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On the mechanism of membrane transport of zinc ions by ZntB

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On the mechanism of membrane transport of zinc ions by ZntB

Artem Stetsenko
2020

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On the mechanism of membrane transport of zinc ions by ZntB

PhD thesis

to obtain the degree of PhD at the
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....
Tho' much is taken, much abides; and tho'
We are not now that strength which in old days
Moved earth and heaven, that which we are, we are;
One equal temper of heroic hearts,
Made weak by time and fate, but strong in will
To strive, to seek, to find, and not to yield.

Ulysses. Alfred Tennyson

...
Уходит многое, но многое пребудет;
Хоть нет у нас той силы, что играла
В былые дни и небом и землею,
Собой остались мы; сердца героев
Изношены годами и судьбой,
Но воля непреклонно нас зовет
Бороться и искать, найти и не сдаваться.

Улисс. Альфред Теннисон

To everyone, whoever loved me
Всем, кто меня когда-либо любил

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Chapter 1

Diversity and similarity in the 2-TM-GxN protein family

A. Stetsenko, A. Guskov

Abstract

Magnesium, cobalt, nickel and zinc are essential microelements for a vast number of bacterial species, and many pathogens rely on the $\text{Mg}^{2+}/\text{Co}^{2+}/\text{Ni}^{2+}$ uptake systems for their pathogenicity and survival. One of the most common classes of transporters of these ions is the 2-TM-GxN family of transporters. Each member of this family is characterized by a large N-terminal cytoplasmic domain, followed by two C-terminal transmembrane (TM) α -helices, containing a conserved GxN motif on the C-terminal end of TM1 and a homopentameric organization. The best-studied member of this family is CorA. Other well-characterized members of this family include ZntB, Mrs2 and Alr1. The CorA is the primary Mg^{2+} channel in over half of all bacteria and archaea. The eukaryotic CorA homologue Mrs2 is a mitochondrial Mg^{2+} uptake system. Mrs2 proteins can be found in plants and other eukaryotes. Alr1 and Mnr are Mg^{2+} transporters found in the plasma membrane of many fungi. ZntB is a bacterial member of the 2-TM-GxN family but most probably mediates influx of Zn^{2+} instead of Mg^{2+} . Recent progress in structural and functional characterization of ZntB have provided novel insight into the molecular mechanism of ZntB action, suggesting that the protein is a transporter. The accumulated structural and biochemical data for bacterial CorA and ZntB indicate that both channels and transporters may

be presented in this family, however this is not clear yet. Here we review the evidence that this is indeed the case.

Introduction

All living organisms require a tight homeostasis of various nutrients, ions and metals, including Mg^{2+} , Zn^{2+} , Co^{2+} and Ni^{2+} [1], [2]. Mg^{2+} is an essential divalent ion for life and is required for the function of many enzymes (e.g. phosphatases, ATPases and RNA polymerases) [3]. Zn^{2+} plays both a functional and a structural role in all enzyme classes and regulates gene expression by zinc-finger proteins [4]. Both Mg^{2+} and Zn^{2+} are essential for the structural integrity of ribosomes [5]. Co^{2+} is an integral part of vitamin B12, which is essential in the metabolism of folic acid and fatty acids. In addition to its role in cobalamin, cobalt is a cofactor of some metalloproteinases that bind cobalt directly such as methionine aminopeptidase-2 and nitrile hydratase [6], [7]. Ni^{2+} plays an important role in the survival of some plants, bacteria, archaea and fungi. For example, nickel is required for several enzymes, such as ureases, NiFe hydrogenases, superoxide dismutases and etc. [8]–[11].

Because of the need for tight regulation of the homeostasis of these ions, several families of transporters capable of their transport evolved, for example, the major facilitator (MFS), cation diffusion facilitator (CDF), P-type ATPases, cyclin M (CNNM) and metal ion transporters (MIT) superfamilies [12]–[16]. Interestingly, some of these transporters and channels are redundant, i.e. can substitute for each other to maintain the homeostasis for the required ion [17]–[20]. One of such protein families is the 2-TM-GxN family that belongs to the MIT superfamily. The main feature of the 2-TM-GxN family is two transmembrane helices, both located at the end of the C-terminus, and connected by a conserved loop containing the signature motif of the family with the amino acid sequence of Gly-x-Asn (GxN), where x could be Met, Val, or Ile (M, V or I). This structural element

is extremely well conserved in all three domains of life and therefore may have formed early in evolution to transport magnesium and other divalent cations across membranes [21]–[23].

The overall sequence similarity between the 2-TM-GxN proteins across all domains of life is very low, except of the GxN motif. Detailed phylogenic research of more than 360 2-TM-GxN members from all three domains of life separated showed four proteobacterial CorA clades with homologues in Archaea, one extended proteobacterial ZntB clade, a fungal and yeast Alr/Mnr clade, and three clades of Mrs2 in plants, vertebrates and fungi (Fig. 1) Also the same study proposed that independent gene duplications have happened in proteobacteria, fungi and plants at diverse phylogenetic depths. Furthermore, there are a few examples that horizontal gene transfers have occurred both in Eubacteria and Archaea. For example, fifteen versions of Mrs2 gene are presented in the plant *Arabidopsis thaliana*, while vertebrates have only one gene of the Mrs2 [21].

structural basis of Mg^{2+} and Zn^{2+} transport, and provided the model structures for their eukaryotic homologues such as Mrs2, Alr1 and Alr2. The structures of CorA and ZntB have revealed the same transmembrane topologies and pentameric organization, but potentially different transport mechanisms. Continuous attempts to further characterize other members of the 2-TM-GxN family have set the stage to better understanding of the structural basis of regulated Mg^{2+} (and similar divalent ions) transport via this wide-spread and ubiquitous system [19,26–30]. This review focuses on the 2-TM-GxN members and characterizes their structures and transport mechanism.

CorA

CorA is one of the most extensively studied members at functional and structural levels of the 2-TM-GxN family. A hypothetical Mg^{2+} channel CorA was initially identified in a cobalt-resistant mutant of *Escherichia coli* [24]. Later, *corA* gene from *Salmonella typhimurium* was cloned and expressed in *E. coli* with the following biochemical characterization *in vivo* [25]. In addition to these two, CorA proteins of *Methanocaldococcus jannaschii*, *Haemophilus influenza* and *Thermotoga maritima* were used as model proteins for biochemical studies [26], [27]. These studies have shown that CorA proteins are able to transport Mg^{2+} , Co^{2+} , and Ni^{2+} with the K_m values of 10–20, 20–40, and 200–400 μM , respectively. The CorA proteins can be divided into at least two subgroups A and B, based on the amino acid sequence alignment. The members within of each subgroup have a higher level of homology at the N-terminus, and subgroup B has a larger N-terminal domain, approximately with 30 amino acids extra. Another difference between these subgroups is a capability to select for Co^{2+} over Mg^{2+} in subgroup B [28]–[30]. However, any conclusions should be made with caution without the sufficient structural and biochemical data for subgroup A.

More than 15 crystallographic and cryo-EM structures are available for CorA, but the full-length structures are only available for CorA from *T. maritima* (TmCorA) and *M. jannaschii* (MjCorA) [23], [31]–[34]. These structures reveal a unique homopentamer organization, where long transmembrane α -helices (TM1 or the “stalk” helices) form a transmembrane pore through which magnesium ions presumably flow. TM2 α -helices form a highly hydrophobic ring surrounding the first transmembrane helices and probably are necessary to stabilize the position of the protein in the lipid bilayer (Fig. 2). TM1 and TM2 are connected via the extracellular loop bearing the signature GMN motif of the 2-TM-GxN family [21], [33]. The rest of the protein sequence is folded into a large N-terminal cytoplasmic domain, which possibly plays a regulatory function.

Figure 2. The pentameric architecture of CorA from (A) *Thermotoga maritima* and (B) *Methanocaldococcus jannaschii*. Single monomer is colored and shown as ribbons, rest four monomers are colored in grey. Transmembrane helices are embedded into the plasma membrane;

cytoplasmic domain carrying divalent cation binding sites at the bottom. Magnesium ions are colored as red spheres.

The pore in TmCorA is long, apolar and contains two hydrophobic constrictions: the so-called “MM stretch” (MM), a 19 Å long constriction site formed by pore-lining residues M291, L294, A298, and M302 with distances of 6.4-7 Å between the opposing side chains, and the “lower leucine constriction” (LC), a shorter steric bottleneck formed by the side chain of L280, where the opposing side chains are 6-7 Å apart [35]. Similar hydrophobic lock composed of the L260-ring, the M257-ring, and the M253-ring is observed in MjCorA. In all crystal structures of CorA, both hydrophobic constrictions are too narrow to be hydrated, suggesting that the channel is in its closed state. In addition to indicated structural hydrophobic parts of the pore, down the funnel an aspartate ring is formed by D277 with the diameter around 8-9 Å, thus only partially hydrated ions can pass through (Table 1). There is an additional second hydrophilic ring down to the stalk, formed by S284 with similar O-O distances as within the aspartate ring (Fig. 3A).

Figure 3. Structural features of TmCorA and MjCorA. **A.** Surface representation of the pore diameter in TmCorA. The lower leucine constriction (LC) and MM stretch (MM) are indicated. The pore is colored by its local diameter, where blue pores are larger than that of hexahydrated magnesium (6.8 Å), red ones are smaller than that of a single water molecule (2.8 Å), or white is intermediate [34]. **B.** The close-up of the metal binding groove between two cytoplasmic domains of monomers A and E showing the binding of Mg^{2+} (red spheres) in MjCorA. The interacting residues are shown as sticks. All distances are in the range of 4–5 Å. *Denotes the residues from adjacent monomer. **C.** MjCorA viewed from the extracellular side, colored by one protomer. Magnesium ions are shown as red spheres **D.** TmCorA viewed from the extracellular side, colored by one protomer. Magnesium ions are shown as red spheres and M1 and M2 sites are indicated between two protomers.

Two metal-binding sites, M1 and M2, were identified in TmCorA near the top of the intracellular domain to which Mg^{2+} , Co^{2+} and probably Ni^{2+} can

bind (Fig. 3D). An ion in the M1 site has distances of about 2 Å to coordinating carboxylates from D89 and D253, indicating a tight binding, whereas the distance to E88 is 4.4 Å suggesting the weak coordination. It therefore can be assumed that the carboxyl groups from D89 and D253 have substituted two water molecules in the first hydration shell, and the carboxyl group of E88 has replaced one water molecule in the second hydration shell. The M2 coordination site includes the carboxyl groups of E88, D175, D253 and the carbonyl groups of L12 and P13. Highly conserved L12 and P13 are in the extended N-terminal part that is found only in the subgroup A [30]. The distances between the cation and the coordinating groups in the M2 site are between 3 and 5 Å, suggesting that the ion holds its first hydration shell and the carboxyl and carbonyl groups replace the water molecules in the second shell [22]. It is noteworthy that Mg^{2+} and Co^{2+} could bind to the same sites with the same coordination.

