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Cloning, purification and preliminary X-ray crystallographic analysis of a hypothetical protein, MJ0754, from *Methanococcus jannaschii* DSM 2661

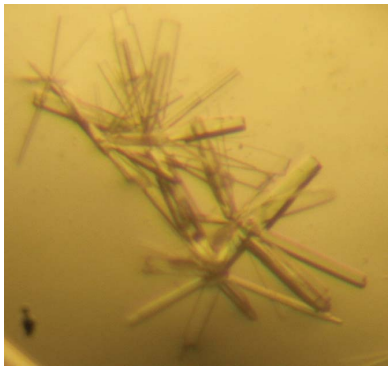
The protein encoded by the *MJ0754* gene from the archaeon *Methanococcus jannaschii* DSM 2661 is an unknown hypothetical protein. Two recombinant proteins, MJ0754 (residues 1–185) and MJ0754t (a truncated form of MJ0754, residues 11–185), were cloned from *MJ0754*, overexpressed as His-tag fusion proteins and purified. The crystals were found to grow under two different conditions and to have two different shapes. The crystal of MJ0754 belonged to space group $P6_1$, with unit-cell parameters $a = b = 127.015$, $c = 48.929$ Å, a calculated Matthews coefficient of 2.85 Å^3 Da^{-1} and two molecules per asymmetric unit. The crystal of MJ0754t belonged to space group $C222_1$, with unit-cell parameters $a = 51.915$, $b = 79.122$, $c = 93.869$ Å, a calculated Matthews coefficient of 2.41 Å^3 Da^{-1} and one molecule per asymmetric unit. The SeMet-labelled $P6_1$ crystal diffracted to a resolution of 3.1 Å, while the native $C222_1$ crystal diffracted to 1.3 Å resolution.

1. Introduction

A *BLAST* search of the NCBI database using the sequence of *Methanococcus jannaschii* MJ0754 revealed a range of hypothetical proteins. According to the results of the search, MJ0754 is considered to be a member of the ferritin-like diiron-carboxylate proteins. The ferritin-like domain is composed of a four-helix bundle surrounding the diiron site that is coordinated by conserved histidines and additional carboxylate residues (Nordlund & Eklund, 1995). Proteins containing a ferritin-like domain possess ferroxidase activity, which enables catalysis of the oxidation of Fe^{2+} to Fe^{3+} by O_2. Ferroxidase function protects living organisms because free iron is toxic to cells; it acts as a catalyst in the formation of free radicals from reactive oxygen species (Orino *et al.*, 2001). Ferritin functions as an iron-storage protein that maintains iron in a soluble and nontoxic form *via* ferroxidase action (Granier *et al.*, 2003). Ferritin also regulates free intracellular iron levels by sequestering up to 4500 atoms of iron in its hollow core (Harrison & Arosio, 1996).

Ferritin-like diiron proteins contain bacterioferrtin and rubrerythrin (Nordlund & Eklund, 1995). Bacterioferritin is a prokaryotic protein that plays a role in iron detoxification and storage. The structure of bacterioferritin is very similar to that of ferritin, which forms a hollow core from a globular complex consisting of 12 or 24 subunits incorporating 12 haem groups (Dautant *et al.*, 1998). Rubrerythrin, which is isolated from anaerobic sulfate-reducing bacteria, is a nonhaem protein that contains an N-terminal diiron-binding domain and a rubredoxin-like C-terminal domain (Van Beeumen *et al.*, 1991). The biomolecular unit of rubrerythrin is a homodimer, thus differing from ferritin. Both proteins have ferroxidase activity, but their physiological roles have not yet been reported. In addition, no studies have been conducted to evaluate the characteristics of MJ0754 and its ferroxidase activity.

To date, two crystal structures of putative ferritin-like diiron-carboxylate proteins have been deposited in the PDB: one from *Thermotoga maritima* (PDB code 1vjx; Joint Center for Structural Genomics, unpublished work) and one from *Bacillus anthracis* strain Ames (PDB code 2qqy; Y. Kim, G. Joachimiak, R. Wu, S. Patterson, P.



