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Zinc-binding and structural properties of the histidine-rich loop of *Arabidopsis thaliana* vacuolar membrane zinc transporter MTP1

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ABSTRACT

The vacuolar Zn^{2+}/H^{+} antiporter of *Arabidopsis thaliana*, AtMTP1, has a cytosolic histidine-rich loop (His-loop). We characterized the structures and Zn^{2+}-binding properties of the His-loop and other domains. Circular dichroism analyses revealed that the His-loop partly consists of a polyproline type II structure and that its conformational change is induced by Zn^{2+} as well as the C-terminal domain. Isothermal titration calorimetry of the His-loop revealed a binding number of four Zn^{2+} per molecule. Numbers of Ni and Co associated with the His-loop were approximately one ion per molecule and the thermodynamic parameters of the association with these ions were different from that of Zn^{2+}. These results suggest the involvement of the His-loop in sensing cytosolic Zn^{2+} and in the regulation of zinc transport activity through Zn^{2+}-induced structural change.

1. Introduction

Metal tolerant protein 1, MTP1, belongs to the cation diffusion facilitator (CDF) family and functions as an active transporter. In addition to MTP family members, mammalian zinc transporters such as ZnT4 (SLC30A4) [1], *Saccharomyces cerevisiae* ZRC1 (ScZRC1) [2] and *Escherichia coli* YiiP [3] are also members of the same family. MTP1 proteins have six transmembrane (TM) domains, an N-terminal domain (NTD), and a C-terminal domain (CTD). NTD is exposed to the cytoplasm and CTD has metal-binding sites and protrudes into the cytoplasm as demonstrated for YiiP [4,5]. The CDF members transport Zn^{2+}, Cd^{2+}, Co^{2+}, and/or Ni^{2+} as a cation/H^{+} exchanger, although the ion selectivity varies with the molecular species [1–3,6,7]. For example, YiiP has the capacity to transport Zn^{2+}, Fe^{2+}, and Cd^{2+} [4]; ScZRC1 transports Zn^{2+} [2]; and ScCOT1 transports Zn^{2+}, Co^{2+}, and Cd^{2+} [8]. Structure-based ion selectivity has been reported for YiiP [4,5], AtMTP1 [9], and *Noccaea goesingense* MTP1 (NgMTP1, formerly *Thlaspi goesingense* MTP1) [10].

Arabidopsis thaliana AtMTP1 functions in the vacuolar membrane as a Zn^{2+}/H^{+} exchanger to sequester cytoplasmic Zn^{2+} with the help of vacuolar proton pumps [7,11,12]. Zinc accumulation in the vacuole has two physiological roles: reservation of Zn^{2+} as a key nutrient and detoxification of excess Zn^{2+} in the cytoplasm. Zinc is an essential micronutrient but is toxic at high concentrations [13–15]. The loss-of-function mutant *atmtp1* is phenotypically sensitive to excess Zn^{2+} in the medium, showing the importance of AtMTP1 in zinc homeostasis, particularly for detoxification of zinc in the cytoplasm [16,17]. Thus, the recognition or sensing of the cytoplasmic Zn^{2+} concentration by AtMTP1 is a key process in maintaining zinc homeostasis in cells.

AtMTP1 has a long, cytosolic histidine-rich loop (His-loop). Other MTPs such as *Arabidopsis halleri* MTP1, *N. goesingense* MTP1t1 (formerly TgMTP1t1), *Populus* MTP1, and ScCOT1 also have a His-loop although the number of histidine residues and the length of the loop differ among each [7,13]. Furthermore, a mutant of AtMTP1 that lacks the first half of the His-loop has enhanced Zn^{2+} transport activity [7]. Therefore, the His-loop of AtMTP1 is not essential for Zn^{2+} transport and is estimated to play roles as a buffering pocket for Zn^{2+} and as a sensor of Zn^{2+} levels [7].