Table 1. List of the properties of magnesium, cobalt, nickel and zinc

	Magnesium	Cobalt	Nickel	Zinc
Atomic radius (Å) _{SEP}	1.50	1.35	1.24	1.37
Ionic (2+) radius (Å)	0.86	0.84	0.69	0.74
Hydrated radius (first shell) (Å)	2.09	2.10	2.07	2.13

In contrast to TmCorA with two Mg^{2+} binding sites between the monomer-monomer interfaces, MjCorA has Mg^{2+} binding grooves. Each binding groove might provide a transient binding area for up to eight Mg^{2+} ions as found within the A-E groove (Fig. 3B and 3C). These grooves are formed by the sidechains of the negatively charged D54*, E68*, E69*, D70*, E142, D219, E215, and D223 residues, the hydroxyl containing Q62*, Y137,

N141, and N216 residues, and the main chain carbonyls of P65*, V67*, and V127 (* stands for the adjacent monomer). Thermostability tests were performed to elucidate the role of these grooves. The presence of Mg^{2+} has no influence on the thermostability of MjCorA. This finding strengthens the argument that the Mg^{2+} in binding grooves does not stabilize the protein and suggests that the binding of Mg^{2+} to these grooves has regulatory purpose.

Two low-resolution (7.1 Å) structures of the CorA from *T. maritima* in the absence of magnesium were recently published [36]. They reveal large-scale conformational changes in the structure, in which the five-fold symmetry seen in previous crystallographic structures is distorted as a result of movements of the cytoplasmic domains (Fig. 4). Four of the five subunits are moved relative to the central axis, and there are large hinge-bending motions of intracellular domains. It is hypothesized that Mg^{2+} stabilizes a closed state, while deficiency in intracellular Mg^{2+} leads to release of Mg^{2+} from CorA subunits followed by large cytoplasmic domain rearrangements. Ligand removal from the CorA subunit interfaces frees the channel to explore asymmetric conformations that seem to unleash a flexibility required to conduct ions. Hence, in contrast to the majority of ligand-gated channels, where ligand stabilize an open state, in CorA the magnesium ions stabilize the closed-state conformation. It appears that the open state in CorA is formed by multiple asymmetric conformations that interconvert to generate transiently conductive states. The loss of the interactions, which are stabilized in the presence Mg^{2+} , results in greater overall flexibility of the protein complex, including in the hydrophobic gate region of the narrow pore. The higher flexibility likely increases the frequency of hydration events that enable Mg^{2+} transport [35]. However, since this study has been performed in magnesium-free conditions, that are unlikely to occur in physiological

environment, the relevance of the given interpretation for the functioning of the protein *in vivo* is questionable.

Figure 4. Structure of TmCorA in the absence of Mg^{2+} (A) side view, (B) top view and (C) bottom view. The large inward and outward movements of the of subunits can be seen compared to symmetrical closed state of TmCorA in Fig. 3D and Fig. 2A.

Recently, another study on the TmCorA transport mechanism, involved two X-ray structures of TmCorA's D89K/D253K and D89R/D253R mutants, has been published [37]. These two structures with resolutions 3.1 and 3.3 Å showed no significant structural alterations compared to the wild type protein, although there was no Mg^{2+} bound at the M1 sites. It means that both mutants are most likely in the closed conformation. However, both structures have Mg^{2+} bound to M2 sites, one Mg^{2+} in the D89K/D253K mutant, and three Mg^{2+} ions in the D89R/D253R mutant [37]. In addition to

these magnesium ions, both structures have two Mg^{2+} ions bound inside the pore. Hence, one of the possible explanations can be that crystal contacts and nonspecifically bound Mg^{2+} support the closed state. If this explanation is correct, then these two structures are artefacts and do not represent the real open state. Detailed future structural and biochemical studies are required to elucidate the actual CorA transport mechanism and the role of M1 and M2 sites.

Existing data for CorA provide some foundation to understand the relation between the structure, function, regulation and transport of magnesium ions in CorA. The possible CorA's transport mechanism is self-regulated. When the cell has enough magnesium, free magnesium ions bind to the cytoplasmic binding sites and convert a putative open state into a stable homopentamer with the narrow closed hydrophobic pore. In general, the assumption is made that its eukaryotic homologs, such as the human mitochondrial Mg^{2+} channel Mrs2, can have the same transport mechanism. A crystal structure of the soluble domain of Mrs2 confirms its high structural similarity with CorA; and the essential GMN-selectivity filter motif of Mrs2 also implies a shared mechanism of ion selectivity and conduction [38]–[40]. However whether it is really true will require full length structures of Mrs2 preferably in different states.

ZntB

Zinc is the second most abundant transition metal in biological systems and is essential for many cellular processes. But due to high toxicity the tolerable free zinc concentration is in the nano- and femtomolar range [41]. All living organisms require multiple transport systems to maintain zinc homeostasis in the cells. For instance, the ZnuABC and ZupT perform zinc

uptake and the ZitB, ZntA and CzcABC transport systems are responsible for its efflux in bacteria [42]–[45]. In addition to the aforementioned systems, there is ZntB that exclusively identified in the protobacteria of the α -, β -, and γ -subgroups. The ZntB-type genes generally seem to be found in fewer taxa compared to *corA* genes. However, the ZntB homologue appears to be the only 2-TM-GxN type protein specified by the genomes of *Silicibacter pomeroyi*, *Idiomarina loihiensis*, the *Vibrio* group and the isolated proteobacterial genus *Magnetococcus*. In these bacterial species, CorA orthologs are apparently lacking [21].

ZntB belongs to the 2-TM-GxN family, but it has GVN motif instead of GMN signature motif. The first study in *S. typhimurium* proposed ZntB (StZntB) as zinc and cadmium, but not magnesium, efflux transport system [46]. However, a follow-up study proposed ZntB as Zn^{2+} importer based on detailed analysis of metal uptake by several *Cupriavidus metallidurans* mutants [17]. Another study of *Agrobacterium tumefaciens* ZntB demonstrated that AtZntB is not involved in Zn^{2+} as well as in Cd^{2+} , Co^{2+} , Cu^{2+} , Fe^{2+} , Mg^{2+} , Mn^{2+} , Ni^{2+} and Pb^{2+} resistance, indicating that AtZntB is probably not a metal exporter. Both wild type and AtZntB knock-out showed similar metal resistance, but it is important to note that AtZntB shares below 20% amino acid identity with StZntB and has the signature motif GxxGMNxxDExP instead of standard ZntB motif GxxGVNxGGxP [21], [47]. This data suggests that AtZntB is possibly a metal importer, however its function in zinc uptake remains elusive.

Up to date, the structures of cytoplasmic domains structures from two ZntB homologues are available [48]–[50]. Both of them are in agreement with the previously demonstrated the 2-TM-GxN's homopentameric organization with the extensive cytoplasmic domain and two transmembrane helices per

monomer. The first crystal structure of the *Vibrio parahemolyticus* ZntB's (VpZntB) cytoplasmic domain with 1.9 Å resolution revealed a funnel-shaped homopentamer, similar to the full-length TmCorA and MjCorA structures (Fig. 5A and 5C). The VpZntB structure did not have any bound substrates as Zn^{2+} , Mg^{2+} or other relevant divalent metal ions, despite the fact that it was crystallized in the presence of 0.2 M MgCl_2 . However 25 well-ordered Cl^- anions in total, with five Cl^- ions per monomer, four external, and one internal to the funnel, were modeled bound to the protein, forming a chloride ring in the middle of a cytoplasmic pentamer [48]. It is puzzling why in this particular case ZntB prefers chloride ions over magnesium, but it is known for other metal channels that electrostatic forces may play an important role in metal transport [51], [52]. Furthermore, since it is a truncated form, such a binding might be an artifact.

Figure 5. Structures of ZntB cytoplasmic domains from (A) *V. parahemolyticus* and (B) *S. typhimurium*. (C) VpZntB cytoplasmic domain, viewed from the extracellular side, colored by one protomer. The bound Cl^- anions are shown as green spheres. (D) ScZntB cytoplasmic domain, viewed from the extracellular side, colored by one protomer. The bound Zn^{2+} cations are shown as yellow spheres.

The other two crystal structures of the soluble domains of *S. typhimurium* ZntB were reported in two different forms. One structure has two antiparallel monomers per asymmetric unit and another structure has five monomers per asymmetric unit, resembling the physiologically relevant

pentamer (Fig. 5B and 5D). Both the pentameric and monomeric structures of the StZntB had three Zn^{2+} ions bound to each monomer (Fig. 5B and 5D). The first Zn^{2+} coordinated by two adjacent H41 most probably is non-physiological due to its position. A second Zn^{2+} is located on the funnel surface, coordinated by C94 in $\beta 5$ and H159 in $\alpha 5$ from the same monomer. The third Zn^{2+} is bound within the wall of the pentamer and is coordinated by H168 in $\alpha 5$ and C246 in $\alpha 7$.

The funnel shape of the StZntB N-terminal domain is significantly different compared to those of CorA and VpZntB. The pore of StZntB within the funnel has a cylindrical shape (12 Å diameter), while for both CorA and VpZntB the interiors of the funnel have rather a conical shape, wide at the cytosolic end and narrow on the funnel top (3.2 Å and 4 Å for TmCorA and VpZntB respectively).

The main goal of this thesis is a structural and biochemical characterization of *E. coli* ZntB. Both *apo* and ligand bound structures of the full-length ZntB are presented in the next chapters. All available data indicate that ZntB is indeed a transporter, hence the 2-TM-GxN family consists both of channels and transporters similar to the CIC protein family [53], [54]. I present the full-length *apo*-structure of EcZntB in **Chapter 2**. In stark contrast to TmCorA, EcZntB maintains its five-fold in the absence of the substrate. I propose that zinc transport via ZntB is stimulated by proton gradient, based on the radioactive and fluorescent uptake assays at various pH conditions. In the next **Chapter 3** I discuss EcZntB structure in the presence of zinc and cadmium, however, it is hard to make any solid conclusion from those structures as they contradict each other. However in the same chapter I confirm the proton:zinc ions coupling via combination of mutagenesis and

transport assays. And in **Chapter 4** I show that CorA and ZntB can transport the same ions, but transport via CorA is not stimulated by proton gradient.

