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

Cloning, expression, purification, crystallization and preliminary crystallographic analysis of NifH2 from *Methanocaldococcus jannaschii*

Kai Huang,[a,b] Jinming Ma,[a,b] Ye Yuan[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 9 October 2010
Accepted 18 November 2010

Nitrogenases are protein complexes that are only found in *Azotobacter* and are required for biological nitrogen fixation. They are made up of a nitrogenase, which is a $NifD_2/NifK_2$ heterotetramer, and a nitrogenase reductase, which is a homodimer of NifH. Many homologues of nitrogenase have been found in various non-nitrogen-fixing prokaryotes; in particular, they are found in all known methanogens. This indicates that these homologues may play a role in methane production. Here, the cloning of NifH2, a homologue of the NifH nitrogenase component, from *Methanocaldococcus jannaschii* (*Mj*NifH2) and its expression in *Escherichia coli* with a polyhistidine tag, purification and crystallization are described. *Mj*NifH2 crystals were obtained by the hanging-drop vapour-diffusion method and diffracted to a resolution limit of 2.85 Å. The crystals belonged to space group $P2$, with unit-cell parameters $a = 64.01$, $b = 94.38$, $c = 98.08$ Å, $\alpha = \gamma = 90$, $\beta = 98.85^\circ$.

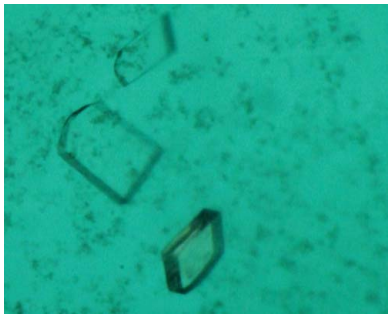
1. Introduction

Nitrogen is an essential element for life. Only fixed nitrogen can be used in metabolism and the major pathways of nitrogen fixation are biological fixation and fixation during lightning discharge. Biologically fixed nitrogen production currently far exceeds production owing to lightning discharge (Falkowski, 1997; Raymond *et al.*, 2004).

All nitrogen-fixing organisms that have been characterized to date are prokaryotes and their capacity for nitrogen fixation is dependent solely on the organism possessing a nitrogenase enzyme system (Simpson & Burris, 1984). A typical nitrogenase complex is made up of two components: a nitrogenase and a nitrogenase reductase (Staples *et al.*, 2007). Nitrogenases, which are also known as FeMo proteins, are $\alpha_2\beta_2$ heterotetramers of the proteins NifD and NifK (Kim *et al.*, 1993). They contain a P cluster (an unusual cluster which contains Fe_8S_7) and an active-site cluster (FeMo-co, containing Fe_8S_7MoN), the function of which is to reduce dinitrogen and produce ammonia (Chan *et al.*, 1993; Tittsworth & Hales, 1993; Peters *et al.*, 1997). Nitrogenase reductase, which is also known as the Fe protein, is a homodimer of NifH and has an Fe_4S_4 cluster bound between two subunits (Georgiadis *et al.*, 1992; Kim & Rees, 1994). Nitrogen reductase is responsible for transferring electrons to the nitrogenase, allowing the reduction of dinitrogen to ammonia.

Recently, many atypical sequences related to *nifH* and *nifD*/*nifK* genes have been uncovered in various non-nitrogen-fixing organisms by analysis of their published complete genomes (Raymond *et al.*, 2004; Staples *et al.*, 2007). These genes were named *nflH* and *nflD* (nfl for Nif-like) and the proteins that they encode were named NflH and NflD, respectively (Staples *et al.*, 2007). These proteins, which are classified as group VI nitrogenases, have been discovered in all known methanogens (Raymond *et al.*, 2004). Nitrogen fixation is not attributed to these proteins and their biological function remains unknown.

