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

Cloning, expression, purification, crystallization and preliminary crystallographic analysis of NifH1 from *Methanocaldococcus jannaschii*

Hao Wu,[a,b] Ye Yuan,[a,b] Jinming Ma[a,b] and Yongxiang Gao[a,b]*

[a]Hefei National Laboratory for Physical Sciences at Microscale and School of Life Sciences, University of Science and Technology of China, 96 Jinzhai Road, Hefei, Anhui 230027, People's Republic of China, and [b]Key Laboratory of Structural Biology, Chinese Academy of Sciences, 96 Jinzhai Road, Hefei, Anhui 230027, People's Republic of China

Correspondence e-mail: yxgao@ustc.edu.cn

Received 7 January 2011
Accepted 26 February 2011

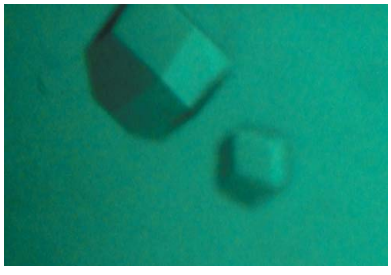
Nitrogen fixation is catalyzed by the nitrogenase complex in *Azotobacter*, which is composed of dinitrogenase and dinitrogenase reductase. Dinitrogenase is an $\alpha_2\beta_2$ heterotetramer of the proteins NifD and NifK. Dinitrogenase reductase is a homodimer of the protein NifH. The expression of NifD/K and NifH nitrogenase homologues (named NflD/K and NflH for Nif-like D and H, respectively) has been detected in the non-nitrogen-fixing hyperthermophilic methanogen *Methanocaldococcus jannaschii*. Solving the structure of *Mj*NifH1 may help in better understanding its function and may supply some clues to understanding the evolution of nitrogenase. The full-length protein with an additional His_6 tag at the C-terminus was expressed, purified and crystallized by the hanging-drop vapour-diffusion method at 287 K. An X-ray diffraction data set was collected to a resolution of 3.3 Å. The crystal belonged to space group $P4_132$, with unit-cell parameters $a = b = c = 139.45$ Å, and was estimated to contain one protein molecule per asymmetric unit.

1. Introduction

Nitrogen fixation, the process that reduces atmospheric nitrogen to ammonia in diazotrophs (Bulen & LeComte, 1966), is an essential process in the global nitrogen cycle and is very important for the metabolism of life on earth (Mancinelli & McKay, 1988; Ferguson, 1998; Halbleib & Ludden, 2000). All nitrogen-fixing organisms are prokaryotic microorganisms such as bacteria and archaea (Raymond *et al.*, 2004).

The key enzymatic reactions in nitrogen fixation are catalyzed by the nitrogenase complex, which is composed of dinitrogenase and dinitrogenase reductase. Dinitrogenase, also called FeMo protein or component I, is an $\alpha_2\beta_2$ heterotetramer of the proteins NifD and NifK (encoded by the genes *nif*D and *nif*K, respectively; Kim *et al.*, 1993). Two unusual [Fe_8S_7] clusters called the P clusters, each of which resides at the α/β subunit interface of dinitrogenase, transfer electrons and protons to the active-site [Fe_7S_8MoN(homocitrate)] cluster (called FeMo-co) in the α subunit (NifD) to reduce dinitrogen and produce ammonia (Chan *et al.*, 1993; Peters *et al.*, 1997). Dinitrogenase reductase, also called Fe protein or component II, the function of which is to transfer electrons to the dinitrogenase, is a homodimer of the protein NifH (encoded by the gene *nif*H) with a [4Fe–4S] cluster bridging the two subunits (Georgiadis *et al.*, 1992).

Another essential process for the metabolism of life on earth is photosynthesis, which is part of the global carbon cycle. In the process of photosynthesis, the enzymatic complex BchLNB is needed during the transformation of protochlorophyllide (pchlide) into chlorophyllide through the reduction of ring D (the fourth pyrrole ring of pchlide) and the BchXYZ system is needed during the transformation of chlorophyllide into bacteriochlorophyllide through the reduction of ring B (the second pyrrole ring of pchlide). BchL and BchX are NifH homologues, and BchNB and BchYZ are NifD and NifK homologues, respectively (Burke *et al.*, 1993; Fujita *et al.*, 1993; Fujita & Bauer, 2000).