Alr1, Alr2 and Mnr2

Another subgroup in the 2-TM-GxN family consists of Alr1, Alr2 and Mnr2 proteins. Alr1 is essential for uptake of Mg^{2+} into yeast cells, while Alr2 is not important for yeast growth, but can compensate Mg^{2+} transport in *alr1*Δ mutants [55]. The homologous Alr1 and Alr2 reside in the yeast and fungus plasma membrane, while Mnr2 is localized in the vacuole membrane [56]. Patch clamp data in yeast suggest that the Alr1 protein acts as an Mg^{2+} -permeable ion channel [14]. Alr1 and Alr2 are closely related proteins with 70% identity and similar polypeptide size. Mnr2 has similar size, but shares only 20% identity with both Alr1 and Alr2. Sequence data suggests that Alr and Mnr2 proteins share similar structural features of the 2-TM-GxN family – pentameric organization, two transmembrane helices, the GMN motif and the large charged N-terminal domain.

Environmental magnesium does not control expression of CorA in bacteria [57]. However, there is an evidence that expression of yeast *alr1* and *alr2* genes is dependent on Mg^{2+} concentrations in the environment, although the mechanism of this regulation is still unknown [55], [58]. Another study has shown that expression of *alr* genes and turnover of Alr1 in yeast is controlled by the Mg^{2+} concentration in the medium [59]. The same study showed that low Mg^{2+} concentration increases Alr1 expression and enhances concentration and stability of the protein in the membrane, while the addition of high amount of Mg^{2+} to the growing cells induces rapid degradation of the protein via the endocytotic pathway, ending in the vacuole [59]. It remains to be demonstrated whether these ion transporters themselves control Mg^{2+}

influx into cells or organelles or whether other factors mediate or contribute to flux control.

A split-ubiquitin assay demonstrated that Alr1 and Alr2 can form not only homo-oligomeric, but also Alr1-Alr2 hetero-oligomeric interactions, similar to Mrs2 in plants, discussed below [58]. One possible hypothesis for function of the formation of Alr1-Alr2 hetero-oligomers is reduction of Mg^{2+} uptake in yeast because of the low activity of Alr2 towards magnesium transport [58], [60]. Additionally, it has been shown that both the N-terminus and C-terminus of Alr1 and Alr2 are in the same compartment, most probably in the cytoplasm. The same orientation of N- and C- termini is present in CorA and ZntB structures, as well as Mrs2 [22], [50], [61].

Overexpression of the genes Alr1 and Alr2 confers tolerance of *Saccharomyces cerevisiae* to trivalent ions such as aluminum and gallium, simultaneously increasing sensitivity to zinc, manganese, nickel, cobalt and copper ions [62]. This demonstrates that Alr proteins are most probably even more promiscuous than ZntB and CorA. Alr1 and Alr2 can transport La^{3+} in contrast to Al^{3+} and Ga^{3+} , suggesting that still some trivalent cations can be effectively imported by Alr proteins. But it is unlikely that Alr proteins contribute under physiological conditions to the uptake of aforementioned trivalent and divalent cations other than Mg^{2+} , due to the insignificant requirement in yeast for these ions [63].

The Alr1 branch of the CorA proteins includes a subgroup represented by Mnr2 [28], a vacuolar membrane protein required for Mg^{2+} export from the vacuole [29]. Mutants lacking Mnr2 display a growth deficiency and accumulate a higher intracellular magnesium content in Mg^{2+} -deficient conditions. As Alr1 and Mnr2 both supply Mg^{2+} to the cytosol, the regulation of these proteins is likely to be of central importance to cytosolic Mg^{2+}

homeostasis. The opposite effect of the *mnr2* and *alr1/alr2* mutations on Mg^{2+} content and the location of the Mnr2 protein is consistent with Mnr2 supplying cytosol with Mg^{2+} from vacuolar stores, rather than from the external environment. Indirect evidence for an important role of Mnr2 in Mg^{2+} transport came from the observation that Mnr2 overexpression appeared to relocate a fraction of the protein to the cell surface, while also partially suppressing the growth defect of an Alr1/Alr2 mutant [56]. Although this experiment did not provide direct evidence for Mg^{2+} transport by Mnr2, it did demonstrate that Mnr2 could still function in the absence of the Alr proteins, suggesting that Mnr2 does not simply act as a regulatory or structural component of these proteins.

A study on Alr2 and Mnr2 from the fungal pathogen *Magnaporthe oryzae* has demonstrated their important roles for fungal development and virulence. It was revealed that MoAlr2 plays a significant role in intracellular Mg^{2+} regulation during *M. oryzae* growth and differentiation. Combined results from the phenotypic defects show that 2-TM-GxN members are involved at all stages of the life and infection cycles of *M. oryzae*. Knockdown of MoMnr2 and especially MoAlr2 causes *M. oryzae* to lose its virulence, suggesting that these proteins could be targets for development of new antifungal agents [64].

Mrs2 and Lpe10

After identification of the first eukaryotic CorA homologs Alr1 and Alr2, further studies identified related proteins in the mitochondrial inner membrane – Mrs2 and Lpe10. Both proteins were required for the Mg^{2+} transport into the mitochondria, and loss-of-function mutations in either gene caused similar reductions in mitochondrial function and Mg^{2+} concentration [65]–[67]. Members of the Mrs2 subfamily exhibit considerable sequence

similarity. Mammals, including *Homo sapiens*, have only one *mrs2* gene and its protein is located in mitochondria [68]. The yeast genome contains two genes – *mrs2* and *lpe10*, while known plants genomes can consists up to several dozen variants of Mrs2, located either in mitochondria, in the plasma membrane or in other cellular membranes [21]. Mrs2 proteins in plants have alternatively been named as Mrs2 or MGT proteins [69], [70], it will be called Mrs2 for convenience in this chapter.

The overall organization of prokaryotic CorA and eukaryotic Mrs2 is most probably similar. Bioinformatics analysis of full-length Mrs2 predicted a large N-terminal domain and transmembrane domain consisting of two α -helices. Up-to-date only one structure of the soluble domain Mrs2 without a signal sequence from yeast *S. cerevisiae* (ScMrs2) is available. It was also crystallized as a monomer similar to the CorA soluble domain. The cytoplasmic domain of ScMrs2 consists of the C-terminal triple coiled coil and the N-terminal compact α - β - α sandwich domains (Fig. 6). While the C-terminal parts of the soluble domains are identical in CorA and ScMrs2, the N-terminal parts have several differences. The central β -sheet is formed by seven strands in CorA versus six strands in ScMrs2. While the last four β -strands are topologically identical in both proteins and form a series of three β -hairpins, the first two β -strands differ topologically. The α -helix that follows the second β -strand and the next whole β -strand are missing in Mrs2, making the eukaryotic soluble domain smaller than its prokaryotic homolog [40]. The α 7 helices inside of the ScMrs2 form the inner pore of the pentameric funnel with facing negatively charged residues. Negative charge inside of the inner pore is common in cation channels and creates an electrostatic sink that helps to transport positively charged ions [71].

Figure 6. Structure of the N-terminal domain of *S. cerevisiae* Mrs2_{248–308}. (A) side view, (B) top view and (C) bottom view.

Although Mrs2 has highly-conserved amino acids D97 and E270 that can potentially form a divalent cation sensor (DCS) similar to CorA proteins, Mg²⁺ and Co²⁺-soaked crystals did not reveal bound metal ions. The absence of cations bound to the monomeric Mrs2 can be explained by that DCS sites are composed of ligands from adjacent subunits in the pentamer, and a single subunit cannot bind divalent ions without adjacent subunit. Furthermore, D97 was mutated to Ala, Phe or Trp in the full length protein, but the cells expressing the mutant proteins did not show growth defects and no significant differences in Mg²⁺ uptake between wild-type Mrs2 and the mutants in mitochondria were observed [40]. It might be that sensing mechanism of Mg²⁺ is different in Mrs2 compared to CorA or similar to MjCorA with magnesium binding grooves. Further studies of the full-length structure Mrs2 are required to understand Mg²⁺ transport and sensing by Mrs2.

Mrs2 has a functional homolog Lpe10, which was recently demonstrated to be involved in mitochondrial magnesium homeostasis [65]. Existing data indicates that Lpe10 is a membrane protein of the inner mitochondrial membrane and that both N- and C-termini are oriented towards the matrix side of the inner membrane. The study shows that the Lpe10 protein is functionally similar to Mrs2, but cannot substitute it, meaning that Lpe10 perhaps is fully functional only as a hetero-oligomer [66]. For example, hetero-oligomerization is common in plants and can adjust ion channels' transport characteristics to suit the needs of cell during various moments of life cycle [72], [73]. Nevertheless further studies are required to confirm this hypothesis.

Mrs2 proteins play a significant role in the transport of magnesium and other divalent metal cations in plants. Up to date, several groups have investigated the role of Mrs2 in the plant model *Arabidopsis thaliana* and in plants important for agriculture such as rice, maize and rapeseed. *A. thaliana* has 10 homologs of Mrs2, maize *Z. mays* has 12 homologs, rice *O. sativa* has 9 members, and in rapeseed *B. napus* the Mrs2 family has as many as 36 members [74]–[77]. The Mrs2 genes in *B. napus* are classified into five groups by the phylogenetic relationships of the Mrs2 genes from *Arabidopsis* and rice [21]. The triplication of Mrs2 genes in *B. napus*, compared with that in *Arabidopsis* occurred after the genome tripling in *B. rapa* and *B. oleracea* [76]. There are several explanations for such big diversity of Mrs2 genes in plants. One of the explanations is that plants can only extract their nutrients from the soil in contrast to other forms of life that can chose their nutrients sources. For this reason, such diversification in the 2-TM-GxN protein family might help plants to adapt to the availability of metal ions in the soil via creating various Mrs2 heteromers. However it also requires confirmation

from detailed biochemical and structural characterizations of Mrs2 plant homologs.

All Mrs2 proteins in *A. thaliana*, *O. sativa*, *B. napus* have two conserved TM regions at the C-terminus, the same as all members in the 2-TM-GxN family. While all the AtMRS2 family members contain the conserved GMN motif, it is modified in some rice and rapeseed Mrs2 proteins [70], [75], [76]. For example, the motif in the OsMrs2-4 and OsMrs2-5 is altered to AMN, OsMrs2-8 has the GIN motif [75]. Furthermore, the analysis of all the BnMrs2 protein sequences showed that the complete GMN motif was also observed in 31 BnMrs2, but it was altered to GMR in BnMrs27c and GIN in BnMrs2-10e, BnMrs2-10f, BnMrs2-10g, and BnMrs2-10h similar to OsMrs2-8 [76]. It can be explained by the result of gene variation during evolution and environmental selection conditions. There is a possibility that ion selectivity in heteromers can be improved or changed by combining different motifs, however, this needs to be tested experimentally.

