 118.4$, $c = 81.2$ Å, $\beta = 100.9^\circ$, and are suitable for data collection.

1. Introduction

[NiFe] hydrogenases contribute to energy metabolism in various microorganisms by catalyzing the reversible formation of molecular hydrogen. The assembly of the $NiFe(CO)(CN)_2$ metal centre of [NiFe] hydrogenase requires a number of specific proteins (Casalot & Rousset, 2001; Blokesch *et al.*, 2002). The *hyp* genes were originally identified as genes necessary for the maturation of the three [NiFe] hydrogenases present in *Escherichia coli* (Lutz *et al.*, 1991; Jacobi *et al.*, 1992). It is now known that products of the *hypABCDEF* genes are directly involved in the functional expression of *E. coli* hydrogenase 3 and that similar sets of genes are conserved in various bacteria and archaea (Vignais *et al.*, 2001). The maturation process of hydrogenases is initiated by the insertion of the Fe atom with diatomic ligands, followed by the insertion of the Ni atom (Maier & Böck, 1996). In the final step of maturation, specific endopeptidases such as HycI remove the C-terminal tail of the precursor of the large subunit to yield the active enzyme (Rossmann *et al.*, 1995; Fritsche *et al.*, 1999). HypA and HypB are involved in the insertion of the Ni atom into the precursor large subunit (Waugh & Boxer, 1986; Olson *et al.*, 2001). An additional auxiliary protein, SlyD, has also been identified as being involved in this step (Zhang *et al.*, 2005). A GTP-dependent conformational change of HypB is presumably coupled to nickel delivery (Gasper *et al.*, 2006). HypE and HypF have been shown to be involved in the synthesis of the cyanide ligand attached to the active-site Fe atom (Reissmann *et al.*, 2003; Blokesch, Paschos *et al.*, 2004). HypF catalyzes the transfer of the carbamoyl group from a carbamoylphosphate to the C-terminal cysteine residue of HypE, coupled with the conversion of ATP to AMP and PP_i. An ATP-dependent dehydration by HypE converts HypE-carboxamide to HypE-thiocyanate (Reissmann *et al.*, 2003). HypC and HypD form a complex that is presumably involved in the insertion of the Fe atom coordinated by diatomic ligands (Blokesch & Böck, 2002). It has been demonstrated that the cyanide moiety of HypE-thiocyanate is transferred to the HypC–HypD complex *in vitro*, provided that the complex is prepared under anaerobic conditions (Blokesch, Albracht *et al.*, 2004). Therefore, it has been assumed that the ternary complex

of HypC, HypD and HypE is an intermediate complex in which the active-site Fe atom is coordinated by two cyanide ligands and that the HypC–HypD complex subsequently transfers the Fe atom to the precursor large subunit. However, the mechanism of the CN transfer to the Fe atom in the ternary complex remains unclear.

In order to gain insight into the mechanism of the cyanation reaction, we have cloned, expressed, purified and crystallized HypC and HypD from the hyperthermophilic archaeon *Thermococcus kodakaraensis* KOD1.

2. Cloning, expression and purification

T. kodakaraensis KOD1 (Atomi *et al.*, 2004; Fukui *et al.*, 2005) was the source of genomic DNA. *E. 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 HypC and HypD genes were amplified from the genomic DNA of *T. kodakaraensis*. The primer sets used were HypC5 (5′-GCG**CATATG**TGCCTTGCAGTTCCTGG-3′; the *Nde*I site is in bold) and HypC3 (5′-GCG**GGATCC**ACTAGTTCAGAACCCCTCCATGGCC-3′; the *Bam*HI site is in bold) for HypC and HypD5 (5′-GGG**TCTAGA**AATAATTTTGTTTAACTTTAAGAAGGAGATATA**CATATG**GAGGAGCCCTTTGAGGC-3′; the *Xba*I and *Nde*I sites are in bold) and HypD3 (5′-GCG**GAATTC**ACTAGTTCAGAACAGCACGCCGTAC-3′; the *Eco*RI site is in bold) for HypD, respectively. The region between the *Xba*I and *Nde*I sites in HypD5 was included for construction of coexpression vectors used elsewhere. After confirming the sequences of the DNA fragments, they were inserted into the *Nde*I/*Bam*HI sites and *Nde*I/*Eco*RI sites of pET21a(+), respectively. After introduction into *E. coli* BL21-CodonPlus(DE3)-RIL cells, gene expression was induced with 0.1 m*M* isopropyl β-D-thiogalactopyranoside (IPTG) at the mid-exponential growth phase, with further incubation for 6 h at 310 K.