Photosynthesis is one of the most important biochemical processes on earth. In this process, solar energy is converted to chemical energy by plants and phototrophs. Interestingly, homologues of Nifs have been linked to two photosynthetic processes (Burke *et al.*, 1993; Fujita



et al., 1993; Fujita & Bauer, 2000). The transformation of protochlorophyllide to chlorophyllide by reduction of ring D is accomplished using the enzymatic complex BchLNB and transformation of chlorophyllide to bacteriochlorophyllide by reduction of ring B is accomplished using a system containing BchXYZ (Fujita & Bauer, 2000). Both BchLNB and BchXYZ are homologues of NifHDK: BchL and BchX are homologues of NifH, while BchNB and BchYZ are homologues of NifDK.

*Mj*NifH2 (coded by gene MJ0685) is one of two homologues of NifH found in *Methanocaldococcus jannaschii* (the other is NflH, which is coded by gene MJ0879). It has only 24% sequence identity to NflH, but they share a conserved domain. Evolutionary research has shown that these nitrogenase homologues may be ancestors of nitrogenase. These homologous proteins are thought to have first occurred in archaebacteria before being transferred into the bacterial domain (Raymond *et al.*, 2004).

Solving the structure of *Mj*NifH2 would help us to better understand the process of methane production. In addition, comparison of the structures of NifH2 from *M. jannaschii*, NifH from *Azotobacter* and BchL and BchX from phototrophs may lead to elucidation of the evolutionary relationship between these homologous proteins.

2. Materials and methods

2.1. Strains and plasmids

The MJ0685 coding sequence was PCR-amplified by the use of *Ex Taq* (TaKaRa) and the primer pair (5′ to 3′) MJ0685/F (CGC**GGATCC**CATATGATTGCTGTGAGTGGAAAAG; *Bam*HI restriction site shown in bold) and MJ0685/R (CCG**CTCGAG**TTAAAATTTTTTATTAATTATCTTCTCAGC; *Xho*I restriction site shown in bold). The amplified DNA was digested with *Bam*HI and *Xho*I and cloned into pET28a (Novagen) to obtain the plasmid pET28a*Mj*NifH2.

2.2. Protein expression and purification

Escherichia coli BL21 (DE3) (Stratagene) was used as the expression host. The recombinant plasmid was transformed into *E. coli* BL21 (DE3) cells. The cells were first cultured in Luria–Bertani medium containing 100 µg ml^{-1} kanamycin at 310 K until an OD_{600} of 0.8 was achieved. To overexpress the protein, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.2 m*M* in the medium. Cells were then cultured for a further 4 h at the same temperature.

The cells were harvested by centrifugation, resuspended in buffer *A* (20 m*M* Tris–HCl, 500 m*M* NaCl pH 8.0) and lysed by sonication. The supernatant containing *Mj*NifH2 was separated from the cell lysate by centrifugation at 12 000*g* for 30 min. The supernatant was then loaded onto a nickel-chelating column (GE Healthcare) pre-equilibrated with buffer *A*. Ten column volumes of buffer *A* were used to wash the column and ten column volumes of buffer *A* containing 50 m*M* imidazole were then used to elute nonspecifically bound proteins. Finally, *Mj*NifH2 was eluted using buffer *A* containing 400 m*M* imidazole.

The eluted *Mj*NifH2 solution was concentrated by ultrafiltration. Upon concentration, the NifH2 solution exhibited a brown colour, indicating the presence of Fe–S cluster-containing proteins. The colour disappeared after the addition of 10 m*M* β-mercaptoethanol to the protein solution. The concentrated *Mj*NifH2 solution was then loaded onto a Superdex 200 16/60 column (GE Healthcare) pre-equilibrated with buffer *A* containing 10 m*M* β-mercaptoethanol. The fraction containing the 30.3 kDa monomer of *Mj*NifH2 was collected, concentrated and stored in 2 m*M* Tris–HCl, 50 m*M* NaCl pH 8.0. SDS–PAGE showed that the purity of NifH2 was greater than 95% (Fig. 1). The concentration of *Mj*NifH2 was 20 mg ml^{-1} as measured using a BCA Protein Assay Kit (Pierce).