A serious agricultural problem is Al^{3+} toxicity in the crops, but the mechanism of toxicity remains elusive [78]. Uptake experiments on ryegrass roots have confirmed that Al^{3+} can inhibit Mg^{2+} uptake [79]. The importance of Mg^{2+} in aluminum toxicity in grasses and cereals was shown by experiments in which increase of Mg^{2+} uptake was shown to reduce Al^{3+} toxicity [80], [81]. A first study in yeast *S. cerevisiae* Alr1 and Alr2 demonstrated that aluminum can inhibit magnesium uptake in the 2-TM-GxN family and subsequent overexpression of Alr1 and Alr2 conferred resistance to aluminum [62], [82]. Later it was confirmed that upregulation of the gene for the rice OsMrs2-1, localized in plasma membrane, is required for conferring Al^{3+} tolerance by increasing Mg^{2+} uptake into the cells [83]. The following investigations showed that even low concentrations of Al^{3+}

effectively inhibit both Mrs2-1 and Mrs2-10 leading to Al^{3+} -induced Mg^{2+} deficiency in *A. thaliana* [74]. Summarizing all studies, the Mrs2 clade of proteins is one of possible molecular targets for Al^{3+} toxicity in higher plants. Further molecular genetics, biochemical and structural studies are needed to confirm Mrs2 proteins as potential targets to improve Al^{3+} tolerance in plants.

Conclusions

The 2-TM-GxN family is widely present in bacteria, archaea, and eukaryotes and is responsible for transport of divalent cations, in particular Mg^{2+} , Ni^{2+} , Co^{2+} and Zn^{2+} [21]. All of them are believed to have pentameric organization, with two transmembrane helices per monomer, signature family motif GxN and large N-terminal cytoplasmic domains, but it appears that they diverged dramatically to serve for several different purposes, such as Mg^{2+} transport via channel-like mechanism, transport of zinc ions in proton-coupled manner, with some members being strictly homopentameric and some able to form heterooligomers to suit needs of an organism. Recent advances in the structural and functional studies on the CorA lead to better understanding of its role in the cellular metal ions homeostasis. Nevertheless, there is a significant lack in the structural characterization of other members of the family. It is essential to solve the full-length structures of Alr1/2, Mrs2 and other aforementioned proteins, as well as to biochemically characterize them thoroughly, to obtain a more comprehensive understanding of their functions and transport mechanisms.

Outline of this thesis

The main goal of this thesis is the study of the transport mechanism of ZntB, one of the members of the 2-TM-GxN protein family, that presumably consists mostly of magnesium channels. When I started my PhD in 2015,

there was no full-length structure of ZntB resolved and biochemical characterization was very scarce. Therefore, it was an open question whether ZntB is a channel or a transporter, and what the mechanism of transport is. Here, I briefly outline content of each chapter with a short description.

In **Chapter 1** I provide a general overview about the 2-TM-GxN family and the clusters defined within the family – namely bacterial CorA and ZntB, fungal Alr1 and Alr2 and eukaryotic Mrs2 proteins. In this chapter I have summarized all known structural data with the description of their role in the cellular metal homeostasis.

I present the first full-length structure of *Escherichia coli* ZntB without bound ligand in **Chapter 2**. Also I demonstrate that ZntB, reconstituted into proteoliposomes, can transport zinc as well as cadmium, nickel and cobalt ions. By combining the radioactive and fluorescent uptake assays with various pH conditions, obtained results show that transport via ZntB is stimulated by a pH gradient across the membrane. In addition to uptake experiments, I have performed isothermal titration calorimetry to determine K_d values for Zn^{2+} , Cd^{2+} , Ni^{2+} and Co^{2+} ions.

In **Chapter 3** I demonstrate another full-length structure of *Escherichia coli* ZntB in the presence of cadmium or zinc ions, however these structures are of low resolution and are hard to interpret. In addition, I have generated several mutations for the amino acids, which are important for zinc and proton binding. According to the transport experiments these amino acids are indeed involved in proton binding and are critical for the proton-coupled transport mechanism, while other amino acids are not crucial for the zinc ion recognition. By combining data from **Chapter 2 and 3**, I propose that ZntB is a Zn^{2+} importer that is driven by a proton gradient.

I investigate cation selectivity in *Escherichia coli* ZntB, bacterial *Thermotoga maritima* and archaeal *Methanocaldococcus jannaschii* CorAs in **Chapter 4**. In this chapter I show that all three proteins can transport Mg^{2+} , Co^{2+} , Ni^{2+} , Zn^{2+} and Cd^{2+} , but not a trivalent Al^{3+} . Furthermore, I demonstrate that the transport via CorA is not stimulated by proton gradient, but by the membrane potential, adding one more piece of evidence that transport mechanisms in CorA and ZntB are different.

In **Chapter 5** I present an overview of the most commonly used detergents for membrane protein structural studies. The aim of this study is to analyze the application of different solubilizing agents based on the published structures of membrane proteins and to describe the main properties of ten most popular detergents, including critical micelle concentration (CMC) value, micelle size and its molecular weight. Apart from detergents, I briefly discuss the alternative agents such as styrene maleic acid (SMA) polymer, amphipols, nanodiscs, calixarens and fluorinated surfactants.

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Chapter 2

The structural basis of proton driven zinc transport by ZntB

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Abstract

Zinc is an essential microelement to sustain all forms of life. However excess of zinc is toxic, therefore dedicated import, export and storage proteins for tight regulation of the zinc concentration have evolved. In *Enterobacteriaceae* several membrane transporters are involved in zinc homeostasis and linked to virulence. ZntB has been proposed to play a role in the export of zinc, but the transport mechanism of ZntB is poorly understood and based only on experimental characterization of its distant homologue CorA magnesium channel. Here, we report the cryo-electron microscopy structure of full-length ZntB from *Escherichia coli* together with the results of isothermal titration calorimetry, and radio-ligand uptake and fluorescent transport assays on ZntB reconstituted into liposomes. Our results show that ZntB mediates Zn²⁺ uptake, stimulated by a pH gradient across the membrane, using a transport mechanism that does not resemble the one proposed for homologous CorA channels.

Introduction

Zinc is one of the few “essential-but-also-toxic” divalent cations required for the cell and is an important ‘token coin’ in host:pathogen interactions¹: whenever host organisms try to sequester all available zinc at the host:pathogen interface to reduce the virulence of invading bacteria², the latter employ highly specific uptake systems to scavenge zinc³. Conversely, if the zinc concentration is elevated in hosts to oppress pathogens⁴, the latter regulate their intracellular zinc concentration by scaling up the export of zinc³. Due to this ambidexterity, the tight regulation of zinc homeostasis is crucial. Different bacteria cope with this task in a variety of ways – for example by storage of zinc by metallothioneins as in cyanobacteria⁵, by assembly of redundant importers as in *Cupriavidus metallidurans*⁶ or via a controlled shunt of zinc export-import as in *Escherichia coli*, where Zinc-iron permeases (ZIP) family transporter ZupT⁷ and the ATP-binding cassette (ABC) transporter ZnuABC^{8,9} are recruited for import, and P-type ATPase ZntA¹⁰ and cation-diffusion facilitator YjiP¹¹ for export of zinc (Supplementary Fig. 1). In addition the zinc transporter ZntB, which belongs to the CorA Metal Ion Transporter (MIT) family is widespread in *Enterobacteriaceae*^{12,13}. There is controversy over the question whether ZntB is an exporter¹² or importer⁶. Furthermore, mechanistic insight is lacking because crystal structures are available of only cytoplasmic parts of ZntB^{14,15}, and scarce transport activity measurements have been performed only in whole cells. We have obtained the structure of full-length ZntB from *E.coli* and performed isothermal titration calorimetry (ITC), radiolabeled zinc uptake and fluorescent transport experiments with ZntB reconstituted into liposomes. This study shows that ZntB mediates Zn²⁺ transport, which is stimulated by a pH gradient across the membrane. The comparison of the full-

length structure of ZntB with previously resolved structures of ZntB soluble domains in different conditions (in the presence and absence of Zn^{2+}) and structures of homologous CorA proteins, is indicative that ZntB and CorA proteins utilize different transport mechanisms.

Results

Structure of ZntB

The *apo* structure of ZntB was obtained by single-particle cryo-electron microscopy (cryo-EM) using n-Dodecyl- β -D-Maltopyranoside (DDM)-solubilized and purified *E.coli* ZntB (EcZntB) (pre-treated with Ethylenediaminetetraacetic acid (EDTA)) and resolved at an overall resolution of 4.2Å (Supplementary Figs. 2 and 3, Table 1). The structure of ZntB revealed a pentameric arrangement (Fig.1a, b), similar to that reported for other members of the CorA family^{13,16,17} (Supplementary Fig. 4) even though CorA and ZntB share very little sequence identity (below 20 %) (Supplementary Fig. 5). Each protomer of ZntB consists of a large N-terminal cytoplasmic domain folded into an $\alpha\beta\alpha$ motif. A long α -helix protrudes from the cytoplasmic domain into the membrane (TM1) and is joined to a second transmembrane helix (TM2) (Fig. 1a) via the only periplasmic loop, which bears the signature motif GxN of the CorA MIT family¹³ (Fig. 1b, c). In the resolved structure of EcZntB, the cytoplasmic domain is very similar to the isolated domain of ZntB from *Vibrio parahaemolyticus* (Vp)¹⁴ (rmsd ~2.5Å), but significantly different from the homologous domain of *Salmonella typhimurium* (St) ZntB¹⁵ (rmsd ~12Å). Taking into account very high sequence conservation between EcZntB and StZntB of 92.6% (see Supplementary Fig. 5) the structural difference is intriguing. This difference could be attributed to the two structures representing two different states in the transport cycle (discussed below). Analysis of the substrate translocation

pore revealed a wider profile in ZntB than in the magnesium channels TmCorA and MjCorA (Supplementary Fig. 6). All three proteins have short extracellular loops between TM1 and TM2, where the family signature motif GxN that forms the selectivity filter (Fig. 1b, c) is located. Whereas in CorA proteins the signature motif has the sequence GMN, ZntBs show a Met to Val substitution, potentially having consequences for the substrate recognition, as in ZntB, the radius of the filter is ~ 4.5 Å vs 3.5-4.0 Å in CorAs (Fig. 1d).

