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Expression, purification, crystallization and preliminary X-ray crystallographic analysis of a novel plant-type ferredoxin/thioredoxin reductase-like protein from *Methanosarcina acetivorans*

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The genome of *Methanosarcina acetivorans* contains a gene (*ma1659*) that is predicted to encode an uncharacterized chimeric protein containing a plant-type ferredoxin/thioredoxin reductase-like catalytic domain in the N-terminal region and a bacterial-like rubredoxin domain in the C-terminal region. To understand the structural and functional properties of the protein, the *ma1659* gene was cloned and overexpressed in *Escherichia coli*. Crystals of the MA1659 protein were grown by the sitting-drop method using 2 *M* ammonium sulfate, 0.1 *M* HEPES buffer pH 7.5 and 0.1 *M* urea. Diffraction data were collected to 2.8 Å resolution using the remote data-collection feature of the Advanced Light Source, Lawrence Berkeley National Laboratory. The crystal belonged to the primitive cubic space group *P*23 or $P2_13$, with unit-cell parameters $a = b = c = 92.72$ Å. Assuming the presence of one molecule in the asymmetric unit gave a Matthews coefficient (V_M) of 3.55 Å^3 Da^{-1}, corresponding to a solvent content of 65%.

1. Introduction

Proteomic and genomic analysis of *Methanosarcina acetivorans* revealed the presence of several novel uncharacterized proteins (Li *et al.*, 2007). *M. acetivorans*, a strictly anaerobic methane-producing organism belonging to the domain Archaea, contains a gene (*ma1659*) encoding a protein consisting of two different conserved domains: a domain similar to the plant-type ferredoxin/thioredoxin reductase (FTR) protein catalytic subunit in the N-terminal region and a domain similar to a bacterial-type rubredoxin protein in the C-terminal region (Fig. 1). It is intriguing that in *M. acetivorans* the gene *ma1659* is annotated as a rubredoxin, while a homologous gene (MM3270) from the closest relative *M. mazei* is annotated as an FTR catalytic subunit.

FTR proteins are mostly found in plants (Buchanan, 1992; Droux *et al.*, 1987; Knaff & Hirasawa, 1991) and the cyanobacterium *Syne-*

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MA1659        MSEEEVDK------VYRRLNQEVEKSGYHLNPDVEFTKELVRGLLANERRYGYWSCPCRL
FTR           MTSSDTQNNKTLAAMKNFAEQYAKRTDTYFCSDLSVTAVVIEGLARHKEELGSPLCPCRH
Rubredoxin    MKK-----------------------------------------------------------

MA1659        SADNKEEDLDII--CPCYYRDPDLNDYGACYCALYVS-DEVIRGEKEVESIPERRPPREK
FTR           YEDKEAEVKNTFWNCPCV----PMRERKECHCMLFLTPDNDFAGDAQ-------DIPMET
Rubredoxin    -----------------------------------------------------------

MA1659        REAIRAEEASRAEMMETMEFTGKLSKPVWRCKVCGYL---------------CAMDEAPG
FTR           LEEVKASMA---------------------------------------------------
Rubredoxin    ----------------------------YVCTVCGYEYDPAEGDPDNGVKPGTAFEDVPA

MA1659        --VCPICKARKERFERFM
FTR           ------------------
Rubredoxin    DWVCPICGAPKSEFEP-A
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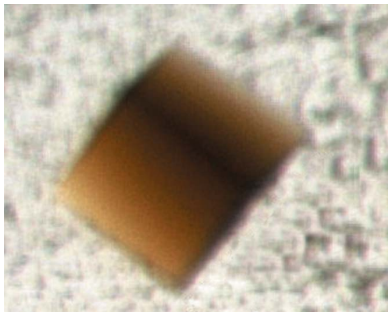
Figure 1
Multiple sequence alignment of *M. acetivorans* MA1659, *Synechocystis* sp. FTR and *D. vulgaris* rubredoxin. Sequence alignment was performed using the *MAFFT* software.



chocystis sp. (Dai *et al.*, 2000; Glauser *et al.*, 2004). FTR is the central regulatory enzyme involved in the reduction of several redox target proteins (Droux *et al.*, 1987). It mainly reduces disulfide bonds present in the target proteins, especially thioredoxins, through a novel two-electron reduction pathway (Knaff, 1989). Ferredoxin (Fd) was identified as the physiological electron donor for this novel FTR protein, hence the name ferredoxin/thioredoxin reductase. Native FTR is composed of two dissimilar subunits: a large catalytic subunit (~30 kDa) and a small variable subunit (~13–16 kDa). The catalytic activity of FTR arises from the presence of a 4Fe–4S cluster and a redox-active disulfide bridge in the catalytic subunit. The function of the variable subunit is still not clear. It is intriguing that without the variable subunit the catalytic subunit is highly unstable and inactive. There are no reports of the characterization of FTR proteins from sources other than plants and *Synecocystis* sp.