The recombinant HypC and HypD proteins were purified using a similar method, although the purification protocol was modified slightly for HypD. After inducing gene expression, cells were washed with 50 m*M* Tris–HCl pH 8.0 and resuspended in the same buffer. Cells were sonicated on ice with an output energy of 30 W. The pulse was 0.4 s on/0.6 s off and continued for a total period of 15 min. The supernatant after centrifugation (20 000*g*, 30 min at 277 K) was subjected to heat treatment at 353 K for 10 min. After centrifugation (20 000*g*, 30 min at 277 K), the supernatant was applied onto an anion-exchange chromatography column (HiTrapQ, GE Healthcare, Little Chalfont, England) equilibrated with 50 m*M* Tris–HCl pH 8.0 and 100 m*M* NaCl (150 m*M* NaCl for HypD). HypC was eluted with a 0.1–0.4 *M* linear gradient of NaCl and eluted at a concentration of 0.11–0.22 *M*. HypD was eluted with a 0.15–0.4 *M* linear gradient of NaCl and eluted at a concentration of 0.26–0.34 *M*. $(NH_4)_2SO_4$ was added to a concentration of 1.5 *M* and the samples were applied onto a hydrophobic interaction chromatography column (Resource ISO, GE Healthcare) equilibrated with 50 m*M* Tris–HCl pH 8.0 and 1.05 *M* $(NH_4)_2SO_4$ [1.125 *M* $(NH_4)_2SO_4$ for HypD]. Proteins were eluted with a 1.05–0.45 *M* linear gradient of $(NH_4)_2SO_4$ (1.125–0.675 *M* for HypD). HypC was eluted at a concentration of 0.645–

