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Crystal structure of a periplasmic solute binding protein in metal-free, intermediate and metal-bound states from *Candidatus Liberibacter asiaticus*

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ABSTRACT

The Znu system, a member of ABC transporter family, is critical for survival and pathogenesis of *Candidatus Liberibacter asiaticus* (CLA). Two homologues of this system have been identified in CLA. Here, we report high resolution crystal structure of a periplasmic solute binding protein from second of the two gene clusters of Znu system in CLA (CLas-ZnuA2) in metal-free, intermediate and metal-bound states. CLas-ZnuA2 showed maximum sequence identity to the Mn/Fe-specific solute binding proteins (SBPs) of cluster A-I family. The overall fold of CLas-ZnuA2 is similar to the related cluster A-I family SBPs. The sequence and structure analysis revealed the unique features of CLas-ZnuA2. The comparison of CLas-ZnuA2 structure in three states showed that metal binding and release is facilitated by a large displacement along with a change in orientation of the side chain for one of the metal binding residue (His39) flipped away from metal binding site in metal-free form. The crystal structure captured in intermediate state of metal binding revealed the changes in conformation and interaction of the loop hosting His39 during the metal binding. A rigid body movement of C-domain along with partial unfolding of linker helix at its C-terminal during metal binding, as reported for PsaA, was not observed in CLas-ZnuA2. The present results suggest that despite showing maximum sequence identity to the Mn/Fe-specific SBPs, the mechanistic resemblance of CLas-ZnuA2 seems to be closer to Zn-specific SBPs of cluster A-I family.

1. Introduction

Metal ion uptake and sequestration is critical for bacterial survival and growth in the environment as well as within various hosts (Waldron and Robinson, 2009). Transition metals such as manganese, zinc, and iron play an important role as enzyme co-factors for a number of biological processes including DNA replication, protein synthesis, respiration, cell wall synthesis and neutralization of reactive oxygen species (Counago et al., 2012). Metal deficiencies greatly inhibit the growth of microorganisms. Therefore, inhibition of metal uptake can serve as a possible strategy towards developing antibacterial agents against the pathogenic bacteria. One of the transport systems facilitating metal ion transport across membrane is ATP-binding cassette-type (ABC-type) transport system. The metal transporting proteins of this super family belongs to the cluster A-I family which includes zinc, manganese and iron transporters. The ABC-type transport systems comprise of three components, that are: a solute-binding protein (SBP) found in the periplasm in Gram-negative bacteria or linked to the cytoplasmic membrane in Gram-positive bacteria, a transmembrane permease and a nucleotide-binding protein (ATPase) (Higgins, 2001). Crystal structures have been reported for zinc, manganese and iron transporting SBPs of the cluster A-I family (Chandra et al., 2007; Gribenko et al., 2013; Lawrence et al., 1998; Lee et al., 1999; Rukhman et al., 2005; Sun et al., 2009; Yatsunyk et al., 2008). The overall structure consists of a pair of N- and C-terminal $(\alpha/\beta)_4$ sandwich domains linked through a long backbone α-helix running across two domains. The cleft of N- and C-terminal domain interface constitutes the metal binding site. Among manganese and zinc transporters, crystal structures of both metal-free open and metal-bound closed forms have been reported (Counago et al., 2014; Lee et al., 2002; Yatsunyk et al., 2008). In case of Zn-transporting SBPs, metal binding and release is accomplished through the conformational changes in specific secondary

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structural elements in the C-domain without any significant change in the relative domain movements and the linker helix (Chandra et al., 2007; Lee et al., 2002; Wei et al., 2007). The Mn/Fe-transporting SBPs, in contrast, exhibit a rigid body movement of C-domain away from the metal binding cleft resulting in an open solvent accessible metal-binding cleft with refolding of the distorted C-terminal end of the linker helix (Counago et al., 2014).

Citrus Huanglongbing (HLB) is an extremely destructive, fast-spreading disease of citrus which causes severe economic losses worldwide. The disease is caused by phloem-limited, unculturable, Gram-negative bacteria *Candidatus Liberibacter* spp. Three species of '*Ca. Liberibacter*' known are '*Ca. L. asiaticus*', '*Ca. L. africanus*', and '*Ca. L. americanus*'. *Ca. L. asiaticus* (CLA) is considered to be the most devastating species and is transmitted by Asian citrus psyllid, *Diaphorina citri*. The current disease control strategies include controlling psyllids chemically and biologically and scouting and eliminating infected trees. However, they have not been able to stop the spread of the disease (Wang and Trivedi, 2013). Vahling-Armstrong et al. (2012) have reported that the CLA encodes two ZnuABC homologous systems, out of which only one system is functional and able to complement Δznu *Escherichia coli* and Δznu *Sinorhizobium meliloti* strains. It was proposed that the second of the two homologous system might possibly be involved in manganese uptake and therefore it was not able to complement Δznu *E. coli* and Δznu *S. meliloti* strains (Vahling-Armstrong et al., 2012).

In this study, we have determined high resolution crystal structures of a periplasmic solute binding protein from second of the two gene clusters of Znu system (CLas-ZnuA2) in CLA in metal-free, an intermediate and metal-bound states. The comparison of CLas-ZnuA2 structures with related metal-free open and metal-bound closed forms of structures showed a unique mechanism for metal binding and release which may be closer to Zn-specific SBPs of cluster A-I family. This is the first report of a crystal structure of cluster A-I SBP from a plant pathogen.

