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

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### **1. Introduction**

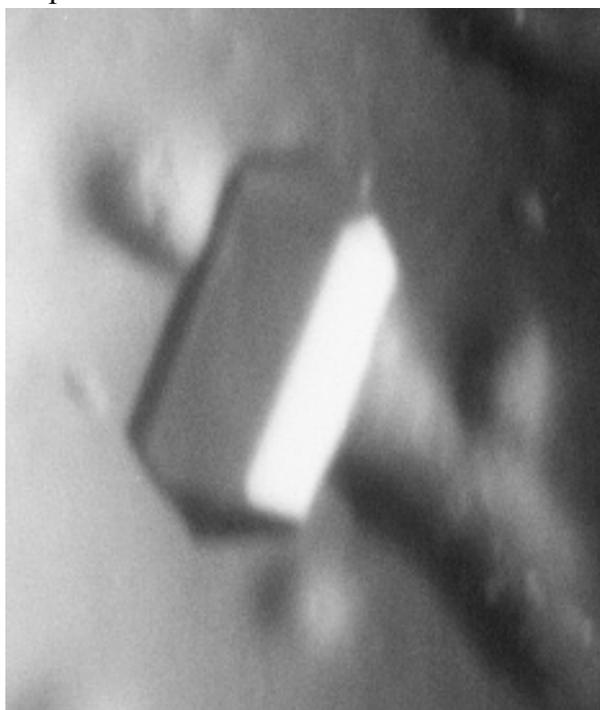
Manganese ions play important catalytic roles in all organisms. In oxygenic photosynthetic organisms, manganese is absolutely required for oxygen-evolving activity (Debus, 1992). In *Synechocystis* sp. PCC 6803, uptake of manganese is carried out by two high affinity manganese transport systems, one of which has been characterized in terms of kinetic parameters and substrate specificity (reviewed in Pakrasi, Ogawa and Bhattacharyya-Pakrasi, 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; Bartsevich & Pakrasi, 1996; Bartsevich & Pakrasi, 1999). Of the three protein components encoded by these genes, MntC encodes the periplasmic solute binding protein (SBP), MntB encodes the integral membrane spanning subunit, and MntA encodes the intracellular ATP binding cassette subunit (Bartsevich & Pakrasi, 1995; Bartsevich & Pakrasi, 1996; Bartsevich & Pakrasi, 1999). The endogenous MntC protein is a membrane anchored lipoprotein. However, removal of the lipid anchor via mutagenesis and expression as an 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 overexpressed 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 structure of two SBPs (PsaA from *Streptococcus pneumoniae* and TroA from *Treponema pallidum*) that are also members of this family of metal transporters have been determined (Lawrence *et al.*, 1998; Lee *et al.*, 1999). Both of these proteins have been crystallized with a zinc 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. Despite the similarities in overall structure, significant differences exist between these two transition metal binding proteins. 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 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.

### 3. Methods and Materials

#### *Overexpression of MntC*

The *mntC* gene from *Synechocystis* 6803 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. This results in the expression of a completely soluble protein lacking the cysteine residue for attachment of the lipid moiety. *E. coli* cells were grown at 37°C to mid-logarithmic phase in LB broth (Maniatis *et al.*, 1982) supplemented with 100 mg ml<sup>-1</sup> ampicillin and then induced with 1mM IPTG for 4h. Cells were harvested by centrifugation (12000 RPM, 10 min, 4°C), resuspended in 100mM NaCl + 10mM Tris-HCl, pH 8.0, 1mM EDTA and disrupted by Yeda



**Figure 1.** MntC crystal. Dimensions are about 0.25mm along the long axis.

press treatment at 20 atmospheres. The lysate was then centrifuged (12000 RPM, 10 min, 4°C), and the MntC protein was found exclusively in inclusion bodies. The inclusion bodies were solubilized in 8M urea, and then protein refolding was performed by fast dilution of the urea solution with slow addition of 20 mM Tris-HCl, pH 8.0, in the presence of 20 mM Mn.

#### *Isolation and purification of MntC for Crystallization*

The overexpressed MntC protein was further purified by ammonium sulfate precipitation followed by anion exchange HPLC (PL-SAX 1000Å, Polymer Laboratories). The protein was eluted with a linear gradient of 10-300 mM NaCl in 50 mM 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 20mM Tris-HCl (pH=8.0) and then concentrated to 10-

20 mg/ml by ultrafiltration on 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 1000Å, Polymer Laboratories) indicating that the MntC forms dimers (data not shown).