Table 1. Data Collection and refinement statistics

<i>Data collection</i>	
<i>Microscope</i>	<i>Titan KRIOS with K2-detector</i>
<i>Voltage</i>	<i>300 kV</i>
<i>Pixel size (Å)</i>	<i>1.43</i>
<i>Micrographs collected (#)</i>	<i>2655</i>
<i>Refinement</i>	
<i>Particles (#)</i>	<i>333,490</i>
<i>Resolution (Å; at FSC = 0.143)</i>	<i>4.2</i>
<i>CC (model to map fit)</i>	<i>0.81 (0.83)</i>
<i>RMS deviations</i>	
<i>Bonds (Å)</i>	<i>0.007</i>
<i>Angles (°)</i>	<i>1.152</i>
<i>Chirality (°)</i>	<i>0.065</i>
<i>Planarity (°)</i>	<i>0.008</i>
<i>Validation</i>	
<i>Clashscore</i>	<i>12</i>
<i>Favored rotamers (%)</i>	<i>98.77</i>
<i>Ramachandran favored (%)</i>	<i>91.69</i>
<i>Ramachandran allowed (%)</i>	<i>8.31</i>

Figure 1: The structure of the full-length ZntB. (a) Side view, four subunits of ZntB pentamer are coloured grey, and one is coloured rainbow from blue (N-terminus) to red (C-terminus); the position of the membrane is indicated, trans membrane helices 1 and 2 as well as $\alpha\beta\alpha$ -motif are labelled (b) Top view (from periplasm) onto ZntB – 10 trans membrane helices are arranged cylindrically, with TM2 ring at the periphery. Experimental density is contoured in blue. The connecting loops provide residues for the selectivity

filter (in magenta), further exemplified in (c) structural comparison of selectivity filters from EcZntB (magenta) and TmCorA (cyan), only three out five monomers are shown (d) The overlay of Asn rings (of GxN motif) from ZntB (magenta), TmCorA (cyan) and MjCorA (yellow). Note that ZntB forms a slightly wider entry point to the pore.

Substrate selectivity of ZntB

One of the puzzling features of the CorA family is substrate selectivity. The geometry of the selectivity filter is thought to define the correct distances between (partially) hydrated cation and amino acid side chains of the filter, and hence recognition^{18,19}. Intriguingly, although the hydrated radii of known transported substrates for the CorA family are very similar (2.10 Å, 2.09Å, 2.10Å, 2.07 Å for Zn²⁺ (ref ²⁰), Mg²⁺ (ref ²¹), Co²⁺ (refs ^{21,22}) and Ni²⁺ (ref ²³) respectively) and all these cations have a similar octahedral arrangement of six water molecules in their first hydration shell in aqueous solution, different subfamilies have distinct substrate specificities. It has been proposed that this selectivity might be based on the rigidity of ion solvation shells and rates of water exchange¹⁸.

Figure 2: ITC profiles of substrate binding to ZntB of (a) Zn^{2+} (b) Cd^{2+} (c) Ni^{2+} (d) Co^{2+}

To characterize the specificity we performed isothermal titration calorimetry (ITC) experiments and fluorescent transport assays. ITC experiments revealed binding of Zn^{2+} , Cd^{2+} , Ni^{2+} and Co^{2+} to ZntB, with K_d values of 11.5 μM , 22.6 μM , 87.7 μM and 175.4 μM respectively in 1:1 stoichiometry (Fig. 2). No binding of Mn^{2+} , Mg^{2+} and Cu^{2+} was detected. The ability of ZntB proteins to select Zn^{2+} over Mg^{2+} , and conversely the ability of CorA proteins to select Mg^{2+} over Zn^{2+} , may be explained by different sizes of the pore – the substitution of a single amino acid in the signature motif (see above) might be enough. However Co^{2+} and Ni^{2+} appear to be substrates of both ZntB and CorA^{24,25}, thus the precise determinants of selectivity are still elusive. We additionally tested the transport of the cations by ZntB reconstituted into the liposomes, and found that the ions that bind to the protein in ITC assay are also transported (see below).

Zinc transport by ZntB is stimulated by pH gradient

To characterize the mode of transport, we performed the $^{65}\text{Zn}^{2+}$ uptake assays with purified ZntB reconstituted into liposomes. Transport was measured using either equal pH on the in- and outside of the liposomes, or using pH gradients. Zn^{2+} was taken up by the liposomes containing ZntB, and uptake was enhanced by a pH gradient with the lumen of the liposomes more basic than the outside. In contrast, uptake was suppressed in the presence of a reverse pH gradient. These experiments suggest that zinc transport is driven by the pH gradient. Consistently, addition of the proton ionophore FCCP at a time point when the uptake had reached a plateau led to efflux of the accumulated $^{65}\text{Zn}^{2+}$ in case of a pH gradient that was basic inside (Fig. 3a), and addition of FCCP to liposomes with the opposite pH gradient stimulated additional uptake. FCCP did not affect the Zn^{2+} accumulation in the absence

of a pH gradient (Fig. 3a). These results were confirmed by performing the transport assays with the specific zinc reporting dye fluozin-1²⁶, encapsulated into liposomes. The fluorescence signal increased upon Zn^{2+} transport into the liposomes and FCCP had a similar effect as in uptake assays with radiolabeled Zn^{2+} (Fig. 3b). The observed stimulation of Zn^{2+} uptake by an inward pH gradient suggests a mechanism in which protons are cotransported with Zn^{2+} . We directly measured Zn^{2+} -dependent proton transport using pH sensitive fluorophore 9-Amino-6-Chloro-2-Methoxyacridine (ACMA) (Fig. 3c) with proteoliposomes that had equal pH in the lumen and exterior. Zn^{2+} uptake into these proteoliposomes was accompanied by generation of a pH gradient, consistent with coupled H^+ - Zn^{2+} transport mechanism. A Na^+ gradient instead of a proton gradient did not stimulate transport (Supplementary Fig. 7). These experiments show that Zn^{2+} transport is stimulated by a proton gradient across the membrane. Finally, the transport of Zn^{2+} saturated with increasing Zn^{2+} concentrations with a K_M of $\sim 7.5\mu\text{M}$ (Figure 3d), again indicative of a transporter mechanism.

Figure 3: Radioactive and fluorescent transport assays (a) $^{65}\text{Zn}^{2+}$ uptake via ZntB reconstituted in liposomes under different conditions (color-coded: black – inward pH flux (7.5 in / 6.5 out); green – outward pH flux (6.5 in / 7.5 out); red and blue – no pH flux at 6.5 and 7.5 pH respectively). Ionophore FCCP was added at the time point of 10 min. Note the opposite effect of FCCP on direct and reverse pH gradient. Error bars represent s.e.m. from more than 3 technical replicates of independent batches of proteoliposomes (b) Changes in fluorescent signal by the reporter dye FluoZin-1 during uptake of Zn^{2+} (added at 1 min time point) via ZntB reconstituted in liposomes under different conditions (color-coding as in (a), additionally the signal from empty liposomes in magenta). FCCP was added at the time point of 6 min. Error bars represent s.e.m. from more than 3 technical replicates of

independent batches of proteoliposomes. **(c)** Zn^{2+} -dependent transport of H^+ via ZntB. Quenching of the pH-dependent fluorophore ACMA at different Zn^{2+} concentrations is shown by unique colours. **(d)** Rate of transport dependence on Zn^{2+} concentration. The solid line represents the fit to the Michaelis–Menten equation with a K_M of $\sim 7.5\mu\text{M}$ (based on FluoZin-1 experiments).

To complement the ITC experiments on substrate binding (see above) we tested whether Ni^{2+} , Co^{2+} , Cd^{2+} could be transported by ZntB reconstituted into the liposomes using the fluozin-1 dye, and observed comparable levels of transport for Ni^{2+} and Cd^{2+} , but not for Co^{2+} . The failure to detect transport of Co^{2+} is likely caused by lower sensitivity of the dye for this cation (Fig. 4).

Figure 4. Transport of different cations by ZntB. Transport of different cations (added at 1 min and colour-coded: black – $20\mu\text{M}$ Zn^{2+} , red – $20\mu\text{M}$ Cd^{2+} , blue – $100\mu\text{M}$ Ni^{2+} , green – $200\mu\text{M}$ Co^{2+} , dark blue – empty liposomes

with 20 μM Zn^{2+} , purple – empty liposomes with 200 μM Co^{2+}) assayed by the fluorophore FluoZine-1 trapped inside the proteoliposomes.

Discussion

ZntB is a distant homologue of CorA proteins, which are well-characterized magnesium (and cobalt) channels^{13,16,17,27}. Based on whole-cell transport experiments on ZntB¹² and structures of soluble domains^{14,15} it has been proposed that CorA superfamily contains both channels (CorA) and transporters (ZntB), the latter possibly using a different transport mechanism¹². A recently reported cryo-EM structure of TmCorA in Mg^{2+} -free conditions, obtained upon EDTA treatment, revealed an unprecedented asymmetry of the pentamer²⁸. It was concluded that CorA proteins might use a transport mechanism that involves a partial loss of the five-fold symmetry in the open state²⁸⁻³⁰ to create a pore wide enough for the transport of partially hydrated magnesium. Our work on ZntB shows that the mechanism cannot be extrapolated to other members of the family, because even after an extensive treatment with EDTA ZntB maintained its symmetrical pentameric state (Supplementary Fig. 8). There are several possible explanations for the differences between CorA and ZntB. First, the structural differences are genuine, and related to mechanistic differences between CorA and ZntB. Second, ZntB can also form a collapsed state, but under different conditions than CorA (perhaps in the membrane). Third, the observed symmetry-collapsed state of CorA is an artefact, possibly induced by the Mg^{2+} -free conditions that were used to obtain the structure. Such conditions are probably never encountered by the protein under physiological conditions, as the intracellular concentration of free Mg^{2+} is estimated to be around 0.5-1 mM³¹. Also, other intracellular divalent and monovalent cations were shown to bind to TmCorA³⁰. Therefore, it is possible that the observed symmetry-collapsed

structure is not part of the mechanism of channel opening. In contrast to stringent removal of Mg^{2+} from CorA channels, the depletion of Zn^{2+} from ZntB is likely to be physiologically relevant because intracellular concentrations of free Zn^{2+} are extremely low (i.e. in the pM-fM range ^{32,33}). Therefore the symmetrical structure of ZntB may better represent the *apo*-state than the asymmetrical structure of CorA.

