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Evidence for zinc and cadmium binding in a CDF transporter lacking the cytoplasmic domain

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ABSTRACT

Cation diffusion facilitators (CDFs) have been described as requiring the C-terminal cytoplasmic domain for their function. With the identification of smaller proteins lacking the cytoplasmic portion but displaying sequential characteristics of CDFs, this assumption should be reconsidered. Here we describe the results showing that the MmCDF3, a 23-kDa protein lacking a C-terminal domain, interacts selectively with zinc and cadmium. Isothermal titration calorimetry (ITC) binding results indicate that the truncated CDF may have an alternative means of acquiring ions from the cytoplasm in the form of an extended N-terminus, a feature common to putative cation efflux proteins of a similar size.

1. Introduction

Zinc is an essential micronutrient for the proper growth of microbial cells. It is responsible for maintaining the integrity of ribosomes, double-stranded DNA and, in Gram-negative bacteria, the cell wall [1]. Zinc has been found to be required for proteins of all classes in both prokaryotes and eukaryotes [2], with zinc-binding proteins being estimated to account for 10% of the human proteome [3]. Due to the number of proteins with which zinc has a function, an intracellular imbalance in zinc levels can have an antimicrobial action. In the case of excess zinc within the cell, it has been suggested that zinc binds to the membrane, extending the time the cell spends in the lag phase of the growth cycle and, as a result, delaying cell division [4]. It is, therefore, essential that the cell has the means to remove a zinc surplus. The ubiquitous cation diffusion facilitator (CDF) family of integral membrane transporters is found in both prokaryotes and eukaryotes [5], and is part of the zinc regulatory system functioning to remove zinc ions from the cytosolic space or mediate their transport from the cytoplasmic space to intracellular organelles [6]. The CDF family was initially identified as a group of transporters for Zn^{2+} and Co^{2+}; however, they have also been shown to interact with other divalent cations including Ni^{2+}, Mn^{2+}, Cd^{2+} and Fe^{2+} [7]. To date, all studies regarding the transport mechanism of CDFs indicate that they function as H^+-linked antiporters [8–10].

Originally, all described CDF proteins exhibited a common architecture, consisting of a transmembrane domain (TMD) of six helices and a cytoplasmic C-terminal domain (CTD). Recently, however, a truncated protein displaying the common features of CDFs, including the highly conserved active site, has been identified [11]. This protein, termed MmCDF3, has been shown to display all the typical sequential traits of the CDF family; however, its function has yet to be defined. Due to the variety of potential substrates for any CDF, isothermal titration calorimetry (ITC) has been employed to identify the substrates for different CDF family members in the past [12,13]. ITC measures the absorbance or release of heat associated with the addition of a ligand solution to a protein solution [14], providing information on the binding constants for a reaction in addition to the energies associated with the bindings [15]. Hence, the use of ITC enables the establishment of a specific protein affinity to a variety of substrates.

Despite a full-length crystal structure for the *Escherichia coli* CDF, YiiP [16,17], and two crystal structures for the CTD of the more classical CDF family members [18,19], there is still no defined mechanism for their function. Therefore, in order to gain a better understanding of their mode of action, further functional studies are required. Here we present an in-depth analysis of the ability of MmCDF3 to bind divalent metal cations as measured by ITC. This

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could further our understanding of transmembrane ion transport in proteins lacking CTD.

2. Materials and methods

2.1. Cloning the mmcdf3 gene

The *mmcdf3* gene (GenBank ID Q0AM69) was amplified by PCR using Phusion DNA polymerase (Finnzymes) from genomic DNA of *Maricaulis maris* DSM 4729 (DSMZ). As there were a large amount of modifications to the 5′ end, involving the addition of a TEV cleavage site and a glycine linker to facilitate this cleavage, PCR was done in two stages, with two separate forward primers; *mmcdf3polyglyf* (5′-GGTGGTGGTGGTGGTAGTTGTTGCCCGAGCGACC-3′) and *mmcdf3tevf* (5′-ATATA*GGATCC*G**GAAAATCTTTATTTTCAAGGT**GGTGGTGGTGGTGGTAGTTGTTGC-3′) were used in successive PCRs, respectively, with gel purification of the first PCR product before commencing with the second reaction. For both PCR stages, the reverse primer used was *mmcdf3r* (5′-GCGCG*AAGCTT*TTACGCTTCTCCTGGGTTGGC-3′). The restriction sites within the primers are marked in italics, the TEV cleavage site is in bold and the sequence complementary to its template is underlined. The final product was *Bam*HI/*Hin*dIII cloned into the pETDuet-1 expression vector (Novagen) yielding pETDuet-1/MmCDF3TEVHis. In-frame cloning into pETDuet-1 yields an N-terminally His-tagged MmCDF3, simplifying detection and purification. The final clone was confirmed by DNA sequencing.

2.2. Expression and purification of MmCDF3

The expression and purification of MmCDF3 was performed as described for the C-terminally His-tagged protein lacking the TEV recognition site [11]. For this study, however, TEV protease was required and, therefore, was expressed using *E. coli* Rosetta (DE3) pLysS transformed with the plasmid pET-28a TEV S219N, N68D, I77V, T17S and purified as described in [20]. TEV protease was stored in 25 μM aliquots (10 μl) at −80 °C after flash-freezing in liquid nitrogen.