Information on the tertiary structure and on the kinetic properties of Zn^{2+} binding of the His-loop is essential to understand the biochemical and structural role of the loop. The fine tertiary structure of the *E. coli* CDF member YiiP in a functional homodimer has been resolved [4,5]. YiiP has six TM domains and a common CTD but lacks the His-loop. Also, the primary structure of the NTD is different from that of AtMTP1. Thus, homology modeling cannot be applied to estimate the 3D structure of the His-loop. In this study, we prepared polypeptides of the His-loop and other domains, including NTD and CTD, to investigate their structures and Zn^{2+}-binding properties. We found

Abbreviations: CTD, carboxyl terminal domain; His-loop, histidine-rich loop; ITC, isothermal titration calorimetry; MTP, metal tolerant protein; NTD, amino terminal domain; PPII, polyproline type II; TFE, trifluoroethanol; TM, transmembrane.

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that the His-loop binds multiple Zn^{2+} ions and changes its secondary structure upon Zn^{2+} binding, and that the NTD has no capacity to bind Zn^{2+}. We discuss the biochemical relevance of the His-loop in the regulation of zinc transport activity and ion selectivity with these results.

2. Materials and methods

2.1. Plasmid construction and protein expression

To determine the physico-chemical properties using a large quantity of highly purified samples, we prepared recombinant polypeptides of NTD and CTD, and the second half of the His loop (His-loop 2nd half), which were tagged with $(His)_6$. Their cDNAs were prepared from a cDNA library by polymerase chain reaction (PCR) using the primer sets listed in Table S1. PCR was performed using KOD-Plus DNA polymerase (Toyobo, Osaka, Japan). The amplified DNA fragments were treated with restriction enzymes *Nde* I and *Eco* RI and then cloned into the vector pET23b (Novagen, Madison, WI, USA) for introduction into *E. coli* DH5α competent cells (Takara Bio, Otsu, Japan). All constructs were sequenced to verify the PCR errors, and then the expression vector was introduced into *E. coli* BL21(DE3) (Novagen). The transformants were grown in Luria–Bertani (LB) broth supplemented with 0.1 mg/ml ampicillin for 18 h at 25 °C for the NTD and the His-loop 2nd half, and for 8 h at 30 °C for CTD after induction with 0.5 mM isopropyl-1-thio-β-D-galactoside.

2.2. Purification of polypeptides

The transformed *E. coli* cells were harvested by centrifugation, resuspended in buffer, and then disrupted by sonication by the method described previously [18]. After removal of cell debris by centrifugation at 100,000*g* for 30 min, the supernatants were applied to a nickel nitrilotriacetic acid Superflow (Ni-affinity) column (1.5 × 12 cm) (Qiagen, Valencia, CA, USA) equilibrated with 20 mM Tris–acetate, pH 7.5, 20 mM imidazole, 20% (v/v) glycerol, and 2 M NaCl, and then washed with 20 mM Tris–acetate, pH 7.5, 20 mM imidazole, and 2 M NaCl. The recombinant proteins were eluted with 20 mM Tris–acetate, pH 7.5, 300 mM imidazole, and 2 M NaCl.

The obtained protein solutions were dialyzed overnight against 20 mM sodium phosphate, pH 7.0, and 150 mM NaCl (TAGZyme buffer) to remove imidazole. The His tag was removed by a TAGZyme kit (Qiagen) according to the manufacturer's manual. The recombinant proteins without a tag were separated from recombinant proteins with a tag by Ni-affinity column chromatography. Highly purified preparations of the CTD and the His-loop 2nd half were obtained after this step. Their purity was checked by SDS–polyacrylamide gel electrophoresis (PAGE) using precast 15% gels (Atto, Tokyo, Japan) in Tris–Tricine buffer.

NTD was further purified by gel filtration with Hiprep 16/60 Sephacryl S-100 HR (GE Healthcare, Piscataway, NJ, USA). The obtained solutions of the CTD and the His-loop 2nd half were subjected to dialysis to exchange the buffer. The polypeptide of the His-loop and its 1st half were synthesized chemically by Genscript-Japan (Tokyo, Japan) and Operon (Tokyo, Japan), respectively, because the polypeptides were not expressed at a high level in *E. coli*. Biochemical properties are listed in Table S2.