Gornicki & A. Joachimiak, unpublished work). These proteins are still considered to be unknown proteins as no biological information regarding them has been published to date. Both proteins were found to have a low sequence identity with MJ0754 of less than 20%. To provide further structural data regarding the function of ferritin-like diiron-carboxylate proteins, we initiated the determination of the three-dimensional structure of MJ0754 from *M. jannaschii*, which is composed of 185 amino-acid residues (M_r = 20 000). MJ0754t (residues 11–185) was also evaluated. In this study, we overexpressed, purified and crystallized two constructs of MJ0754 and then conducted a preliminary X-ray crystallographic analysis.

2. Experimental procedures

2.1. Cloning, overexpression and purification

The *MJ0754* gene was amplified *via* PCR using the *M. jannaschii* strain DSM 2661 genomic DNA as a template and the following primers: 5′-GGAATTC**CATATG**ATGCTTGAATATATAAGCTCATTACCAAAA-3′ (forward) and 5′-CCG**CTCGAG**CCTATTCATTCCCCTCTCCGTAGA-3′ (reverse). The bases in bold indicate the *Nde*I and *Xho*I digestion sites, respectively. The amplified DNA was then inserted into the *Nde*I/*Xho*I-digested expression vector pET-22b (Novagen), which contains LEHHHHHH at the C-terminus for ease of purification. MJ0754t (residues 11–185) was amplified by PCR using the primers 5′-GGAATTC**CATATG**CAACCAATAAGTGAAGAGGAAAAAGAGG-3′ (forward) and 5′-CCG**CTCGAG**TCACCTATTCATTCCCCTCTCCG-3′ (reverse). The amplified DNA was then inserted into the *Nde*I/*Xho*I-digested expression vector pET-28a (Novagen) to produce recombinant protein with a hexahistidine tag and a thrombin cleavage site at the N-terminus (MGSSHHHHHHSSGLVPRGSHM). The sequences of both proteins were confirmed by nucleotide sequencing.

For protein expression, transformed *Escherichia coli* BL21 (DE3) cells (Novagen) were grown at 310 K to an OD_{600} of 0.6 in LB medium containing antibiotics (50 µg ml^{-1} ampicillin for MJ0754 and 50 µg ml^{-1} kanamycin for MJ0754t). Protein expression was then induced by the addition of 0.5 m*M* isopropyl β-D-1-thiogalactopyranoside (IPTG), after which cell growth was continued at 295 K overnight. The cells were then harvested by 15 min centrifugation at 4200*g* and 277 K. Next, the harvested cell pellets were resuspended in ice-cold buffer *A* [20 m*M* Tris–HCl pH 7.5, 500 m*M* NaCl, 4 m*M* $MgCl_2$, 5%(*v*/*v*) glycerol and 5 m*M* imidazole] and then disrupted using a sonicator (Sonics, USA). The cell debris was removed by centrifugation at 21 000*g* (Hanil Supra 21K rotor) for 40 min at 277 K. Next, the supernatant was loaded onto a HisTrap HP column (GE Healthcare) equilibrated with buffer *A* and the protein was eluted using a gradient to 500 m*M* imidazole in buffer *A*. Eluted fractions containing MJ0754 were pooled and concentrated at 2000*g* and 277 K. Gel filtration was performed using a Superdex 200 16/60 (GE Healthcare) prep-grade column equilibrated with 20 m*M* Tris–HCl pH 7.5 and 100 m*M* NaCl. The protein applied onto the gel-filtration column was at a concentration of 42 mg ml^{-1} as estimated by the Bradford assay (Bradford, 1976). The homogeneity of the purified protein was then assessed by SDS–PAGE, after which the protein was concentrated to a final concentration of 20 mg ml^{-1} using a Centriprep centrifugal filter (Millipore) for crystallization trials. The final protein yield was 20 mg per litre. Preparation of SeMet-labelled protein was conducted with *E. coli* B834 (DE3) cells (Novagen) using the same procedures as used for MJ0754.