Rubredoxins are small soluble nonhaem iron–sulfur proteins (~6 kDa) which are used as electron carriers in anaerobic and aerobic species from the domains Bacteria (Aurich *et al.*, 1976; Gerard *et al.*, 2000; Kok *et al.*, 1989) and Archaea (Fourn *et al.*, 2008). The active site of rubredoxins contains a single Fe atom coordinated to four conserved cysteine S atoms, with a common motif of two pairs of Cys-*X*-*X*-Cys. There are a few exceptions in which high-molecular-weight 1Fe and 2Fe rubredoxins are found (Lee *et al.*, 1997; Silaghi-Dumitrescu *et al.*, 2003). The physiological relevance of rubredoxins is not clearly understood, but they are generally assumed to serve as electron donors. The role of rubredoxin as the physiological electron donor for superoxide reductases and desulfoferredoxin proteins in anaerobes has been reported (Jenney *et al.*, 1999). Rubredoxin can also act as an electron carrier in the alkane-hydroxylation system in aerobic bacteria (Eggink *et al.*, 1987, 1990; Kok *et al.*, 1989). Generally, in anaerobes the rubredoxin gene is often present in the same operon as the superoxide reductases, which are usually located some base pairs downstream of the rubredoxin (Bult *et al.*, 1996; Das *et al.*, 2001; Lumppio *et al.*, 2001; Silva *et al.*, 2001). In *M. acetivorans*, a gene encoding an uncharacterized superoxide reductase (MA3737) is present in an operon or gene cluster containing oxidative-stress proteins (Lessner & Ferry, 2007). The MA1659 gene is located in a different operon away from the superoxide reductase. However, no detailed investigation has been reported for these uncharacterized genes and their functional role has yet to be studied in detail. Here, we present the crystallization and preliminary X-ray analysis of the MA1659 protein.

2. Materials and methods

2.1. Protein expression and purification

The *ma1659* gene of *M. acetivorans* was cloned and overexpressed in *Escherichia coli*. The gene was ligated into pET Blue plasmid vector along with an N-terminal 6×His-tag sequence linked to the *ma1659* gene to give the expression plasmid pMA1659. The expression vector pMA1659 was transformed into *E. coli* Rosetta Blue placI cells. A 10 ml overnight-grown culture of the bacterial cells was used to inoculate a 1 l culture of Luria–Bertani medium containing 100 µg ml^{-1} ampicillin and 30 µg ml^{-1} chloramphenicol and was grown at 298 K. The cells were grown to an optical density of 0.6–0.7 followed by induction with 1 m*M* IPTG and 200 µg ml^{-1} ferric ammonium citrate. After 6 h induction, the cells were harvested by centrifugation at 7000*g* for 15 min at 277 K. HEPES buffer pH 7.5 was used in all purification protocols. The cell pellet was resuspended in 15 ml 50 m*M* buffer. The cell suspension was passed three times through a French pressure cell at a pressure of 1.11 × 10^8 Pa. The resulting cell lysate was centrifuged at 74 400*g* for 30 min at 277 K and the supernatant was diluted to 30 ml with a solution consisting of 500 m*M* NaCl and 20 m*M* imidazole pH 7.5 to give the following composition: 20 m*M* HEPES buffer pH 7.5, 500 m*M* NaCl, 5 m*M* imidazole and cell extract. This solution was loaded onto a 25 ml column (15 mm diameter) of nickel–nitrilotriacetic acid (Ni–NTA) Superflow agarose (GE Healthcare) pre-equilibrated with a 80 ml solution consisting of 20 m*M* HEPES buffer pH 7.5, 500 m*M* NaCl and 5 m*M* imidazole (buffer *A*). The column was washed thoroughly with buffer *A* to remove the weakly bound contaminants; MA1659 protein was eluted from the column with a 100 ml linear gradient of 5–500 m*M* imidazole in 20 m*M* HEPES buffer pH 7.5 and 500 m*M* NaCl. Fractions at about 200 m*M* imidazole contained most of the MA1659 protein and were pooled and concentrated on a YM-3 membrane (Millipore). The protein retained on the membrane was washed three times with 5 ml 20 m*M* HEPES buffer pH 7.5 and recovered in 1 ml of the same buffer. The concentrated and desalted protein was loaded onto a 25 ml column (15 mm diameter) of HiTrap DEAE Sepharose pre-equilibrated with 100 ml 20 m*M* HEPES buffer pH 7.5 and the protein was eluted with a 100 ml linear gradient of 0–500 m*M* NaCl. The protein was eluted in the fractions containing 100–150 m*M* NaCl. These fractions were pooled, concentrated, desalted using a gel-filtration column and diluted in 1 ml 20 m*M* HEPES buffer pH 7.5 as described above.