2.3. Circular dichroism spectroscopy

The ellipticity was followed with a J-700 spectropolarimeter equipped with a PTC-348 WI temperature controller (Jasco, Tokyo, Japan) at 25 °C [19]. Far-UV CD spectra of samples (0.4 ml) in 20 mM Tris–acetate, pH 7.5, were monitored in a range of 200–250 nm with a light path length of 1 mm in the presence or absence of $ZnSO_4$. All spectra are shown as the average of five scans. In some experiments, protein samples were dissolved in 10–50% (v/v) of trifluoroethanol (TFE). In all cases, the background signal for the buffer was subtracted from that of the sample spectra. The detailed measurement conditions are listed in Table S3.

The content of α helices (f_H) in a polypeptide was calculated with the following Eqs. (1) and (2),

$$[\theta]_{222} = \frac{[\theta]_{obs}}{(d \times c)} \tag{1}$$

$$f_H = \frac{([\theta]_{222} + 2340)}{30,300} \tag{2}$$

where $[\theta]_{222}$ represents the molar ellipticity at 222 nm, $[\theta]_{obs}$ the observed value of ellipticity (degree), d light path (m), and c amino acid molar concentration (M) [20].

2.4. Isothermal titration calorimetry

Isothermal titration calorimetric (ITC) analysis was performed using an isothermal titration calorimeter Nano-ITC LV (TA Instruments, New Castle, PA, USA). Protein samples in 20 mM Tris–acetate, pH 7.5, were diluted with the same buffer and degassed in a vacuum. Aliquots (2 or 3 μl) of 2 or 4 mM $ZnSO_4$ were injected into a 0.190 ml sample cell containing 0.2 mM (for His-loop), 0.4 mM (for the 1st half), and 0.15 mM (for the 2nd half) at 25 °C. The total volume of zinc solution was 50 μl. In all cases, the background titration signal for the buffer was subtracted from that of the sample spectra. The data were analyzed using NanoAnalyze (TA Instruments). The calculation was based on the assumption that each polypeptide has one set of identical binding sites for the metal ion. The detailed measurement conditions are listed in Table S4. The enthalpy change (ΔH), the association constant (K_a), and the stoichiometry (n) were determined using the ITC titration curve of the binding of Zn^{2+} to the corresponding protein, and the free-energy change (ΔG) and the entropy change (ΔS) on Zn^{2+} binding was then calculated with the following Eq. (3),

$$\Delta G = -RT \ln K_a = \Delta H - T\Delta S \tag{3}$$

where R represents the gas constant and T the absolute temperature.

3. Results

3.1. Preparation of polypeptides with domain sequences

A relatively large amount of proteins was prepared for biochemical analyses. The recombinant polypeptides of NTD (54 residues, Met-1 to Ser-54) (Fig. S1), CTD (79 residues, Met-320 to Arg-398), and the His-loop 2nd half (42 residues, Glu-217 to Asn-258) tagged with $(His)_6$ were well expressed in *E. coli*. After removal of the $(His)_6$ tag with exoprotease, these polypeptides were purified by affinity column chromatography and then gel filtration column chromatography. The obtained samples were pure as shown in SDS–PAGE (Fig. S2). The His-loop (77 residues, His-182 to Glu-251) and its 1st half (35 residues, His-182 to His-216) were chemically synthesized, because the expression levels in *E. coli* were not high. It should be noted that CTD shows a band at 16 kDa in addition to a monomer band of 8 kDa. The 16-kDa band is a homodimer of the CTD polypeptide. CTD has a single cysteine residue (Cys-362) and tends to form a dimer as shown in Fig. S2. The dimer formation is due to a disulfide bond between the monomers, because dimer formation was stimulated by Cu(II)-$(1,10\text{-phenanthroline})_3$, which is an oxidizing agent that stimulates the formation of disulfide bonds [21], and was cleaved by dithiothreitol (Fig. S3). The apparent molecular sizes on SDS–PAGE, the calculated molecular masses, and the p*I* values of the prepared polypeptides are summarized in Table S2.