Recently, studies have shown that *nif*H-like and *nif*D/*nif*K-like genes (also named *nfl*H and *nfl*D, where *nfl* stands for *nif*-like) are scattered among all known methanogens and some phototrophs (Raymond *et al.*, 2004), but the proteins that they encode (named NflH and NflD) do not have nitrogen-fixation function. Two proteins from the methanogen *Methanocaldococcus jannaschii* have been shown to be NflH proteins and were named *Mj*NifH1 (MJ0879, NCBI reference sequence NP_247874.1) and *Mj*NifH2 (MJ0685, NCBI reference sequence NP_247669.1), respectively (Staples *et al.*, 2007). Although *Mj*NifH1 and *Mj*NifH2 share 24% and 51% sequence identity with functional NifH from *Azotobacter vinelandii* (*Av*NifH; Schmid *et al.*, 2002), respectively, they do not have nitrogen-fixation function. According to evolutionary research, these nitrogenase homologues may be ancestors of nitrogenase and may play important roles in the evolution of nitrogenase. It is interesting that *Mj*NifH1 has two conserved residues (Cys94 and Cys130) that participate in liganding the [4Fe–4S] cluster in *Av*NifH and other dinitrogenase reductases, while *Mj*NifH2 does not have these residues. *Mj*NifH2 has recently been crystallized in our laboratory (Huang *et al.*, 2011).

In the present study, we report the cloning, expression, purification, crystallization and preliminary crystallographic analysis of *Mj*NifH1. Solving the structure of *Mj*NifH1 may help us to better understand its function and may supply some clues to understanding the evolution of nitrogenase.

2. Materials and methods

2.1. Protein expression and purification

The gene (GeneID 1451768) encoding full-length *Mj*NifH1 (residues 1–279) was amplified by polymerase chain reaction (PCR) from *M. jannaschii* genomic DNA with the primer pair 5′-CGC**CATATG**ATGAGAAAATTTTGTGTCTATGG-3′ (*Nde*I restriction site shown in bold) and 5′-CCG**CTCGAG**TCCTTTAACACTCTCTTTTAAAAG-3′ (*Xho*I restriction site shown in bold). The PCR product was digested with *Nde*I and *Xho*I and inserted into the pET22b (Novagen) vector opened using the same enzymes. The resulting plasmid was confirmed by DNA-sequencing analysis.

The recombinant plasmid pET22b*Mj*NifH1 was transformed into *Escherichia coli* Rosetta (DE3) cells (Novagen). The cells were cultured in Luria–Bertani medium supplemented with 100 µg ml^{-1} ampicillin at 310 K until the $OD_{600\ nm}$ reached 0.6 and were then induced with 1 m*M* isopropyl *β*-D-1-thiogalactopyranoside (IPTG) for a further 4 h at the same temperature. After harvesting by centrifugation at 8000*g* for 8 min, the cells were resuspended in lysis buffer (20 m*M* Tris–HCl pH 8.0, 200 m*M* NaCl) and lysed by ultrasonication on ice. The supernatant was separated from the cell fragments by centrifugation at 15 000*g* for 30 min at 277 K and then loaded onto a nickel-chelating column (GE Healthcare) pre-equilibrated with lysis buffer. The column was washed with 40 ml washing buffer (20 m*M* Tris–HCl pH 8.0, 200 m*M* NaCl, 70 m*M* imidazole). The target protein containing a His_6 tag was eluted with elution buffer (20 m*M* Tris–HCl pH 8.0, 200 m*M* NaCl, 350 m*M* imidazole). After concentration by centrifugal ultrafiltration (Millipore, 10 kDa cutoff), the target protein was further purified using a HiLoad 16/60 Superdex 200 size-exclusion column (GE Healthcare) pre-equilibrated with lysis buffer. The pure target protein was concentrated to 26 mg ml^{-1}. All steps were performed at 277 K and the homogeneity of *Mj*NifH1 was determined by SDS–PAGE (Fig. 1).