2.2. Crystallization and preliminary X-ray analysis

Initial crystallization screening was conducted at 295 K *via* 96-well sitting-drop Intelli-Plates (Art Robbins) using the Hydra II Plus One system (Matrix Technology) with approximately 500 conditions and a ratio of 300 nl precipitant solution to 300 nl protein solution (20 mg ml^{-1}, 20 m*M* Tris–HCl pH 7.5, 100 m*M* NaCl) over 70 µl well solution. Crystallization trials were performed using the following commercial screening kits: Crystal Screens 1 and 2, Index Screen, SaltRx, PEG/Ion Screen (Hampton Research) and Wizard Screens 1 and 2 (Emerald BioSystems). Of the 500 conditions used, needle-shaped crystals were produced from Hampton Research Index Screen condition D7 [0.1 *M* bis-tris pH 6.5, 25%(*w*/*v*) PEG 3350]. Therefore, this crystallization condition was further optimized *via* the hanging-drop vapour-diffusion method using 24-well VDX plates (Hampton Research). Commercial additive-screening kits (Hampton Research) were used for optimization. The best crystals (Fig. 1*a*) were obtained by the hanging-drop method using drops consisting of 2 µl MJ0754 mixed with 2 µl 0.1 *M* bis-tris pH 6.9 and 22%(*w*/*v*) PEG 3350 containing 10%(*v*/*v*) 0.1 *M* hexammine cobalt(III) chloride after 3 d. A crystal formed by MJ0754 was cryoprotected by soaking it for 10 s in 10 µl cryoprotectant solution consisting of 0.1 *M* bis-tris pH 6.9, 22%(*w*/*v*) PEG 3350 and 30%(*v*/*v*) glycerol and flash-frozen in liquid nitrogen. SeMet-labelled MJ0754 crystals were obtained using the condition used for the native crystal. For data collection, a single crystal was mounted on a goniometer in a stream of cold nitrogen at 100 K. Single-wavelength anomalous diffraction (SAD) data were

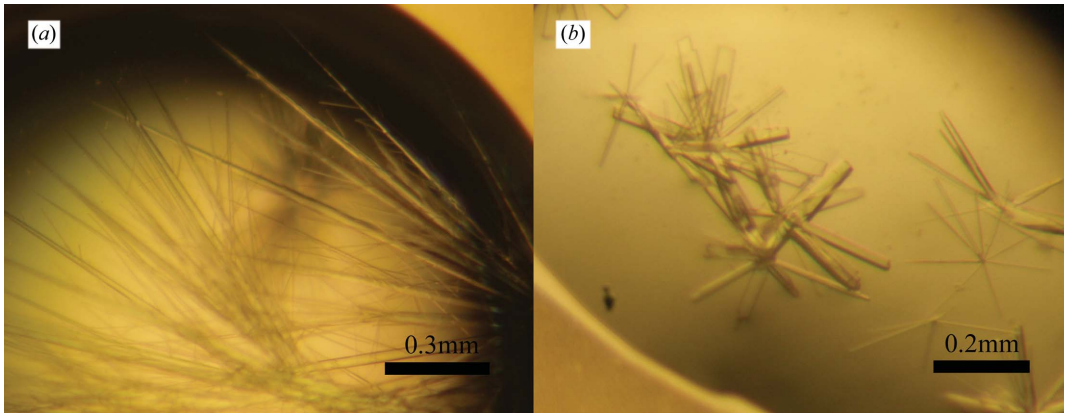


Figure 1
Crystals of MJ0754 and MJ0754t. (*a*) Needle crystals of native MJ0754. The largest crystal had dimensions of approximately 0.4 × 0.05 × 0.05 mm. (*b*) Orthorhombic crystals of native MJ0754t. The largest crystal had dimensions of approximately 0.2 × 0.1 × 0.1 mm.

Table 1
Data-collection statistics for MJ0754 and MJ0754t.

Values in parentheses are for the highest resolution shell.