2.2. Crystallization

For crystallization, the purified His-tagged MA1659 protein was concentrated to 20 mg ml^{-1} in 20 m*M* HEPES buffer pH 7.5. The protein concentration was estimated using the bicinchoninic acid assay method (Wiechelman *et al.*, 1988). Initial crystallization drops were set up with sitting-drop vapour-diffusion geometry using a Phoenix robot (Art Robbins Instruments, USA). The commercial screens tested for crystallization were PEG/Ion, Index HT, Crystal Screen, SaltRX HT and Additive Screen from Hampton Research and Wizard I and II screens from Emerald BioSystems. Prior to drop setup, the protein solution was incubated with 10 m*M* NAD(P)H and 1 m*M* ferrous ammonium sulfate for 24 h at 277 K. This incubation step was crucial to obtain crystals. Each robotic crystallization trial drop consisted of 0.3 µl protein solution mixed with an equal volume of precipitant solution. The plates were incubated in constant-temperature chambers at 294 K. Small brown-coloured crystals (indicative of the presence of Fe) grew within three weeks of setup in condition Nos. 4 and 5 of Index screen. These crystals were reproduced and optimized manually. Larger drops consisting of 4 µl

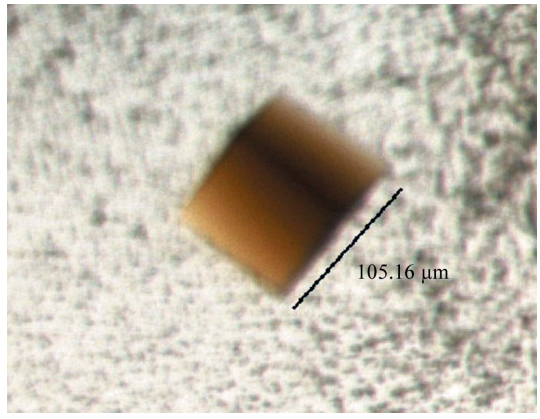


Figure 2
A representative crystal of MA1659 protein.

protein solution and 4 µl precipitant solution were set up with a reservoir volume of 0.5 ml. The best condition for the growth of crystals was condition No. 5 of Index screen: 0.1 *M* HEPES buffer pH 7.5, 2 *M* ammonium sulfate. Crystal soaking times and cryoconditions were optimized to give the best X-ray diffraction. We found that incubation of the crystals in mother liquor with 55%(*v*/*v*) fructose for at least 60 min was required. Upon preliminary X-ray data analysis, these crystals were found to be twinned and diffracted to 3.4 Å resolution. Additive Screen (Hampton Research) was set up with 4 µl protein, 4 µl precipitant (Index screen condition No. 5) and 0.2 µl additive. Several additives (barium chloride dehydrate, calcium chloride dehydrate, L-proline, taurine, sarcosine, urea and xylitol) yielded crystals. Improved crystals were obtained with 0.1 *M* urea as an additive (Fig. 2). Crystal twinning was absent and improved X-ray diffraction to 2.8 Å resolution was observed when using urea as an additive (Fig. 3).

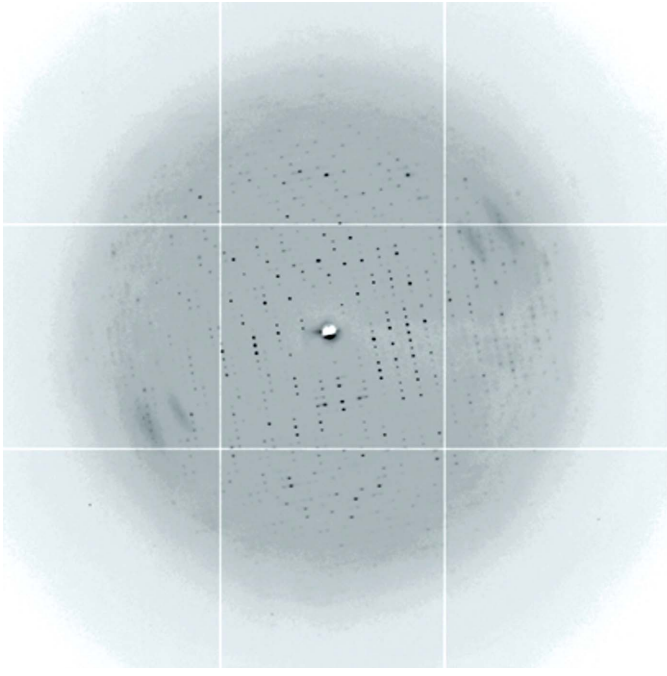


Figure 3
A representative X-ray diffraction image from the MA1659 protein crystal.