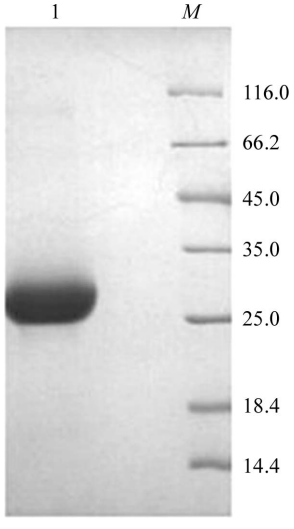


Figure 1
SDS–PAGE of *Mj*NifH1. Lane 1, purified *Mj*NifH1 (about 30 kDa); lane *M*, molecular-weight markers (kDa).

Table 1
Crystal parameters and data-collection statistics for the crystal of *Mj*NifH1.

Values in parentheses are for the last shell.

Resolution range (Å)	50.00–3.30 (3.36–3.30)
Wavelength (Å)	0.9194
Space group	$P4_132$
Unit-cell parameters (Å, °)	$a = b = c = 139.45$, $\alpha = \beta = \gamma = 90$
Unique reflections	7470
Multiplicity	9.4 (8.1)
Mean $I/\sigma(I)$	24.1 (3.1)
Completeness (%)	99.4 (99.7)
B factor from Wilson plot (Å^2)	90.8
R_{merge}† (%)	8.3 (60.0)

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the *i*th observation of reflection *hkl* and $\langle I(hkl)\rangle$ is the weighted average intensity of all observations *i* of reflection *hkl*.

2.2. Crystallization

Initial crystallization experiments were set up by a Mosquito Crystallization Robot (TTP LabTech) using the hanging-drop vapour-diffusion method at 287 K. Each hanging drop consisted of 0.15 µl reservoir solution and 0.15 µl protein solution (26 mg ml^{-1} in buffer consisting of 20 m*M* Tris–HCl pH 8.0, 200 m*M* NaCl) and was equilibrated against 150 µl reservoir solution. Microcrystals appeared in 2 d in several conditions and the crystals obtained using 40%(*v*/*v*) 2-methyl-2,4-pentanediol (MPD), 0.1 *M* Tris–HCl pH 8.0 had the best shape. After several rounds of optimization of buffer pH, precipitant concentration and protein concentration, a well diffracting single crystal was obtained using reservoir solution consisting of 38%(*v*/*v*) MPD, 0.12 *M* Tris–HCl pH 8.2 at 277 K; the concentration of the target protein was 20 mg ml^{-1}.

2.3. Diffraction data collection and processing

The crystal of *Mj*NifH1 was harvested and soaked in cryoprotectant consisting of 20 m*M* Tris–HCl pH 8.0, 200 m*M* NaCl, 20%(*v*/*v*) glycerol for several seconds. The crystal was then flash-cooled to 100 K in a stream of cold nitrogen gas and subjected to X-ray diffraction data collection at 100 K on beamline BL17U of the SSRF (Shanghai Synchrotron Radiation Facility). A complete diffraction data set consisting of 165 images was collected from a single crystal with an oscillation angle of 1° per image. The diffraction data were indexed, integrated and scaled using *HKL*-2000 (Otwinowski &

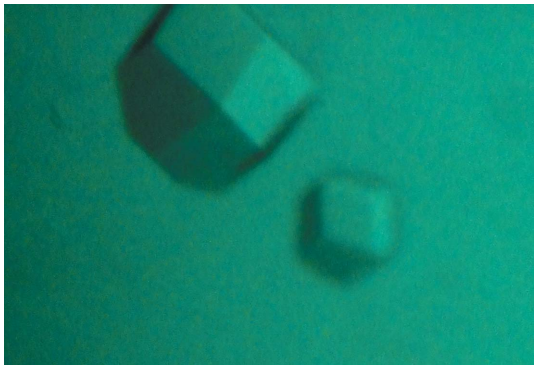


Figure 2
Crystals of *Mj*NifH1. The crystal dimensions were 0.2 × 0.2 × 0.2 mm.

Figure 3
X-ray diffraction pattern collected from a crystal of *Mj*NifH1.

Minor, 1997). Data-collection and processing statistics are listed in Table 1.