To understand the mechanism of transport used by ZntB, the structures of the different states are essential. A comparison of our full-length structure with the structure of the soluble domain of StZntB provides a first indication of the movements that may occur within the symmetrical scaffold to provide a pathway for the transported zinc (Fig. 5). Whereas our structure was obtained in the absence of Zn^{2+} , StZntB was crystallized in the presence of Zn^{2+} . Calculation of the surface potentials revealed dramatic differences between full-length EcZntB and soluble StZntB (Fig. 5a, b). The cytoplasmic domain of full length EcZntB has a strong positive electrostatic surface potential (resembling VpZntB (Fig. 5c)) whereas the potential in the isolated domain of StZntB is negative¹⁵. Threading of the EcZntB sequence in the StZntB produced a similar result of more negative surface potential (Fig. 5d) and the reverse threading (StZntB sequence in EcZntB model) produced a positive surface potential (Fig. 5e). Furthermore, the shape of the internal pore between two forms is different (Fig. 5) possibly resembling two conformational states. The charge inversion of the pore surface (Fig. 5f) between the two symmetrical states might be caused by helical rotation of TM1, which bears a patch of highly conserved basic and acidic residues on the adjacent faces of the helix.

Figure 5: Possible mechanism of Zn²⁺ transport by ZntB (a) Calculated electrostatic potential (± 5 kT/e) of ZntB (cross-section of the pore is shown) (b) same for Zn²⁺-bound soluble domain of StZntB and (c) Zn²⁺-free soluble domain of VpZntB (d) Phyre2-based model of putative Zn²⁺-bound EcZntB using StZntB as a template (pdb id 3NWI) (e) Phyre2-based model of full-length *apo* StZntB using EcZntB as a template (f) Putative mechanism of Zn²⁺ transport via ZntB. ZntB cross-section is shown schematically; Zn²⁺ and H⁺ are shown as a yellow and cyan spheres respectively; arrows indicate possible movements of trans membrane helix 1, which are possibly caused by Zn²⁺ and /or H⁺ binding, eventually leading to the change in electrostatic potential (from positive (blue) to negative (red)) within the pore that stimulates ion advancement through it.

It has been debated whether ZntB is primarily used for import or export. Whole cell assays have indicated that ZntB is a Zn²⁺ and Cd²⁺

exporter¹². This conclusion was based on experiments using a knock-out strain, from which it was assumed that all zinc transporters had been deleted. However, recent studies revealed that additional transporters, such as PitA⁶, HoxN, ActP³⁴ and the STM0353 gene product (homologous to CadA³⁵), might contribute significantly to zinc transport. Nevertheless, these results showed that ZntB at least affected the transport of Zn²⁺ and Cd²⁺. Interestingly, the analysis of regulation of ZntB expression in *C. metallidurans* revealed that it was down-regulated in the presence of high concentrations of Zn²⁺, Cd²⁺ and Cu²⁺⁶, which suggests that it is an importer, rather than an exporter. Additionally the expression of homologous ZntB from *Agrobacterium tumefaciens* was not induced by treatments with Zn²⁺ in a range from 100 to 750μM³⁶. Our experiments show that ZntB most likely mediates Zn²⁺/H⁺ cotransport, and thus indicate that ZntB is an importer for zinc. This indeed confirms that the same fold within CorA superfamily can be used either as a channel (CorA) or a transporter (ZntB).

In conclusion, we have resolved the full-length structure of a ZntB transporter, a member of the CorA MIT family. We have performed ligand-binding and ligand-transport assays that unambiguously show that ZntB is involved in zinc transport. By combining all available data from us and other groups, we conclude that ZntB is a zinc importer that is driven by a proton gradient. Its transport mechanism appears distinct from that of CorA Mg²⁺ channels. Unlike CorA, ZntB does not collapse into a highly asymmetrical state upon depletion of divalent cations. The elucidation of different conformational states of ZntB will be essential to describe its transport mechanism in greater detail.

Methods

Protein expression and membrane vesicle preparation

Expression of ZntB was performed in a 5-l flask containing 2 l of LB medium (10 g l⁻¹ Bacto trypton, 5 g l⁻¹ Bacto yeast extract, 10 g l⁻¹ NaCl), supplemented with 50 ug ml⁻¹ kanamycin and 34 ug ml⁻¹ chloramphenicol. The *E. coli* BL-21(DE3) cells with pNIC_BSA4_ZntB (pNIC28-Bsa4 was a gift from Opher Gileadi (Addgene plasmid # 26103)³⁷) were grown at 37 °C, 200 rpm to an OD₆₀₀ of 0.8, with an induction by addition of 0.1 mM IPTG. After 3 h of expression the cells were collected by centrifugation (15 min, 7,446g, 4 °C), washed in buffer A (50 mM Tris/HCl, pH 8.0) and resuspended in the buffer B (50 mM Tris/HCl, pH 8.0, 150 mM NaCl, 10 mM imidazole, 10% glycerol). Membrane vesicles were either prepared immediately, or the resuspended cells were stored at -80 °C after flash freezing in liquid nitrogen.

Before membrane vesicle preparation, 1 mM MgSO₄ and 50–100 ug ml⁻¹ DNase were added to the cells. The cells were lysed by high-pressure disruption (Constant Cell Disruption System Ltd, UK, two passages at 25 kPsi for *E. coli* cells, 5 °C) and cell debris was removed by low-speed centrifugation (30 min, 12,074g, 4 °C). Membrane vesicles were collected by ultracentrifugation (120 min, 193,727g, 4 °C), and resuspended in buffer C (50 mM Tris/HCl, pH 8.0, 150 mM NaCl, 15% glycerol) to a final volume of 5 ml per 1 l of cell culture. Subsequently, the membrane vesicles were aliquoted, flash frozen in liquid nitrogen and stored at - 80 °C. The total protein concentration in the membrane vesicles was determined by Bradford Protein Assay (Bio-Rad).

Protein purification

Membrane vesicles were thawed rapidly and solubilized in buffer D (50 mM Tris/HCl, pH 8.0, 150 mM NaCl, 10 mM imidazole, 10% glycerol,

1% (w/v) n-dodecyl- β -D-maltopyranoside (DDM, Anatrace)) for 1 h at 4 °C, while gently rocking. Unsolubilized material was removed by centrifugation (30 min, 442,907g, 4 °C). The supernatant was incubated for 1 h at 4 °C under gently rocking with Ni²⁺-sepharose resin (column volume of 0.5 ml), which had been equilibrated with 20 CV of buffer E (50 mM Tris/HCl, pH 8.0, 150 mM NaCl, 15 mM imidazole, 0.03% DDM). Subsequently, the suspension was poured into a 10-ml disposable column (Bio-Rad) and the flow through was collected. The column material was washed with 10 ml of buffer E. ZntB was eluted in three fractions of buffer F (50 mM Tris/HCl, pH 8.0, 250 mM NaCl, 500 mM imidazole, 0.03% (w/v) DDM) of 200, 750 and 500 μ l, respectively. 2 mM of EDTA was added to the second elution fraction to remove co-eluted Ni²⁺ ions and any residual zinc. Subsequently, the second elution fraction was purified by size-exclusion chromatography using a Superdex 200 10/300 gel filtration column (GE-Healthcare), equilibrated with buffer G (50 mM Tris/HCl, pH 8.0, 250 mM NaCl, 0.03% (w/v) DDM). After size-exclusion chromatography, the fractions containing ZntB were combined and used directly for proteoliposome reconstitution, or concentrated by the use of a Vivaspin 500 concentrating device with a molecular weight cut-off of 100 kDa (Sartorius stedim) to a final concentration of 3–6 mg ml⁻¹ with or without additional 1mM EDTA added when prepared for Cryo-EM.

Reconstitution into proteoliposomes

Purified ZntB was reconstituted in proteoliposomes as follows (please see ref³⁸ for details): *E. coli* polar lipids and egg phosphatidylcholine (3:1 (w/w) ratio) were dissolved in chloroform, dried in a rotary evaporator, resuspended to 20 mg ml⁻¹ in buffer containing 50 mM KPi, pH 7.5 and after three freeze-thaw cycles large unilamellar vesicles (LUVs) were stored in

liquid nitrogen until use. For proteoliposomes preparation LUVs were extruded through a 400-nm-diameter polycarbonate filter (Avestin, 11 passages). Liposomes were diluted to 4 mg ml⁻¹ in buffer H (50 mM HEPES, pH 7.5) or buffer I (50 mM HEPES, pH 6.5) and destabilized beyond R_{sat} with Triton X-100. Purified ZntB was added to the liposomes at a weight ratio of 1:250 (protein/lipid), and detergent was subsequently removed by the addition of Bio-beads (50 mg ml⁻¹, four times after 0.5 hours, 1 hour, 2 hours and overnight incubation). Proteoliposomes were harvested by centrifugation (25 min, 285,775g, 4 °C) and resuspended in buffer H or buffer I to a lipid concentration of 10 mg ml⁻¹. After three freeze-thaw cycles, proteoliposomes were stored in liquid nitrogen until use.

Radiolabelled ⁶⁵Zn²⁺ transport assay

To use in the transport assay proteoliposomes with desired pH were thawed and extruded through a 400-nm pore size polycarbonate filter (Avestin, 9 passages). Subsequently, two active units of ProTev Plus (Promega) were added to the protein sample and incubated overnight. The proteoliposomes were diluted 10 times to a final volume of 2 ml in the same buffer. After centrifugation (25 min, 285,775g, 4 °C), the proteoliposomes were resuspended in buffer H or I to a final concentration of 0.5 ug ul⁻¹ ZntB. For each time point in the transport assays, a reaction volume of 200 ul of buffer with desired pH supplemented with 22 uM of ⁶⁵ZnCl₂ was incubated at 30°C while being stirred. Transport was started by adding 1 ug of ZntB, reconstituted in proteoliposomes. At the indicated time point, 2 ml of stop buffer (ice-cold outside buffer) was added and the reaction was rapidly filtered over a BA-85 nitrocellulose filter. After washing the filter with another 2 ml of stop buffer, the levels of radioactivity were determined using a Packard Cobra II 5010 Gamma counter.

