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Crystal structure of *E. coli* ZinT with one zinc-binding mode and complexed with citrate

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ABSTRACT

The ZnuABC ATP-binding cassette transporter found in gram-negative bacteria has been implicated in ensuring adequate zinc import into Zn(II)-poor environments. ZinT is an essential component of ZnuABC and contributes to metal transport by transferring metals to ZnuA, which delivers them to ZnuB in periplasmic zinc recruitment. Although several structures of *E. coli* ZinT have been reported, its zinc-binding sites and oligomeric state have not been clearly identified. Here, we report the crystal structure of *E. coli* ZinT at 1.76 Å resolution. This structure contains one zinc ion in its calycin-like domain, and this ion is coordinated by three highly conserved histidine residues (His167, His176 and His178). Moreover, three oxygen atoms (O_1, O_6 and O_7) from the citrate molecule interact with zinc, giving the zinc ion stable octahedral coordination. Our EcZinT structure shows the fewest zinc ions bound of all reported EcZinT structures. Crystallographic packing and size exclusion chromatography suggest that EcZinT prefers to form monomers in solution. Our results provide insights into the molecular function of ZinT.

1. Introduction

Zinc (Zn) is an essential nutrient for all living microorganisms because it influences diverse physiological processes, including DNA synthesis, cell division and other important biological functions [1]. However, high concentrations of Zn inhibit essential enzymes through non-specific interactions with their polypeptide chains, thus resulting in high toxicity against all types of cells [2]. Maintenance of zinc homeostasis is therefore of utmost importance. To meet this crucial requirement, cells finely control cellular zinc concentrations by coordinating the activity of export and import systems [3]. Previous studies have suggested that the activity of the Zn import and export systems is controlled by Zur and ZntR. These two proteins regulate gene transcription depending on their metalation states [4,5]. Zur controls the expression of a high-affinity Zn uptake system, ZnuABC, when the Zn concentration in the medium is low, and this system is used by gram-negative bacteria to transport Zn from the periplasmic space to the cytosol [3].

ZnuABC is a high-affinity ATP-binding cassette transporter, and as with all such systems, it is typically assembled from three essential components: a soluble periplasmic protein (ZnuA), an ATPase (ZnuC), and a membrane permease (ZnuB) [5]. ZnuA is a ZntR-regulated protein that captures Zn(II) ions and delivers them to ZnuB, whereas ZnuC provides the energy necessary for ion transport through the inner membrane. The Zur-regulated protein ZinT is an auxiliary component of the high-affinity ZnuABC zinc transporter [3]. Increased ZinT and ZnuABC expression thus leads to

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increased zinc import [5,6]. ZinT binds Zn(II) ions in the periplasmic space and affords a defense mechanism in response to zinc shortage [7]. This protein contributes to metal transport by transferring the metal to *E. coli* ZnuA, which delivers it to ZnuB under conditions of very low metal availability [7]. Several crystal structures of ZinT protein homologs have been solved in the presence of different metal cofactors [2]. The sequences of these proteins are highly homologous to that of a domain of AdcA, suggesting that ZinT could cooperate with ZnuA in zinc uptake [2,8]. Although these results suggest that ZinT is involved in zinc homeostasis, the molecular function and the action mechanism of the ZinT protein have not been elucidated.

Several crystal structures of ZinT from *Escherichia coli* (named EcZinT) have been determined (PDB codes: 1TXL, 1OEK, 1S7D, and 5AQ6). We have confirmed that not only the positions of bound zinc in these structures differ but also the electron density maps near the residues that coordinate zinc in most structures are not clear (Fig. S1). Moreover, David et al. proposed that EcZinT is experimentally observed as a monomer, while Colaco et al. proposed that EcZinT is a functional dimer based on its crystallographic packing with a 2-fold symmetry [9,10]. As a result, further studies on the zinc-binding site and oligomeric state of EcZinT are required.

Here, we report the crystal structure of EcZinT at 1.76 Å resolution. This structure reveals that one zinc metal is in a cavity between a helical domain and a calycin-like domain and exhibits stable octahedral coordination by highly conserved histidine residues and a citrate molecule. Crystallographic packing and size exclusion chromatography studies suggest that EcZinT prefers the monomeric state. Our previously unseen zinc-binding mode of EcZinT will provide new insights into the zinc recognition mechanism of ZinT.

