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Preliminary X-ray crystallographic analysis of a soluble form of MntC, a periplasmic manganese-binding component of an ABC-type Mn transporter from *Synechocystis* sp. PCC 6803

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Manganese is recruited in microorganisms by way of ABC-type transporter systems. Here, the expression, purification and preliminary crystallographic analysis of a soluble form of the MntC solute-binding protein component of the MntABC manganese-import system from the cyanobacterium *Synechococystis* sp. PCC 6803 is reported. The protein (321 amino-acid residues) was expressed exclusively in inclusion bodies, which required unfolding and refolding in the presence of manganese prior to purification. The purified protein was crystallized in the presence of PEG and zinc. The crystals belong to space group $P6_222$, with unit-cell parameters $a = b = 128.1$, $c = 90.0$ Å and a single molecule in the asymmetric unit. The crystals diffract to 2.6 Å under cryoconditions using synchrotron radiation.

1. Introduction

Manganese ions play important catalytic roles in all organisms and are an absolute requirement in oxygenic photosynthetic organisms for oxygen-evolving activity (Debus, 1992). In the cyanobacterium *Synechocystis* sp. PCC 6803, uptake of manganese is carried out by two high-affinity manganese-transport systems, one of which has been characterized in terms of kinetic parameters and substrate specificity (reviewed in Pakrasi *et al.*, 2001). We have previously identified three structural genes for a manganese ABC (ATP-binding cassette) type transporter system (Linton & Higgins, 1998), the first such protein complex for manganese identified in any organism (Bartsevich & Pakrasi, 1995). These genes are organized in the *mntCAB* operon and encode one of the high-affinity manganese-transport systems in *Synechocystis*. This transporter is expressed under submicromolar concentrations of manganese and is dependent on the presence of light for activity (Bartsevich & Pakrasi, 1995, 1996, 1999). These genes encode three protein components: *mntC*, which encodes the MntC periplasmic solute-binding protein (SBP), *mntB*, which encodes the integral membrane-spanning subunit, and *mntA*, which encodes the intracellular ATP-binding cassette subunit (Bartsevich & Pakrasi, 1995, 1996, 1999). The endogenous 36.1 kDa MntC protein is a membrane-anchored lipoprotein, a form typically found in Gram-positive bacterial ABC transporter systems. Removal of the lipid anchor *via* mutagenesis and expression as a 35.4 kDa unanchored soluble protein has no discernible effect upon the transport function of this complex *in vivo*, nor does it affect the binding of manganese to purified over-expressed MntC *in vitro* (Bartsevich *et al.*, manuscript in preparation). MntC has homologues in a number of bacterial species, including those pathogenic to humans and animals, and has been placed in a novel class of solute transporters (Pakrasi *et al.*, 2001). The structures of two metal-transporter SBPs have been determined: PsaA from *Streptococcus pneumoniae* and TroA from *Treponema pallidum* (Lawrence *et al.*, 1998; Lee *et al.*, 1999). Both of these proteins have been crystallized with a Zn atom as the bound ligand. While TroA is a zinc-binding SBP, PsaA is predicted to be a manganese-binding SBP from *in vivo* metal-transport analysis data (Dintilhac *et al.*, 1997). These two crystal structures show similarities in overall structure; however, significant differences exist between these two transition-metal-binding proteins. It can be predicted that the MntC protein will be structurally similar to TroA and PsaA on the basis of their sequence homology. The amino-acid sequence homology is about 30% between each pair of these proteins (TroA/PsaA, TroA/MntC and PsaA/MntC). Most of the homologous residues are spread throughout the protein sequence and not clustered in certain regions. Determination of the MntC structure will help to understand both general structural differences in this family and also the characteristics of metal specificity and preferences in this protein family. Ultimately, knowledge of the three-dimensional structure of MntC will

help not only to understand how manganese is bound and delivered into the cell, but also to elucidate some of the steps necessary for manganese being mobilized into photosystem II and may also serve as a target for the development of novel antibacterial agents.

2. Materials and methods

2.1. Overexpresssion of MntC

The *mntC* gene from *Synechocystis* sp. PCC6803 was cloned into the vector pET-3XC (Novagen) for expression as a soluble protein without the lipid-anchoring domain. Specifically, the first 25 amino acids of MntC were replaced by 16 amino acids coded by the pET-3XC vector itself, thus resulting in a protein truncated by nine residues in comparison to the wild-type protein. This results in the expression of a completely soluble protein lacking the cysteine residue for attachment of the lipid moiety, without loss of manganese-binding affinity (Bartsevich & Pakrasi, unpublished work). *E. coli* cells were grown at 310 K to mid-logarithmic phase in LB broth (Maniatis *et al.*, 1982) supplemented with 100 mg ml^{-1} ampicillin and then induced with 1 m*M* IPTG for 4 h. Cells were harvested by centrifugation (at 12 000 rev min^{-1}, 10 min, 277 K), resuspended in 100 m*M* NaCl plus 10 m*M* Tris–HCl pH 8.0, 1 m*M* EDTA and disrupted by Yeda press treatment at 2 MPa. The lysate was then centrifuged (12 000 rev min^{-1}, 10 min, 277 K) and the MntC protein was found exclusively in the inclusion bodies. The inclusion bodies were solubilized in 8 *M* urea and then protein refolding was performed by fast dilution of the urea solution by addition of 20 m*M* Tris–HCl pH 8.0 in the presence of 20 m*M* Mn.