3. Results and discussion

*Mj*NifH1 was cloned, expressed and purified to about 95% purity as monitored by SDS–PAGE (Fig. 1). When *Mj*NifH1 was purified by gel filtration both monomer and dimer peaks were observed. A similar phenomenon was observed with purified *Mj*NifH2 (Huang *et al.*, 2011). Crystal screening was performed for both monomeric and dimeric *Mj*NifH1, but only the monomer could be crystallized. After several rounds of optimization, a single crystal of dimensions 0.2 × 0.2 × 0.2 mm was obtained (Fig. 2) and diffracted to a resolution of 3.3 Å (Fig. 3). Matthews analysis suggested that there was one molecule per asymmetric unit in the *Mj*NifH1 crystal (Matthews coefficient 3.52 $Å^3$ Da^{-1}, solvent content 65.12%; Matthews, 1968). The crystal of *Mj*NifH1 belonged to the primitive cubic Bravais lattice type. Because of the high crystal symmetry, it was difficult to determine the final space group. Molecular replacement was performed using the program *Phaser* (McCoy *et al.*, 2007) with the structure of *Av*NifH (PDB entry 1m34; Schmid *et al.*, 2002) as the search model. A single correct solution was only found when data scaled in $P4_132$ were used, which indicated that the crystal of *Mj*NifH1 belonged to space group $P4_132$.

Phaser produced a single solution with a translation-function *Z* score (TFZ) of 15.4. Obvious and continuous density for the secondary structure could be observed. The indications above both demonstrated that the molecular-replacement solution was genuine. Refinement of the structure of *Mj*NifH1 is ongoing.

We appreciate the assistance of Drs Ling Tang and Jianhua He at beamline BL17U of SSRF during data collection. This work was supported by grants from the Chinese National Natural Science Foundation (grant No. 10979039), the Chinese Ministry of Science and Technology (grant Nos. 2006CB806500, 2006CB910200 and 2006AA02A318) and the Chinese Academy of Sciences (grant No. KSCX2-YW-R-60).

References

Bulen, W. A. & LeComte, J. R. (1966). *Proc. Natl Acad. Sci. USA*, **56**, 979–986.
Burke, D. H., Hearst, J. E. & Sidow, A. (1993). *Proc. Natl Acad. Sci. USA*, **90**, 7134–7138.
Chan, M. K., Kim, J. & Rees, D. C. (1993). *Science*, **260**, 792–794.
Ferguson, S. J. (1998). *Curr. Opin. Chem. Biol.* **2**, 182–193.
Fujita, Y. & Bauer, C. E. (2000). *J. Biol. Chem.* **275**, 23583–23588.
Fujita, Y., Matsumoto, H., Takahashi, Y. & Matsubara, H. (1993). *Plant Cell Physiol.* **34**, 305–314.
Georgiadis, M. M., Komiya, H., Chakrabarti, P., Woo, D., Kornuc, J. J. & Rees, D. C. (1992). *Science*, **257**, 1653–1659.
Halbleib, C. M. & Ludden, P. W. (2000). *J. Nutr.* **130**, 1081–1084.
Huang, K., Ma, J., Yuan, Y. & Gao, Y. (2011). *Acta Cryst.* F**67**, 133–135.
Kim, J., Woo, D. & Rees, D. C. (1993). *Biochemistry*, **32**, 7104–7115.
Mancinelli, R. L. & McKay, C. P. (1988). *Orig. Life Evol. Biosph.* **18**, 311–325.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
McCoy, A. J., Grosse-Kunstleve, R. W., Adams, P. D., Winn, M. D., Storoni, L. C. & Read, R. J. (2007). *J. Appl. Cryst.* **40**, 658–674.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Peters, J. W., Stowell, M. H., Soltis, S. M., Finnegan, M. G., Johnson, M. K. & Rees, D. C. (1997). *Biochemistry*, **36**, 1181–1187.
Raymond, J., Siefert, J. L., Staples, C. R. & Blankenship, R. E. (2004). *Mol. Biol. Evol.* **21**, 541–554.
Schmid, B., Einsle, O., Chiu, H. J., Willing, A., Yoshida, M., Howard, J. B. & Rees, D. C. (2002). *Biochemistry*, **41**, 15557–15565.
Staples, C. R., Lahiri, S., Raymond, J., Von Herbulis, L., Mukhophadhyay, B. & Blankenship, R. E. (2007). *J. Bacteriol.* **189**, 7392–7398.