	MJ0754	MJ0754t
Wavelength (Å)	0.97958	1.0000
Beamline	BL17A, PF	4A, PLS
Resolution (Å)	50.0–3.10 (3.24–3.10)	50.0–1.34 (1.39–1.34)
Space group	$P6_1$	$C222_1$
Unit-cell parameters (Å)	$a = b = 127.015$, $c = 48.929$	$a = 51.915$, $b = 79.122$, $c = 93.869$
No. of observations	124495	231084
No. of unique reflections	9543	40164
$R_{\rm merge}$† (%)	14.7 (39.2)	5.6 (19.2)
Completeness (%)	91.8 (93.4)	91.5 (52.7)
Average $I/\sigma(I)$	17.3 (2.3)	54.0 (3.2)
Redundancy	7.1	5.8

† $R_{\rm merge} = 100 \times \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the observed intensity of an individual reflection and $\langle I(hkl)\rangle$ is the mean intensity of that reflection.

collected from an SeMet crystal using an ADSC Quantum 315 CCD camera on beamline BL-17A of the Photon Factory at the High Energy Acceleration Research Organization (PF; Japan). The crystal was oscillated by 1.0° per frame over a total range of 240° at the SeMet peak wavelength of 0.9795 Å.

Rod-like crystals (Fig. 1*b*) of MJ0754t were obtained after two weeks using the conditions 0.1 *M* bis-tris propane pH 7.0, 22% PEG 3350. The crystals were cryoprotected by soaking them for 10 s in 10 µl cryoprotectant solution containing 0.1 *M* bis-tris propane pH 7.0, 22%(*w*/*v*) PEG 3350 and 25%(*v*/*v*) glycerol. X-ray diffraction data were collected from the cooled crystals using an ADSC Quantum CCD 210 detector on beamline 4A MXW at Pohang Light Source (PLS; South Korea). The crystal was oscillated by 1.0° per frame over a total range of 200° at a wavelength of 1.0000 Å, the range being limited by the available beam time. The data were processed using the *HKL*-2000 program (Otwinowski & Minor, 1997).

3. Results and discussion

Two forms of the MJ0754 protein, MJ0754 and MJ0754t, were cloned from the genomic DNA of *M. jannaschii* DM2661, overexpressed using PET vector and purified by metal-affinity chromatography. Protein crystals were generated under different conditions and showed different diffraction qualities. Specifically, the MJ0754 crystal showed poor diffraction quality, while the MJ0754t crystal had high diffraction quality. The two crystals belonged to different space groups and had different solvent contents. An SeMet-labelled MJ0754 crystal diffracted to a maximum resolution of 3.1 Å. Based on the autoindexing and scaling by *HKL*-2000, the space group of the MJ0754 crystal was determined to be $P6_1$ or $P6_5$, with unit-cell parameters $a = b = 127.015$, $c = 48.929$ Å, $\alpha = \beta = 90$, $\gamma = 120°$. These results indicate that two molecules are likely to be present in the asymmetric unit, which has a corresponding calculated Matthews coefficient ($V_{\rm M}$) of 2.85 Å^3 Da^{-1} and a solvent content of 56% (Matthews, 1968). The space group of MJ0754 was assigned as $P6_1$ after structure calculation. The best MJ0754t crystal diffracted to 1.34 Å resolution. The MJ0754t crystal belonged to space group $C222_1$, with unit-cell parameters $a = 51.915$, $b = 79.122$, $c = 93.869$ Å, $\alpha = \beta = \gamma = 90°$. Assuming that one molecule is contained in the asymmetric unit, the $V_{\rm M}$ was calculated to be 2.41 Å^3 Da^{-1} and the solvent content was determined to be 49%. Detailed data-processing statistics are provided in Table 1.

The SAD method was used for phase calculation. In addition, ten selenium sites were located using the *SOLVE* program (Terwilliger & Berendzen, 1999). Refinement of the heavy-atom parameters and phase calculation were also performed using the *SOLVE* program. Currently, approximately half of the residues have been automatically built using the *RESOLVE* program (Terwilliger, 2003). Manual rebuilding is currently in progress.

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