2.2. Isolation and purification of MntC for crystallization

The refolded MntC protein was further purified by ammonium sulfate precipitation followed by anion-exchange HPLC (PL-SAX 1000 Å, Polymer Laboratories). The protein was eluted with a linear gradient of 10–300 m*M* NaCl in 50 m*M* Tris pH 8.0. The MntC-containing fractions were detected by SDS–PAGE (data not shown). MntC protein eluted as two fractions, which were pooled separately, dialysed against 20 m*M* Tris–HCl pH 8.0 and then concentrated to 10–20 mg ml^{-1} by ultrafiltration on a Centricon-30 concentrator (Amicon). The calculated molecular weight of the purified protein was found to be ∼60 kDa by size-exclusion chromatography on HPLC (PL-GFC 1000 Å, Polymer Laboratories) indicating that the MntC forms dimers (data not shown).

Table 1
Summary of crystal parameters and data-collection statistics.

X-ray diffraction data were collected at ESRF using beamline ID-14 on an ADSC Quantum-4 CCD detector with X-ray radiation at λ = 0.933 Å. Values in parentheses are for the highest resolution shell (2.9–2.8 Å).

Space group	$P6_222$
Unit-cell parameters (Å, °)	$a = b = 128.1$, $c = 90.0$, $\gamma = 120$
Resolution range (Å)	25–2.8
No. of reflections	204033
No. of unique reflections	11170
$R_{\rm merge}$†	0.084
Completeness (%)	87.9 (93.6)
Multiplicity	4.7 (6.1)
$I/\sigma(I)$	9.7 (3.5)

3. Results and discussion

Crystallization trials were performed using both hanging-drop and sitting-drop vapour-diffusion methods at 293 K with drops being mixed as 4 µl protein with 4 µl precipitant from 1 ml precipitant well solution. We screened for suitable crystallization conditions using the Crystal Screen I and II kits (Hampton Research, USA). The trials yielded crystals after 3 weeks in the presence of 10% PEG 8000, 0.1 *M* ZnAc, 0.1 *M* cacodylic acid pH 6.5. The original conditions were modified with use of 10–15% PEG 4000 and 0.05–0.1 *M* ZnAc to improve crystal quality. Subsequently, crystals were further improved by microseeding. Crystals reach a maximum size of 0.3–0.5 mm (Fig. 1)

Crystals were found to be amenable to flash-freezing after a brief incubation (less than 1 min) in silicon-based mineral oil (Riboldi-Tunnicliffe & Hilgenfeld, 1999) and X-ray diffraction data collection was performed at the Cornell High Energy Synchrotron Source (CHESS) beamline A1 and at the European Synchrotron Radiation Facility (ESRF Grenoble) beamline ID14-2. The crystals diffracted to a maximum resolution of 2.6 Å. Analysis of the diffraction pattern using *DENZO* (Otwinowski, 1993) indicated that the MntC crystals belong to space group $P6_222$, with unit-cell parameters of 128.1 × 128.1 × 90.0 Å (Table 1). Assuming a single molecule in the asymmetric unit, the $V_{\rm M}$ value is 3.0 Å^3 Da^{-1} (Matthews, 1968) and the solvent content is 59%. A native data set, 87.9% complete in the range 25–2.8 Å was collected (Table 1). Phasing by molecular replacement (*AMoRe*; Navaza, 1994) using PsaA (PDB code 1psz) was performed. The homology between the MntC and PsaA proteins is about 30% and is distributed rather evenly throughout most of the protein sequence. One short segment was identified that has a 50% homology (between residues 32–93) and this section of the PsaA protein structure served as the original search model. Six distinct solutions, 13.7σ over the background (3.8σ better than the next best set of solutions) were identified by the rotation function. This led to a solution by the translation function which by rigid-body refinement had a correlation coefficient of 30 and an *R* factor of 51.8%. Crystal lattice packing showed little overlap between symmetry-related molecules (Fig. 2). Difference electron-density omit maps were calculated and provided further evidence that the protein had been

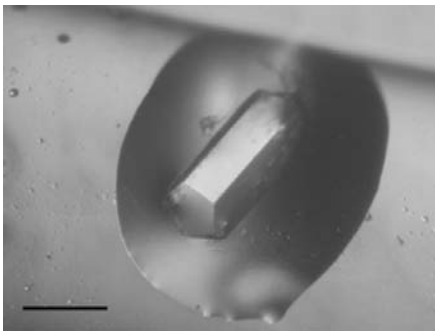

Figure 1
Single crystal of *Synechocystis* sp. PCC6803 MntC, mounted in a 1 mm quartz capillary tube. The bar indicates 0.25 mm.

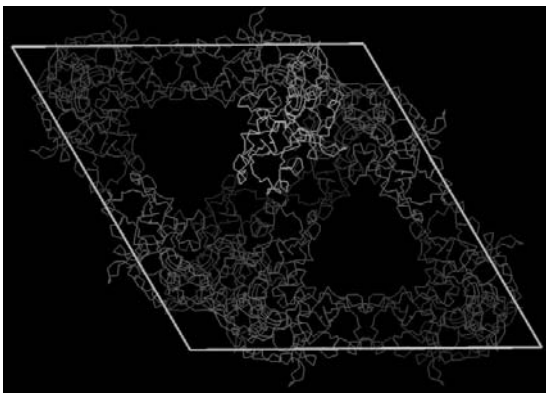

Figure 2
Crystal packing of the temporary MntC model (C^α trace) in the hexagonal unit cell, showing the crystal contacts. The figure was prepared with *Insight*II (MSI).

positioned correctly (data not shown). We are now in the process of elongation and extensive rebuilding of the model, which will be followed by further refinement.

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