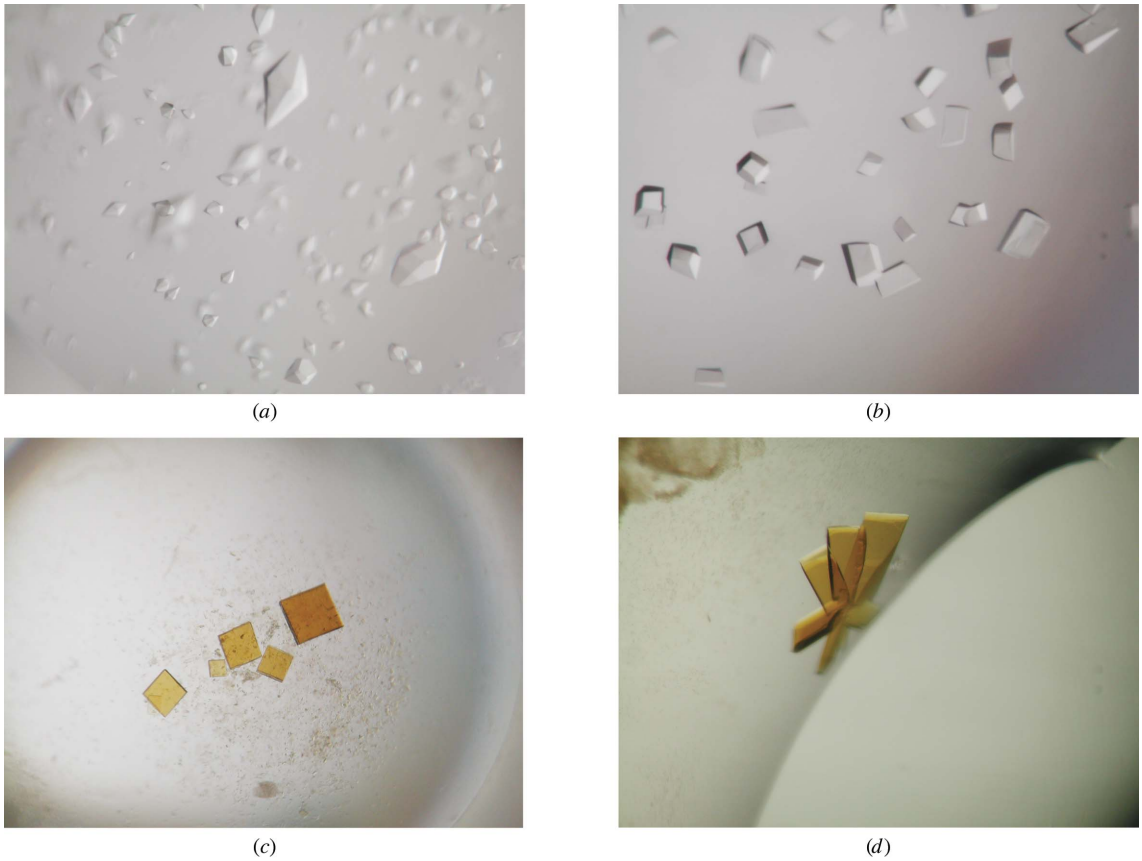

Figure 1
Crystals of HypC and HypD. (*a*) Form I crystals (tetragonal) of HypC. (*b*) Form II crystals (monoclinic) of HypC. (*c*) Form I crystals (tetragonal, plates) of HypD. (*d*) Form II crystals (monoclinic, clusters) of HypD.

Table 1
Data collection from native crystals of HypC and HypD.

Values in parentheses are for the highest resolution shell.

	HypC (type II)	HypD (type II)
Space group	*C*2	$P2_1$
Unit-cell parameters		
a (Å)	78.24	42.26
b (Å)	59.13	118.39
c (Å)	53.97	81.17
β (°)	109.0	100.9
Wavelength (Å)	1.0000	1.0000
Resolution (Å)	50–1.80 (1.86–1.80)	50–2.07 (2.14–2.07)
Total reflections	69772	179006
Unique reflections	21035	47481
Completeness ($I > 1\sigma$) (%)	90.3 (67.8)	94.3 (82.7)
Redundancy	3.3	3.8
$I/\sigma(I)$	31.3 (4.3)	20.5 (5.3)
R_{sym}† (%)	3.5 (21.3)	5.7 (25.4)

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

0.45 *M* $(NH_4)_2SO_4$ and HypD was eluted at 0.9–0.675 *M* $(NH_4)_2SO_4$. After desalting with a HiPrep26/10 column (GE Healthcare), the sample was applied onto a gel-filtration chromatography column (Superdex75 HR 10/30, GE Healthcare) equilibrated with PBS buffer (8.0 g NaCl, 0.2 g KCl, 2.9 g $Na_2HPO_4.12H_2O$ and 0.2 g KH_2PO_4 per litre). 1 m*M* DTT was added to all buffers. The elution peak of HypC was observed at 12.9 ml and that of HypD at 10.2 ml, indicating monomeric states of both proteins in solution. A minor peak corresponding to a dimeric form of HypC was observed at 11.5 ml, but was not collected. The final samples for crystallization were concentrated to 10–50 mg ml^{-1} in buffer containing 20 m*M* Tris–HCl pH 7.6 (pH 8.0 for HypD) and 1 m*M* DTT.