Fluorescent transport assays

Zinc transport was measured with the Zn^{2+} -sensitive fluorophore FluoZin-1 (ThermoFisher, USA). To avoid bleaching of the fluorophore, the sample was shielded from the direct light as much as possible. FluoZin-1 (stock concentration 3 mM in H_2O) was added to a final concentration of 5 μM to the proteoliposomes with desired pH. FluoZin-1 encapsulation was performed by three freeze–thaw cycles and subsequent extrusion through 0.4- μm polycarbonate filters. Extravesicular dye was removed from approximately 500 μl of liposome suspension by size exclusion chromatography on a 2 ml Sephadex G-75 column equilibrated with buffer H or I. Proteoliposomes were collected by ultracentrifugation (25 min, 285,775g, 4 °C), and the supernatant was removed. Proteoliposomes were resuspended with 10 μl buffer H or I per 2.5 mg of proteoliposomes (protein to lipid ratio 1:250). Transport assays with or without proton gradient were initiated by the addition of 10 mM stock solution of zinc acetate to the desired final concentration. For each measurement, 0.3 mg of proteoliposomes (protein to lipid ratio 1:250) was diluted in 1 ml of desired buffer. A fluorescence time course was measured in a 1 ml cuvette with a stirrer using an excitation wavelength of 490 nm and an emission wavelength of 525 nm. Experiments with empty liposomes were performed in parallel as controls. Initial transport rates ($\Delta F \text{ s}^{-1}$) were calculated by performing a linear regression on the transport data between 1 and 10 s after addition of zinc acetate. The resulting data was fitted to a Michaelis-Menten equation. All measurements were at least triplicated.

For H^+ transport assays, the luminal buffer of the proteoliposomes was exchanged for buffer J (5 mM HEPES pH 6.7) by resuspension of the liposomes in this buffer followed by three freeze-thaw cycles and extrusion

through 0.4- μ m polycarbonate filters. Proteoliposomes were collected by ultracentrifugation (25 min, 285,775g, 4 °C), and the supernatant was removed. Proteoliposomes were resuspended with 10 μ l buffer J per 2.5 mg of proteoliposomes (protein to lipid ratio 1:250). For each measurement, 0.3 mg of proteoliposomes was diluted in 1 ml of buffer K (5mM HEPES, pH 6.7, 150 nM ACMA). A fluorescence time course was measured in a 1ml cuvette with a stirrer using an excitation wavelength of 419 nm and an emission wavelength of 483 nm; zinc was added after 3 minutes of equilibration time. Experiments with empty liposomes were performed in parallel as controls. All measurements were triplicated.

Isothermal Titration Calorimetry

ITC experiments were performed using an ITC200 calorimeter (MicroCal). Different substrates in buffer G were titrated into the thermally equilibrated ITC cell filled with 280 μ l of ZntB in the same buffer (10–15 μ M). Temperature was kept constant at 25 °C. Data were analyzed using the Origin-based software provided by MicroCal.

Single particle cryo-electron microscopy

The purified ZntB sample was adjusted to a final concentration of approximately 10 mg ml⁻¹. Aliquots of 3 μ l were applied to a freshly glow-discharged holey carbon grids (Quantifoil Au R1.2/1.3, 300 mesh), excess liquid was blotted for 4-5 s using a FEI Vitrobot Mark IV and the sample was plunge frozen in liquid ethane at a temperature of approximately 100 K. TEM grids were transferred into a Titan Krios 300 keV microscope (FEI, Netherlands), equipped with a K2 direct-electron detector. Zero-loss images were recorded semi-automatically, using the UCSF Image4 script³⁹. The GIF-Quantum energy filter was adjusted to a slit width of 20 eV. Images were collected at a nominal magnification of 81,000 x (yielding a pixel size of 1.43

Å) and a defocus range of -1.5 to -3.0 μm . A total of 2655 movie images were collected with 24 frames dose-fractionated over 18 s, in super-resolution counting-mode.

Beam-induced motion in the raw movie frames was corrected for using whole-frame motion correction with MOTIONCORR 1.0⁴⁰, followed by CTF estimation using gctf⁴¹. All subsequent data processing steps were performed using the RELION 2.0 software suite⁴². References for template-based particle picking were obtained from 2D classes obtained from manually picked particles from a subset of micrographs. The initial run of template-based algorithm picked 1 million particles from all 2655 images. To reduce the number of false positive particle picks from the initial template-based particle picking, several rounds of 2D classification were applied to the full extracted dataset, resulting in a subset of 333,490 particle projections. The resulting particles were submitted to 3D auto-refinement, particle-based motion correction and damage-based weighting of individual frames⁴³. An additional round of 2D classification was performed on these ‘polished’ particles to further discard false positive or low quality particles. The obtained map was used for manual model building in Coot⁴⁴ using the previously published CorA structure (pdb id: 4I0U) as a reference model. Refinement was performed in Phenix⁴⁵ with the final validation check in Molprobity⁴⁶. The ‘Zn²⁺-bound’ model of EcZntB and ‘Zn²⁺-free’ model of StZntB were obtained by threading EcZntB sequence into StZntB model with the Phyre2 server⁴⁷. Electrostatic potentials were calculated with APBS⁴⁸ after the initial preparation of files at PDB2PQR server⁴⁹. Images were prepared with the open source version of PyMol (<https://sourceforge.net/projects/pymol/>).

Data availability

Atomic coordinates and the corresponding electron microscopy density map are deposited in the Protein Data Bank and the Electron Microscopy Data Bank under accession number 5N9Y and EMD-3605 respectively. Other data are available from the corresponding authors upon reasonable request.

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Author contributions A.G. conceived the project. Expression, purification, ITC, radioactive zinc uptakes and transport assays were performed by A.S. Cryo-EM was carried out by C.G. and S.H.W.S. Model building and refinement was done by A.G. A.S., C.G., D.J.S., S.H.W.S and A.G. analysed the data. A.G. and D.J.S. wrote the manuscript with input from all other authors.

Competing financial interests The authors declare no competing financial interests.

Supplementary

Supplementary Figure 1. Schematic overview of membrane transporters involved in Zn²⁺ homeostasis in Gram-negative bacteria. Zn²⁺ shown as yellow spheres, arrows pointing down and up show import and export respectively. OM and IM indicate outer and inner membranes respectively.

Supplementary Figure 2 Cryo-EM reconstruction. (a) Representative reference-free 2D class averages (b) Resolution estimation of the EM structure (c) Colour-coded resolution variations

Supplementary Figure 3 ZntB purification. (a) Size-exclusion chromatography profile (b) SDS-PAGE gel, where M is a marker, FT – flow through, W – wash, E1-E3 – elution fractions and A6-A9 collected fractions.

a **b** **c** **d**

Supplementary Figure 4 Structural comparison with (RMSD values in Å) (a) soluble domain of StZntB (~12) (b) soluble domain of VpZntB (~2.5) (c) MjCorA (~5.8)(d) TmCorA (~12)

a

b

Supplementary Figure 5 Phylogenetic tree and sequence analysis of ZntB proteins. (a) ZntB proteins form a distinct clade of proteins, which are highly conserved as seen in (b) Sequence alignment of ZntB proteins, note the high sequence identity among EcZntB, StZntB and VpZntB

Supplementary Figure 6 Calculated pore profiles of (a) EcZntB, (b) TmCorA, (c) MjCorA. Proteins are shown as grey semi-transparent surface, pores in red. Note that the profile in ZntB is generally wider compared to CorAs.

Supplementary Figure 7 Fluorescent transport of Zn^{2+} into proteoliposomes. Sodium gradient (black line, $\text{K}^+_{\text{inside}} / \text{Na}^+_{\text{outside}}$) does not stimulate uptake of Zn^{2+} when compared to the standard condition (red line, $\text{K}^+_{\text{inside}} / \text{K}^+_{\text{outside}}$). Zn^{2+} and FCCP were added at 1 min and 6 min time points

respectively.

Supplementary Figure 8 ZntB maintains its pentameric state upon additional EDTA treatment. Cryo-EM reconstruction of ZntB with an extra 1mM EDTA added prior sample freezing. ZntB in yellow, Cryo-EM map in blue.

Chapter 3

Structural and biochemical analyses of ZntB transport mechanism

A. Stetsenko, A. Guskov

Abstract

ZntB is the member of 2-TM-GxN protein family, which is mostly responsible for the transport of magnesium in all life domains. In contrast to other members, ZntB is primarily zinc transporter with proposed zinc / proton co-transport mechanism. Previously we have resolved the *apo* structure of ZntB from *E. coli*, but the transport mechanism remains rather elusive. Here, we attempted to gain mechanistical insight by solving a ZntB structure in the presence of the transported substrates Zn^{2+} and Cd^{2+} , however we obtained only low resolution cryo-EM structures of ZntB in the presence of these ions at 7.5 and 8.4 Å resolution respectively. In addition, we performed fluorescent transport assays and isothermal titration calorimetry on variants of ZntB, with mutations in amino acids which are potentially involved in proton and zinc recognition. Our obtained structures are of limited value as the resolution is too low to observe detailed conformational changes and the structures might be affected by the non-physiologically high concentrations of divalent cations. Our functional assays indicate that D253 is crucial for H^+ coupling mechanism, and V289 in the GVN signature motif is not responsible for zinc recognition.

Introduction

Zinc is the second abundant vital trace metal after iron that is essential for all the species of all phyla [1]–[3]. Stable association of zinc ions with binding sites in proteins and their coordination flexibility as Lewis acid render zinc ions as an excellent cofactors for enzymes that require activation of a poor nucleophile [4]. Zinc is commonly found in the structurally important and active sites in about 5-6% of all bacterial proteins [5], [6]. For example, the activity of virulence-related proteins such as class B metallo- β -lactamases and extracellular metalloproteinases relies on zinc ions [7], [8]. Nevertheless, despite zinc importance to biological homeostasis, even micromolar concentrations of free zinc are toxic as zinc can interact with thiols and occupy essential binding sites for other ions in the proteins [9]. Thus, the zinc concentration in the cell must be tightly regulated to prevent such uncontrolled binding.

Zinc homeostasis in bacteria is tightly controlled by the coordinated cooperation of its uptake, redistribution, storage and efflux. Gram-negative bacteria rely on the TonB-dependent receptor ZnuD to transport zinc through outer membrane and the ATP-binding cassette (ABC) transporter ZnuABC to deliver zinc in the cytoplasm [10], [11]. In addition to the high affinity transporter ZnuABC, bacteria can transport zinc via low affinity ZIP (ZIR/IRT-like proteins) in zinc excess conditions [12]. Cytoplasmic zinc is mostly stored by cysteine-rich metallothionines [13]. Finally bacteria have several types of exporters: a P-type ATPase ZntA, cation diffusion facilitator (CDF) family transporters ZitB and YiiP, the periplasm-spanning “efflux guns” CzcD and CzcBCA to efflux the zinc’s excess [14]–[17]. In addition to these proteins, γ -proteobacteria possess an additional zinc transporter ZntB with an elusive role [18].