MmCDF3 was concentrated to 50 μM to reduce the sample volume. DTT and EDTA were added to final concentrations of 5 mM and 2 mM, respectively. TEV protease was introduced at 1 μM (final concentration) and the reaction was left overnight at 20 °C, after which TEV protease was removed from the MmCDF3 sample by subtractive IMAC. The flowthrough containing MmCDF3 was collected, concentrated to 50 μM and stored at −80 °C after flash freezing in liquid nitrogen.

2.3. Protein quantitative and qualitative analyses

Protein samples were analyzed for purity on 16% SDS–PAGE. For determination of the presence of the His-tag before and after TEV cleavage, Western Blotting was employed with monoclonal anti-polyhistidine-peroxidase antibody (Sigma–Aldrich Cat. #A7058). Protein concentration was determined by absorbance spectroscopy at 280 nm (Nanodrop spectrophotometer ND-1000) using an extinction coefficient as determined by the ProtParam bioinformatics tool ($\varepsilon = 30730\ M^{-1}\ cm^{-1}$; http://web.expasy.org/ProtParam) and using the BCA method.

2.4. Isothermal titration calorimetry

The ITC experiments were performed at 25 °C on a MicroCal™ iTC_{200} isothermal titration calorimeter (GE Healthcare). Divalent ion sources (chloride salts of zinc, iron and potassium and a sulfate salt of cadmium) were dissolved in the SEC buffer (50 mM Tris–Cl, pH 8.0 and 0.03% DDM) to final ionic concentrations of 500 μM. Titrations were carried out with MmCDF3 concentrations between 40 and 50 μM, stirring at 1000 rpm with a filter time constant of 2 s. The titrant (metal ions) was added in 1 μl injections every 90–120 s. Negative controls were performed by performing the injections into the SEC buffer, resulting only in signals from heat of salt dilution. The ITC data was analyzed using the Microcal Origin software (GE Healthcare).

2.5. Functional complementation assay

According to the method described recently by Goswami et al. [13], *E. coli* GG48 was transformed with pTTQ18-*mmcdf3* plasmid and single colonies were incubated overnight at 37 °C in 5 ml LB broth as a seed culture. 25 ml of LB broth was then inoculated to a final OD_{600} of 0.05. IPTG was then added to a final concentration of 0.2 mM and the cultures were grown in varying $ZnCl_2$ concentrations (0–800 μM) at 30 °C. After 12 h the OD_{600} of the cultures was measured to determine the *E. coli* GG48 growth. A control experiment was run with *E. coli* GG48 containing pTTQ18 plasmid without insert. *E. coli* Top 10 was also tested without plasmid to determine the zinc tolerance of wild type *E. coli*.

3. Results and discussion

3.1. Purification of MmCDF3 for ITC analysis

Histidine is well known to bind divalent metals, including nickel, cobalt and zinc, and is consequently exploited for IMAC. Hence, after purification of MmCDF3 by IMAC and SEC, it was necessary to remove the His-tag from the N-terminus of the protein using TEV protease. The cleaved protein was further purified by subtractive IMAC. The purity and tag removal of MmCDF3 was confirmed by SDS–PAGE and Western Blot (Fig. 1). The protein could also be converted into a homogenous form by means of a detergent exchange in a 4:1 M ratio of DDM: FC12 (Fig. 1C).

3.2. Titrations of MmCDF3 with zinc ions

The energetic effect of the addition of zinc to MmCDF3 was measured using ITC (Fig. 2). In the presented figures, the top sections represent the raw data collected from the titrations. The lower sections consist of a plot of the integrated heat per mole of titrant as a function of the molar ratio of titrant to MmCDF3.

The ΔH profile for zinc binding shows a negative slope followed by a positive trend approaching zero. The negative portion of this curve is indicative of an endothermic reaction, as can be seen in the raw data profile (Fig. 2A, upper section), in which the positive spikes consistently follow the shown negative bands. By further analysis of the energetics of this section of the curve, the enthalpy change (ΔH^o) was determined to be 3.21 ± 0.22 kcal/mol and the entropy change (ΔS_o) was 38.5 cal/mol/deg with a binding constant (K_a) of 1.16 ± 0.09 μM^{-1}. This positive ΔH value is strongly indicative of an endothermic reaction as it implies that the system is absorbing energy. By contrast, the ΔS_o value cannot provide much insight to the reaction as, when introducing low molecular weight ligands, this can be a positive figure simply as a result of desolvation of the cation and the ligands coordinating groups [21]. This positive value can be attributed to desolvation of the cation and the coordinating groups of the ligand [13].

The stoichiometry of this binding indicated that 0.48 ± 0.01 zinc ions would bind per protomer. When establishing protein concentration, the monomeric molecular weight was used. Since all studied members of the CDF family including MmCDF3 function as homodimers, it can be assumed that the stoichiometric values