3. Crystallization

Crystallization of HypC (75 amino acids, 8.2 kDa) was carried out using the sitting-drop vapour-diffusion method at 293 K. Screening kits from Hampton Research (Aliso Viejo, CA, USA) and Emerald BioSystems (Bainbridge Island, WA, USA) were used to determine initial crystallization conditions. Drops were prepared by mixing 0.5–1 µl protein solution with an equal volume of reservoir solution and were equilibrated against 100 µl reservoir solution. Initial crystals of HypC were obtained in two different forms. Tetragonal crystals (form I) of HypC grew in 2–7 d from a number of screens containing buffer in the pH range 6–7 (MES, HEPES, Tris *etc*) and polyethylene glycol (PEG) 3350–8000 with various salts or 2-propanol. Monoclinic crystals (form II) of HypC appeared in several screens containing buffer in the pH range 4–5 (citric acid), PEG 4000 and $(NH_4)_2SO_4$ or NaBr. After optimization of the crystallization conditions, form I crystals grew to final dimensions of about 0.2 × 0.1 × 0.1 mm (Fig. 1*a*) in a few days by mixing 3 µl protein solution at 10 mg ml^{-1} with 3 µl precipitant solution containing 0.1 *M* MES pH 6.4, 15–18%(*w*/*v*) PEG 4000, 200 m*M* $MgCl_2$ and 20%(*v*/*v*) glycerol and equilibrating against 500 µl precipitant solution. The best crystals of form II with final dimensions of about 0.2 × 0.1 × 0.1 mm (Fig. 1*b*) were obtained in 7 d by mixing 3 µl protein solution at 10 mg ml^{-1} with 3 µl precipitant solution containing 0.1 *M* citric acid pH 4.0–4.5, 20–23%(*w*/*v*) PEG 4000, 200 m*M* $(NH_4)_2SO_4$ or 100–600 m*M* NaBr and 20%(*v*/*v*) glycerol and equilibrating against 500 µl precipitant solution.

The crystallization of HypD (372 amino acids, 42 kDa) was performed in the same manner as that of HypC. Initial small crystals were obtained using PEG or organic compounds [2-methyl-2,4-pentanediol (MPD), 1,6-hexanediol, ethanol, 2-propanol, 1,2-propanediol or *t*-butanol]. Because HypD contains a [4Fe–4S] cluster (Blokesch, Albracht *et al.*, 2004), which was in the oxidized state under aerobic conditions, the crystals of HypD exhibited a yellowish brown colour (Figs. 1*c* and 1*d*). Initial crystals (form I) grew using Crystal Screen condition No. 1 and Grid Screen MPD (Hampton Research). After optimization of the crystallization conditions, plate-shaped crystals (form I) grew to dimensions of approximately 0.3 × 0.3 × 0.02 mm (Fig. 1*c*) in 7 d from drops in which 5 µl protein solution at 8 mg ml^{-1} was mixed with 4 µl precipitant solution [0.1 *M* MES pH 5.6–6.0, 18%(*v*/*v*) MPD] and 1 µl additive solution (2 *M* nondetergent sulfobetaine 201). Initial monoclinic crystals (form II) appeared using Crystal Screen Cryo condition No. 6 (Hampton). After optimization of the crystallization conditions, monoclinic crystals (form II) were obtained as clusters by mixing 2 µl protein solution at 10 mg ml^{-1} with 2 µl precipitant solution [80 m*M* Tris–HCl pH 8.5, 160 m*M* MgCl, 20%(*w*/*v*) PEG 4000 and 20%(*v*/*v*) ethylene glycol] and equilibrating against 100 µl reservoir solution [30 µl buffer solution (20 m*M* Tris–HCl pH 8.0 and 1 m*M* DTT) and 70 µl precipitant solution were mixed]. The form II crystals grew in 10 d to dimensions of approximately 0.4 × 0.2 × 0.2 mm (Fig. 1*d*).

4. X-ray diffraction study

All crystals were taken directly from the mother liquor and flash-cooled in a nitrogen stream. X-ray diffraction experiments were performed at the BL41XU and BL44B2 beamlines with a MAR CCD detector and an ADSC CCD detector at SPring-8 and at the NW12 beamline with an ADSC CCD detector at PF-AR. All data sets were processed using the *HKL*-2000 package (Otwinowski & Minor, 1997).

The form I crystals of HypC only diffracted to 4–5 Å resolution. The form II crystals of HypC diffracted to 1.8 Å resolution. They belong to space group *C*2, with unit-cell parameters *a* = 78.2, *b* = 59.1, *c* = 54.0 Å, β = 109.0°. Assuming the presence of three HypC molecules in the asymmetric unit, the value of the Matthews coefficient (Matthews, 1968) was 2.4 Å^3 Da^{-1}. Diffraction data from the form II native crystal were successfully collected to 1.80 Å resolution at the NW12 beamline for subsequent structure determination (Table 1).