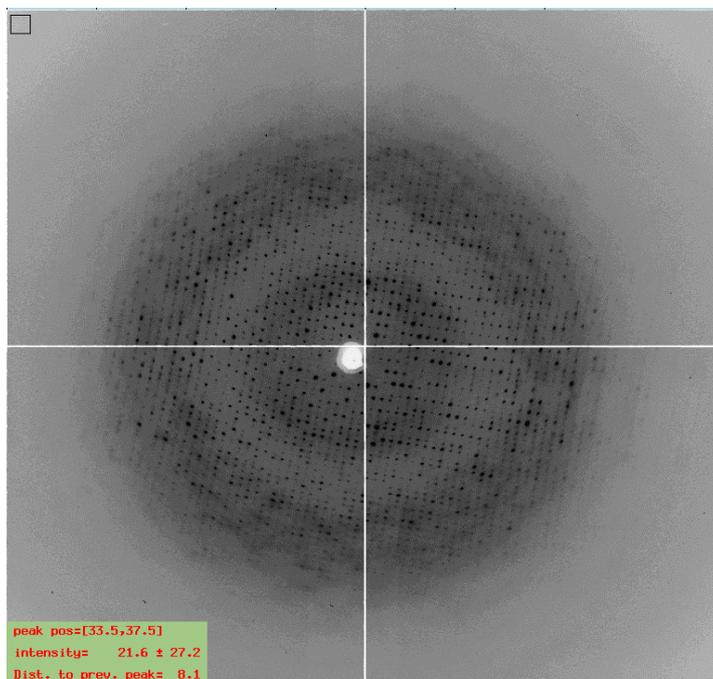
### 3. Results and discussion

#### *Crystallization and preliminary X-ray analysis*

Crystallization trials were performed using both hanging and sitting drop vapor-diffusion methods, at 293 K, with drops being mixed as 4µl protein with 4µl precipitant from 1ml 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, 0.1M ZnAc, 0.1M cacodylic acid (pH 6.5). The original conditions were modified with use of 10%-15% PEG 4000 and 0.05- 0.1M ZnAc to improve crystal quality. Subsequently crystals were further improved by microseeding. Crystals reach a maximum size of 0.3-0.5mM (Fig. 1)

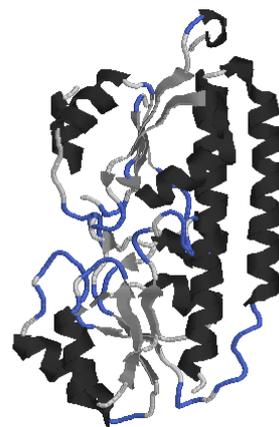
The crystals diffract to a maximum resolution of 3.3Å on an R-Axis IIC diffractometer. Recently, crystals were found to be amenable to flash-freezing after a brief incubation in



**Figure 2.** 1° oscillation diffraction pattern (10 sec. Exposure) from a frozen MntC crystal recorded with an ADSC Quantum-4 CCD detector on beamline A1 at CHESS.

replacement using the *Streptococcus pneumoniae* PsaA structure ((Lawrence et al., 1998), Protein data bank code 1PSZ) is being attempted. While the primary sequence homology between these two proteins is only about 30%, a theoretical homology based model was built (using the 3D-PSSM Protein fold recognition server, <http://www.bmm.icnet.uk/>), which serves as the molecular replacement search model. The model obtained had a high level of confidence between residues 50 to 320, and had a much higher correlation score than the only other similar transport protein, TroA.

silicon-based mineral oil (Riboldi-Tunnicliffe & Hilgenfeld, 1999), and X-ray diffraction was performed at the Cornell High Energy Synchrotron Source (CHESS) beamline A1. The crystals diffracted to a maximum resolution of 2.6Å (Fig. 2). Analysis of the diffraction pattern using either DENZO (Otwinowski, 1993) or Mosfilm (CCP4, 1994) indicated that the MntC crystals belong to a hexagonal space group with unit cell dimensions of 126Å x 126Å x 88Å. A number of native data sets, 100% complete to 3.0Å were collected. Utilization of the self-rotation function in AMORE (CCP4, 1994) did not show the presence of non-crystallographic symmetry, indicating a single molecule in the asymmetric unit. Phasing by molecular



**Figure 3.** Homology based molecular model of the MntC protein folded according to the structure of the PsaA protein from *Streptococcus pneumoniae*

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