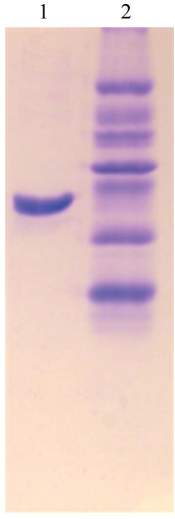


Figure 4
SDS–PAGE of the DEAE-purified protein (lane 1) and molecular-weight markers (lane 2).

Table 1
Data-collection statistics for MA1659.

Values in parentheses are for the highest resolution shell.

Wavelength (Å)	0.9765
Beamline	5.0.3, ALS
Detector	3 × 3 CCD array [ADSC Q315R]
Crystal-to-detector distance (mm)	350
Rotation range per image (°)	0.5
Total rotation range (°)	90.0
Space group	*P*23 or $P2_13$
Unit-cell parameters (Å)	$a = b = c = 92.724$
Resolution range (Å)	50.0–2.80 (2.85–2.80)
Measured reflections	14125
Unique reflections	7531
Mosaicity (°)	0.91
Multiplicity	6.3 (6.3)
Completeness (%)	100 (100)
Mean $I/\sigma(I)$	25.5 (2.0)
$R_{\rm merge}$† (%)	6.9 (67.4)

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

2.3. X-ray data collection

For diffraction experiments, crystals of 0.1 × 0.1 × 0.05 mm in size were immersed for 60 min in precipitant solution (0.1 *M* HEPES pH 7.5, 2 *M* ammonium sulfate) containing 55%(*v*/*v*) saturated fructose solution for cryoprotection. Crystals were frozen in a stream of liquid nitrogen for in-house screening. Crystals shipped to the synchrotron were flash-cooled by plunging them into liquid nitrogen and were stored in uni-puck cassettes. X-ray screening and data collection were carried out remotely on beamline 5.0.3 at the Advanced Light Source (ALS), Lawrence Berkeley National Laboratory. Diffraction data images were recorded at 100 K with a φ step of 0.5° using an ADSC Q315R CCD detector; the wavelength was set to 0.9765 Å. Data were processed using the *HKL*-2000 software suite (Otwinowski & Minor, 1997).

3. Results and discussion

N-terminally His-tagged MA1659 protein was successfully over-expressed in *E. coli* and purified to homogeneity. SDS–PAGE analysis was performed to confirm the purity of the protein before crystallization experiments (Fig. 4). The best conditions for crystal growth (0.1 × 0.1 × 0.05 mm) and diffraction were found to be 0.1 *M* HEPES buffer pH 7.5, 2 *M* ammonium sulfate and 0.1 *M* urea. The crystals diffracted to a resolution of 2.8 Å (Fig. 3). The crystal belonged to the primitive cubic space group *P*23 or $P2_13$, with unit-cell parameters $a = b = c = 92.724$ Å. Assuming the presence of one molecule in the asymmetric unit gave a Matthews coefficient ($V_{\rm M}$) of 3.55 Å^3 Da^{-1}, corresponding to a solvent content of 65% (Matthews, 1968). Data-collection and processing statistics are summarized in Table 1. We are attempting phase determination by molecular replacement using the *Phaser* program, which is part of the *PHENIX* package (Adams *et al.*, 2010). The template models that have been tried so far are the structures of *Synechocystis* sp. FTR (PDB entry 1dj7; Dai *et al.*, 2000) and of rubrerythrin (PDB entry 2hr5; B. D. Dillard, S. M. Clarkson, K. R. Strand, J. R. Ruble, L. Chen, Z.-J. Liu, F. E. Jenney Jr, M. W. W. Adams, J. P. Rose & B.-C. Wang, unpublished work). These models were used based on the domains indicated to be conserved in the MA1659 protein sequence by a *BLAST*

search. In the case that we are not successful in solving the structure by molecular-replacement methods, we will solve the structure using the multiple-wavelength anamalous dispersion (MAD) or single-wavelength anamalous dispersion (SAD) methods using the anomalous signal of the Fe atom.

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