2.3. Crystallization and data collection

Crystallization experiments were performed using the hanging-drop vapour-diffusion method. ProPlex (Molecular Dimensions) was used to screen initial crystallization conditions. Microcrystals were observed in condition 2.2 of the ProPlex kit [0.1 *M* sodium citrate, 8%(*w*/*v*) PEG 8000 pH 5.0]. Further optimization of the crystallization conditions was carried out using 1 µl protein solution mixed with 1 µl reservoir solution (0.1 *M* sodium citrate, 8% PEG 8000 pH 5.0) and equilibrated against 150 µl reservoir solution. After 10 d, single crystals which showed good diffraction properties appeared at a constant temperature of 277 K (Fig. 2). The largest crystal obtained was approximately 0.2 × 0.2 × 0.3 mm in size and was colourless.

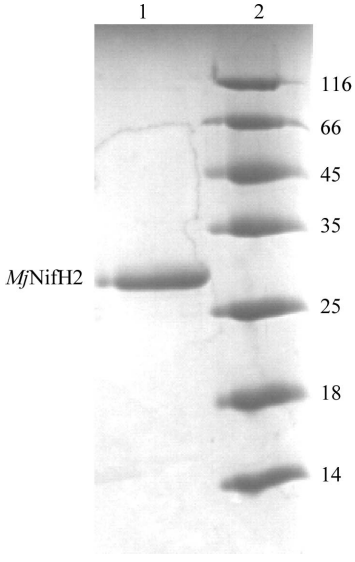


Figure 1
SDS–PAGE of *Mj*NifH2. Lane 1, purified *Mj*NifH2 (after β-mercaptoethanol treatment). Lane 2, molecular-weight markers (kDa).

Figure 2
Photomicrograph of crystals of *Mj*NifH2 formed using the hanging-drop method with 0.1 *M* sodium citrate, 8% PEG 8000 pH 5.0.

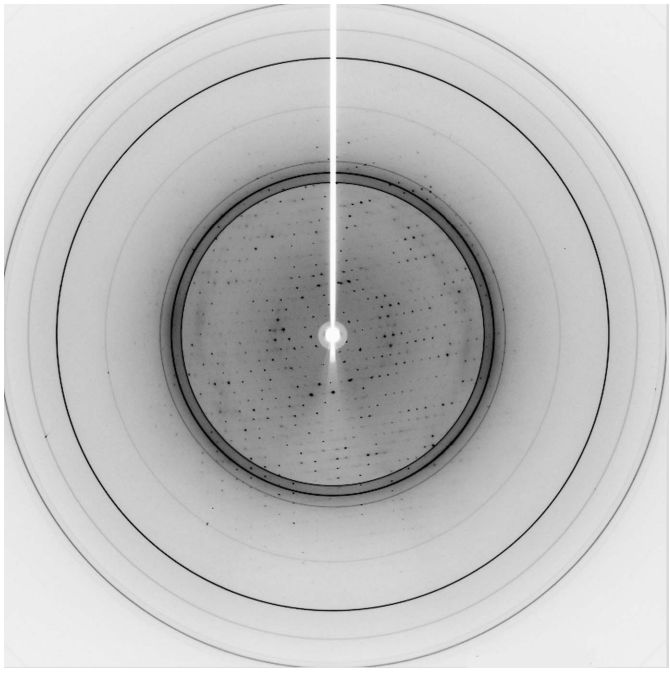


Figure 3
Image of the diffraction to highest resolution.

Crystals were mounted on a CrystalCap with a cryoprotectant consisting of 0.1 *M* sodium citrate pH 5.0, 8% PEG 8000, 20% glycerol and then directly flash-cooled at 100 K in a stream of nitrogen gas. X-ray diffraction data were collected using an MX-225 CCD image-plate detector (MAR Research) on BL17U of the SSRF (Shanghai Synchrotron Radiation Facility; Fig. 3). The oscillation angle was 1° per image and the exposure time was 2 s. A complete diffraction data set consisting of 180 images was collected using one crystal at a crystal-to-detector distance of 200 mm.