2. Materials and methods

2.1. Protein preparation

The gene encoding EcZinT (residues 25–216, excluding its signal peptide) was amplified from *E. coli* K12 genomic DNA and was then cloned into a pPROEX-HTA vector (Invitrogen, USA) between the *Nco*I and *Xho*I restriction sites. The resulting plasmid contains a hexa-histidine tag-encoding region and a Tobacco etch virus (TEV) protease recognition site at the N-terminus. Recombinant DNA was transformed into *E. coli* BL21 (DE3) cells, which were then grown in LB broth with 100 μg/ml ampicillin at 37 °C until reaching an OD_{600} of 0.6–0.8. Protein expression was induced by the addition of 0.5 mM isopropyl-β-D-thiogalactoside (IPTG), and the culture was then incubated at 30 °C for 6 h. The cells were harvested by centrifugation and resuspended in a lysis buffer containing 20 mM Tris-HCl, pH 8.0, 150 mM NaCl and 2 mM β-mercaptoethanol. The cells were disrupted by sonication on ice, and the lysate was centrifuged at 14000 rpm for 30 min at 4 °C. The supernatant was mixed with Ni-NTA affinity resin (GE Healthcare, USA) and then preincubated for 30 min at 4 °C. After the resulting slurry was loaded into the column, the resin was washed with a wash buffer containing 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM β-mercaptoethanol and 20 mM imidazole. The protein samples were subsequently eluted with elution buffer containing 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM β-mercaptoethanol and 250 mM imidazole, and β-mercaptoethanol was then added to a final concentration of 10 mM. Next, the hexa-histidine tag was removed by TEV protease at 25 °C overnight. These proteins were diluted 4-fold with buffer containing 20 mM Tris-HCl (pH 8.0) and then loaded onto a HiTrapQ column (GE Healthcare). The protein was eluted from the column using a linear gradient from 0 to 1 M NaCl in 20 mM Tris-HCl (pH 8.0). The fractions containing EcZinT were concentrated and then separated by a HiLoad Superdex 200 gel filtration column (GE Healthcare) preequilibrated with a buffer containing 20 mM Tris-HCl, pH 8.0, 150 mM NaCl and 2 mM β-mercaptoethanol.

2.2. Crystallization

Prior to crystallization, the purified EcZinT protein was concentrated to 10 mg/ml using a Vivaspin centrifugal concentrator (Millipore, USA). Initial crystallization screening was performed with Crystal Screen HT, a high-throughput sparse-matrix screening kit (Hampton Research, USA), using the sitting-drop vapor-diffusion method. Crystals of EcZinT were grown under conditions of 0.2 M ammonium acetate, 0.1 M Na-citrate, pH 4.6, and 30% polyethylene glycol 4000 at 14 °C. After optimization, crystals were obtained by the sitting-drop method by mixing 1 μl of protein solution with 1 μl of reservoir solution. The reservoir solution consisted of 100 mM Na-citrate, pH 4.6, 135 mM ammonium acetate, 4 mM zinc chloride and 22.5% (w/v) polyethylene glycol 4000.

2.3. Data collection

Crystals were transferred into a cryoprotectant solution consisting of reservoir solution supplemented with 25% (v/v) glycerol and were then flash-cooled in liquid nitrogen. X-ray diffraction data were collected using an ADSC Q310 CCD detector on beamline BL17U1 of the Shanghai Synchrotron Radiation Facility (SSRF) (Shanghai, People's Republic of China) at 100 K using a nitrogen stream [11]. The intensity data were processed and scaled using the *HKL*-2000 program and CCP4 suites [12,13]. Matthews calculations suggested that the corresponding Matthews coefficient [14] and solvent content are 2.01 $Å^3\ Da^{-1}$ and 38.72%, respectively. The data collection statistics are given in Table 1.

2.4. Structure determination

1. The crystal structure of EcZinT was solved by molecular replacement (MR) with Phaser [15] using the crystal structure of ZinT from *Salmonella enterica* (PDB code: 4AW8) as the search

Table 1
Crystallography data and refinement statistics.

Data collection	
Space group	*P* 1 21 1
Cell dimensionsrowhead	
a, *b*, *c* (Å)	41.04, 65.87, 73.54
α, *β*, *γ* (°)	90.00, 91.35, 90.00
Resolution range (Å)	41.03–1.76
R_{sym} (%)	10.70(25.1)
Completeness (%)	93.49 (83.27)
Multiplicity	5.5
Average *I*/*σ*(*I*)	13.26
Model refinementrowhead	
R_{factor}/R_{free} (%)	18.75(22.30)/23.41(26.30)
No. of atoms	
Protein	6425
Water	493
Average B factor ($Å^2$)	28.0
R.m.s.d. from idealrowhead	
Ramachandran plot	
Preferred (%)	98.0
Outliers (%)	0.0
PDB code	5YXC

$\sum_{hkl}|F_o\text{-}F_c|/\sum_{hkl}|F_o|$ for all data with $F_o > 2\sigma(F_o)$, excluding data used to calculate R_{free}.§Rfree = ∑hkl|Fo-Fc|/∑hkl|Fo| for all data with Fo > 2σ(Fo) that were excluded from refinement.