The plate crystals (form I) of HypD diffracted X-rays to beyond 2.8 Å resolution. They belong to space group $P4_22_12$, with unit-cell parameters *a* = *b* = 140.0, *c* = 102.0 Å. However, the diffraction spots of the form I crystals were highly diffuse along the *c* axis, indicating that they were not suitable for data collection. In the case of the form II crystals of HypD, fragments separated from the clusters could be used for X-ray diffraction experiments. The diffraction quality of these fragments of the form II crystals was usually poor, but a few fragments exhibiting data-quality diffraction could be found, the best of which diffracted X-rays to beyond 2.1 Å resolution. The form II crystals of HypD belong to space group $P2_1$, with unit-cell parameters *a* = 42.3, *b* = 118.4, *c* = 81.2 Å, β = 101.0°. Assuming that the form II crystals of HypD contain two HypD molecules in the asymmetric unit, the Matthews coefficient is calculated to be 2.6 Å^3 Da^{-1}. Diffraction data from the form II native crystal were successfully collected at 2.07 Å resolution at the NW12 beamline and were suitable for further structure determination (Table 1).

We thank Drs M. Kawamoto, N. Shimizu, K. Hikima and T. Matsu of SPring-8 and Drs N. Matsugaki, N. Igarashi, Y. Yamada and S. Wakatsuki of the Photon Factory for their help with X-ray data collection. This work was supported by a grant from the National Project on Protein Structural and Functional Analyses from the

Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

Atomi, H., Fukui, T., Kanai, T., Morikawa, M. & Imanaka, T. (2004). *Archaea*, **1**, 263–267.

Blokesch, M., Albracht, S. P. J., Matzanke, B. F., Drapal, N. M., Jacobi, A. & Böck, A. (2004). *J. Mol. Biol.* **344**, 155–167.

Blokesch, M. & Böck, A. (2002). *J. Mol. Biol.* **324**, 287–296.

Blokesch, M., Paschos, A., Bauer, A., Reissmann, S., Drapal, N. & Böck, A. (2004). *Eur. J. Biochem.* **271**, 3428–3436.

Blokesch, M., Paschos, A., Theodoratou, E., Bauer, A., Hube, M., Huth, S. & Böck, A. (2002). *Biochem. Soc. Trans.* **30**, 674–680.

Casalot, L. & Rousset, M. (2001). *Trends Microbiol.* **9**, 228–237.

Fritsche, E., Paschos, A., Beisel, H.-G., Böck, A. & Huber, R. (1999). *J. Mol. Biol.* **288**, 989–998.

Fukui, T., Atomi, H., Kanai, T., Matsumi, R., Fujiwara, S. & Imanaka, T. (2005). *Genome Res.* **15**, 352–363.

Gasper, R., Scrima, A. & Wittinghofer, A. (2006). *J. Biol. Chem.* **281**, 27492–27502.

Jacobi, A., Rossmann, R. & Böck, A. (1992). *Arch. Microbiol.* **158**, 444–451.

Lutz, S., Jacobi, A., Schlensog, V., Böhm, R., Sawers, G. & Böck, A. (1991). *Mol. Microbiol.* **5**, 123–135.

Maier, T. & Böck, A. (1996). *Biochemistry*, **35**, 10089–10093.

Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.

Olson, J. W., Mehta, N. S. & Maier, R. J. (2001). *Mol. Microbiol.* **39**, 176–182.

Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.

Reissmann, S., Hochleitner, E., Wang, H., Paschos, A., Lottspeich, F., Glass, R. S. & Böck, A. (2003). *Science*, **299**, 1067–1070.

Rossmann, R., Maier, T., Lottspeich, F. & Böck, A. (1995). *Eur. J. Biochem.* **227**, 545–550.

Vignais, P. M., Billoud, B. & Meyer, J. (2001). *FEMS Microbiol. Rev.* **25**, 455–501.

Waugh, R. & Boxer, D. H. (1986). *Biochimie*, **68**, 157–166.

Zhang, J. W., Butland, G., Greenblatt, J. F., Emili, A. & Zamble, D. B. (2005). *J. Biol. Chem.* **280**, 4360–4366.