Diffraction data were processed using *HKL*-2000 (Otwinowski & Minor, 1997). Data-collection and processing statistics are listed in Table 1.

Table 1
Statistics of data collection and reduction.

Values in parentheses are for the highest resolution shell.

No. of crystals	1
Wavelength (Å)	1.0
Temperature (K)	100.0
Rotation range per frame (°)	1
Total rotation range (°)	180
Exposure per frame (s)	2
Crystal-to-detector distance (mm)	200
Mosaicity (°)	0.89
Multiplicity	3.6
Space group	*P*2
Unit-cell parameters (Å, °)	a = 64.01, b = 94.38, c = 98.08, β = 98.85
Molecules per asymmetric unit	4
Resolution limits (Å)	40–2.85 (2.90–2.85)
Observations	93684
Independent reflections	26743
Completeness† (%)	93.4 (98.6)
$\langle I/\sigma(I)\rangle$	14.7 (2.61)
$R_{\rm merge}$‡	0.083 (0.354)

† The completeness is the ratio of the number of reflections to the number of possible reflections. ‡ $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the observed intensity of reflection *hkl* and $\langle I(hkl)\rangle$ is the mean intensity of reflection *hkl*.

3. Results and discussion

Previous research has shown that NifHs form a homodimer in solution with the iron ion located between two subunits (Kim & Rees, 1994; Lawson & Smith, 2002). Superdex 200 16/60 chromatography indicated that the *Mj*NifH2 protein was present as a mixture of two oligomeric states in solution, with both a dimeric and a monomeric state occurring. The chromatogram showed that these two forms were not fully separable under the chromatographic conditions used. SDS–PAGE and mass-spectrometric analysis confirmed the presence of two oligomeric states of the *Mj*NifH2 protein. After 10 m*M* β-mercaptoethanol was added to the protein solution, the dimer form disappeared and all protein was present as the monomer. This homogeneous protein was used for crystallization.

*Mj*NifH2 crystallized in space group *P*2, with unit-cell parameters a = 64.01, b = 94.38, c = 98.08 Å, $\alpha = \gamma = 90$, β = 98.85°. The X-ray data were alternatively scaled in space group $P2_1$, but analysis of the systematic absences showed that about two-thirds of the intensities of systematic absences were near zero, while the other third were nonzero. Therefore, it cannot be determined whether the crystal belongs to space group $P2_1$ or *P*2. Matthews analysis suggested that there were four molecules per asymmetric unit (Matthews coefficient 2.44 Å^3 Da^{-1}, solvent content 49.6%). Analysis of a self-rotation function shows the same result. This data analysis laid the foundation for a full structure solution, which will be presented in a subsequent report.

Financial support for this project was provided by research grants from the Junior Scientist Funds of USTC (grant No. KA207000007), the Chinese National Natural Science Foundation (grant Nos. 30025012 and 10979039) and the Chinese Ministry of Science and Technology (grant Nos. 2006CB806500, 2006CB910200 and 2006AA02A318). This article was prepared with the help of International Science Editing.

References

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.
Falkowski, P. G. (1997). *Nature (London)*, **387**, 272–275.
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.
Kim, J. & Rees, D. C. (1994). *Biochemistry*, **33**, 389-397.
Kim, J., Woo, D. & Rees, D. C. (1993). *Biochemistry*, **32**, 7104–7115.
Lawson, D. M. & Smith, B. E. (2002). *Met. Ions Biol. Syst.* **39**, 75–119.
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.
Simpson, F. B. & Burris, R. H. (1984). *Science*, **224**, 1095–1097.
Staples, C. R., Lahiri, S., Raymond, J., Von Herbulis, L., Mukhophadhyay, B. & Blankenship, R. E. (2007). *J. Bacteriol.* **189**, 7392–7398.
Tittsworth, R. C. & Hales, B. J. (1993). *J. Am. Chem. Soc.* **115**, 9763–9767.