2. Materials and methods

2.1. Cloning, expression and purification of CLas-ZnuA2

The genomic DNA of CLA was isolated from HLB infected sweet orange plants (*Citrus sinensis*) at Nagpur, Maharashtra. PCR amplification of 16S rDNA was carried out using primers OI1/OI2c to confirm the presence of the genomic DNA (Jagoueix et al., 1996). CLas-ZnuA2 gene (CLIBASIA_02120) encoding a protein of 275 amino acids lacking signal sequence was amplified using primers ZnuA1-F/ZnuA-R (Table S1). The amplified product was cloned into the expression vector pET-28c with His6-tag and TEV protease cleavage site. The CLas-ZnuA2 protein was over-expressed in *E. coli* BL21 DE3 host cells by induction with 0.4 mM IPTG at 37 °C. The protein was purified to homogeneity using HIS-Select HF Nickel Affinity column (Sigma Aldrich). The purity of the protein was confirmed by SDS–PAGE. His-tag was removed by TEV protease treatment and His-tag cleaved protein was further purified.

2.2. Sequence analysis

Sequence search was carried out using the BLAST search tool at the NCBI web site (www.ncbi.nlm.nih.gov). Putative signal sequence was predicted by SignalP 4.1 server (Petersen et al., 2011). Multiple sequence alignments were made using Clustal Omega Webserver (http://www.ebi.ac.uk/Tools/msa/clustalo/) taking default parameters and ESPript 3.0. Phylogenetic analysis was done by MEGA 5 program from amino acid alignments using the Maximum Likelihood method based on the JTT matrix-based model (Tamura et al., 2011). The reliability of the branching was tested by bootstrap statistical analysis (1000 replications).

2.3. Crystallization and data collection

The purified protein was dialysed in Tris–HCl buffer, pH 8.0 (buffer A) and concentrated to 7–10 mg/ml before crystallization. The native protein was crystallized using sitting drop vapour diffusion method in 96 well plates at 20 °C and 4 °C. The drops were prepared by mixing 1 μl of protein solution with 1 μl of reservoir solution and equilibrated against 50 μl reservoir solution. 1 mM $MnCl_2$ solution was added to protein before crystallization in order to enhance the crystallization prospects. Crystals were obtained in 0.1 M sodium acetate trihydrate buffer, pH 4.6 containing 2.0 M ammonium sulphate at 4 °C. The intermediate state of metal binding was captured in the crystals obtained in above conditions. Later, the metal-free and metal saturated states of protein were prepared following reported methods (Counago et al., 2014; Sun et al., 2009). The metal-free protein was prepared by dialysing the protein twice in 1 L sodium acetate buffer pH 4.0 containing 20 mM EDTA. The EDTA was then removed by dialysing protein in buffer A. The metal-free protein was centrifuged at 10,000 rpm for 10 min to remove any insoluble material. It was concentrated and crystallized in presence of 0.5 mM EDTA in similar crystallization conditions. For metal-bound state, apo-protein was dialysed in buffer A containing 100 μM $MnCl_2$ and excess $MnCl_2$ was removed by again dialysing the protein in buffer A. The metal saturated protein was crystallized using above mentioned conditions. However, data collection and structure analysis showed that metal was not present in the metal-binding site. The crystals of metal saturated protein were then soaked in precipitant solution containing 50 mM $MnCl_2$ for 5 min to obtain metal-bound state.

Crystals were cryoprotected by briefly exposing them to well solution containing 20% glycerol and mounted in the cryo-loops prior to the collection of X-ray diffraction data. Data of intermediate state were collected on a MAR 345 image-plate system using Cu Kα radiation generated by a Bruker Microstar-H rotating-anode generator. The data of metal-free and bound states were collected on a MAR345 image plate detector mounted on Rigaku MicroMax-007HF rotating anode generator. The crystal and data collection parameters are given in Table 1. The diffraction data were processed and scaled with iMOSFLM and SCALA program in CCP4i suite (CCP4, 1994).

2.4. Structure solution and refinement

A molecular replacement solution for intermediate state was obtained with automated molecular replacement pipeline BALBES (Long et al., 2008), using MtsA structure (PDB ID: 3HH8) which shares 32% sequence identity with CLas-ZnuA2, with an initial R_{factor} of 0.36. The initial models were subsequently rebuilt manually using COOT (Emsley and Cowtan, 2004; Emsley et al., 2010) and refined using REFMAC 5.7 (Murshudov et al., 1997; Winn et al., 2001) and PDB_REDO web server (http://xtal.nki.nl/PDB_REDO/). The quality of the final models was validated by PROCHECK (Laskowski et al., 1993) and MOLPROBITY (Chen et al., 2010). Structural alignments were done using Superpose (Krissinel and Henrick, 2004). Structure figures were prepared using PyMOL (DeLano, 2002) and Chimera (Pettersen et al., 2004). The metal-free and metal-bound structures were solved by molecular replacement by Molrep (Vagin and Teplyakov, 1997) using intermediate state as search model.

2.4.1. Accession number

The coordinates have been deposited in the Protein Data Bank with accession codes 4UDN (metal-free state), 4UDO (metal-bound state) and 4CL2 (intermediate state).

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