ZntB belongs to the 2-TM-GxN protein family [19]. Recently we have reported a ZntB structure in the absence of Zn^{2+} (after treatment with EDTA) that appears to be in the closed state, which in combination with biochemical characterization of transport suggests a $\text{Zn}^{2+}/\text{H}^{+}$ symport mechanism [20]. For another member of the 2-TM-GxN family, the Mg^{2+} channel CorA, structures of Mg^{2+} -bound closed and putative open Mg^{2+} -free states at resolutions of 2.7 Å and 7.06 Å respectively are available [21], [22]. The low resolution (7.06 Å) cryo-EM structure of *Thermotoga maritima* CorA in the absence of Mg^{2+} (after treatment with EDTA) revealed a transition from the five-fold symmetric conformation observed in the Mg^{2+} bound state, to an asymmetric conformation, where subunits experienced variable hinge-like motions at the stalk helix [22]. However, since the complete depletion of Mg^{2+} in the cell is unlikely, this structure might be an artefact of EDTA treatment. If such transition is nevertheless correct, the loss of 5-fold symmetry is not a conserved mechanistical feature of the entire superfamily, as Zn^{2+} depleted ZntB maintains five-fold symmetry. Another proposed mechanism of transport via CorA suggests an iris-like movement of TM1 α -helices, resulting to spontaneous hydration of the hydrophobic neck [23]. Although the exact details of movements and turns are not entirely clear, this mechanism has got certain experimental support from mutagenesis, EPR and electrophysiology measurements [21], [24], [25].

Differences between CorA and ZntB might be caused by the different structural constraints imposed by channels (CorA) and presumed transporters (ZntB) [19], [26]. Hence, it is essential to resolve a ZntB structure with a bound substrate in an attempt to decipher its transport mechanism and compare it with those aforementioned proposed for CorA proteins. In this report we present a low resolution cryo-EM structure of ZntB presumably

with bound Cd^{2+} ions together with an additional biochemical characterization of the ZntB mutants through ITC measurements and uptakes assays.

Results & Discussion

Structure of EcZntB in the presence of Cd^{2+} and Zn^{2+} ions

Single-particle cryo-EM analysis of *E. Coli* ZntB in the presence of cadmium ions revealed its structure at an overall resolution of 7.5 Å. The structure of EcZntB in the presence of 8 mM CdCl_2 showed significant conformational differences compared to *apo* structure of EcZntB (Fig. 1). The five-fold symmetry observed in the closed state is disrupted as a result of movements of the cytoplasmic domains (Fig. 1) similar to TmCorA in the absence of Mg^{2+} ions [22]. However, this result is most probably an artefact of excessive binding of Cd^{2+} ions at monomer-monomer interfaces in ZntB. This proposal is reinforced by the structure of ZntB in presence of Zn^{2+} ions. In this case the symmetry is mostly maintained, however there is no density for one out of five stalk helices, despite the cytoplasmic part is clearly pentameric (Fig. 1). Hence at this stage unfortunately new structural information on ZntB is of limited value.

Figure 1. Cryo-EM analysis of ZntB in the presence of Cd^{2+} (A-C) and Zn^{2+} (D-F). **A** and **D**, representative micrographs, some of ZntB single particles are encircled in red. **B** and **E**, the representative 2D classes. **C** and **F**, the obtained cryo-EM maps. Individual protomers of ZntB are labelled A to E.

To further characterize the transport mechanism even in the absence of high or medium resolution structures of ZntB with bound substrate, we created mutants that we functionally characterized. Several amino acids based on the *apo* structure of ZntB and the analysis of its sequence were mutated to

investigate their roles for zinc transport in EcZntB. Transport was measured with pH gradients (the wild type was also measured without pH gradient) in an attempt to decouple H^+ and Zn^{2+} transport (Fig. 2) as we have observed previously [20]. In the previous experiments we built the proton gradient with pH 6.5 and 7.5 outside / inside liposomes and the other way around. In the condition of the inward proton flux with pH 6.5 outside and pH 7.5 inside proteoliposomes, transport of Zn^{2+} was twice higher compared to conditions with the equal pH inside and outside (pH 6.5 / 6.5 or 7.5 / 7.5). When the proton gradient was reversed, Zn^{2+} uptake was only around 25% from the level of transport at the inward proton flux [20]. The current analysis revealed that one of the key amino acids for possible proton binding is D253. The D253N mutant was constructed and the transport was assayed. The uptake of D253N mutant in the presence of pH gradient drops to the level of the wild type EcZntB uptake in the absence of pH gradient. The addition of the FCCP does not change the uptake level revealing that this mutant is insensitive for pH gradient. The kinetics of Zn^{2+} transport seems to be changed too. The saturation rate was increased compared to the wild type protein with and without proton gradient. We have also analysed the involvement of conserved (in ZntB) R191 and R198 as they might be the gating residues preventing the movement of positively charged Zn^{2+} down the pore due to the charge field created by positively charged guanidinium groups of Arg.

Figure 2. Fluorescent-based transport assays on ZntB mutants reconstituted into proteoliposomes with the FluoZin-1 dye in presence of pH gradient (pH 7.5_{in}/pH 6.5_{out}) (Zn²⁺ added at 1 min time point; FCCP added at 6 min time point). Colour-code: black – ZntB_{WT}, red – ZntB_{V289M}, blue – ZntB_{R191E}, cyan – ZntB_{D253N}, green – ZntB_{R198E}, orange – ZntB_{WT} without pH gradient (pH 7.5_{in/out}), purple – ZntB_{R191E_R198E}, dark cyan – ZntB_{M270H}, magenta – empty liposomes (EL)). Error bars represent s.e.m. from more than three technical measurements.

To investigate the role of Arg191 and Arg198 and whether they face the pore during the transport events we constructed two mutants R191E and R198E. If these residues indeed face the pore, then the mutants should demonstrate the enhanced transport as glutamate side chains will be negatively charged at given pH values, hence creating a favourable negative

charge along the translocation pathway. The single mutation R191E does not have significant impact on the transport, except it reduces the sensitivity to disruption of proton gradient, while the R198E mutation significantly reduces zinc uptake level and leads to the almost complete loss of sensitivity to proton gradient. The double mutation R191E_R198E almost completely abolishes zinc uptake. This might seem very similar to the so-called “dead” mutant we constructed by the M270H mutation in the narrow methionine ring, which will bind substrate and block the pore, however for R198E it is rather unlikely for several reasons. First of all, the size of the pore at the position of R198 is 10 Å wider than at the position of M270 (~17 Å vs 7Å), hence even if all glutamate side chains in R198E mutant bind Zn^{2+} ions simultaneously (and glutamate side chains are shorter than arginine side chains), there is still some room left. One might argue that five bound zinc ions can create a strong energetic barrier for the other incoming ions, but the charges on these ions are likely shielded by water molecules and the incoming ions will knock off the already bound ones as suggested for example for Mg^{2+} transport via CorA [27]. Furthermore, the affinity of Zn^{2+} to glutamate side chains is considerably less than for histidine side chains. Thus, it looks very unlikely that R198E and R191E_R198E double mutants are the dead mutants. In the absence of any other experimental evidence we may only speculate about the possible scenarios.

One of such scenarios is that the protonation at D253 (in long stalk helix) favors the conformational change in the $\alpha 5$ helix adjacent to it. Since there is a patch of conserved basic and acidic residues on adjacent helices (R191, R198, D239, D242) it might be that the charge inversion takes place and by creating R191E_R198E mutant the protein is locked in a certain

conformation when this rotation is not possible anymore. However, to test this hypothesis a more extensive mutagenesis study is required.

Additionally, we investigated the influence of point mutations on cation recognition by conducting isothermal titration calorimetry experiments on EcZntB mutants (Fig. 3). We have shown previously that ZntB can bind Zn^{2+} , Cd^{2+} , Ni^{2+} and Co^{2+} with K_d values of 11.5, 22.6, 87.7 and 175.4 μM , respectively, and no detectable binding of Mn^{2+} , Mg^{2+} and Cu^{2+} [20]. We presumed that mutation of the signature motif of ZntB from GVN to GMN, which is found in CorA, might improve Mg^{2+} binding or change affinity of Zn^{2+} binding. We have tested V289M mutant with both Zn^{2+} and Mg^{2+} ions, but no significant difference was detected compared to the wild type protein. The K_d value for Zn^{2+} binding was 5 μM and no detectable binding was observed for Mg^{2+} . We have also tested the mutants D253N, and R191E_R198E, and in both cases binding of Zn^{2+} was similar compared to the wild type protein, with K_d values of 8.5 μM and 2.2 μM , respectively.

Figure 3. ITC profiles of substrate binding of (A) Zn^{2+} to $\text{ZntB}_{\text{V289M}}$, (B) Mg^{2+} to $\text{ZntB}_{\text{V289M}}$, (C) Zn^{2+} to $\text{ZntB}_{\text{D253N}}$, (D) Zn^{2+} to $\text{ZntB}_{\text{R191E_R198E}}$

In conclusion we have identified that residue D253 is the most probable coupler of H^+ transport, as a mutation introduced at this position

leads to the decoupling of H^+/Zn^{2+} symport. The double mutation of conserved arginines 191 and 198 is indicative that these residues might be involved in the network of interactions ultimately causing the conformational changes leading to the transport via ZntB but to make any solid conclusion, further mutagenesis studies are required. Furthermore, a higher resolution structure of ZntB with the bound substrate is essential to understand the transport mechanism and the further attempts to obtain it are ongoing.

Materials and methods

Cloning and mutagenesis. ZntB from *E. coli* was cloned into pET StrepII TEV LIC cloning vector (gift from Scott Gradia (Addgene plasmid #29664), encoding TEV-cleavable Strep II fusion tag. The full-length ZntB gene were amplified from our pNIC28_ZntB construct [20]. The expression vector was constructed, using ligation independent cloning with primers for EcZntB (forward 5'-TACTTCCAATCCAATGCAATGGTCGTCATCTACCTGC-3' and reverse 5'-TTATCCACTTCCAATGTTATTATTAATTAAAGAGCTCTCCG-3'). Single mutations were introduced in recombinant EcZntB using site-directed mutagenesis [28]. DNA sequencing confirmed the presence of only the desired mutations.

Primer	Sequence 5'-3'
Fw_V289M	TGACAGGGTTATTTGGCATGAACCTTGGTGGGATCCC
Rev_V289M	GGGATCCCACCAAGGTTTCATGCCAAATAACCCTGTCA
Fw_D253N	CTGGCGTGATGGCGAATGAAATCGCTCAGG

Rev_D253N	CCTGAGCGATTTTCATTCGCCATCACGCCAG
Fw_R191E	GTGGATTCCTGGCTCTGCTGGAGAAACAATTAATCGTGATGCG
Rev_R191E	CGCATCACGATTAATTGTTTCTCCAGCAGAGCCAGGAATCCAC
Fw_R198E	CGCAAACAATTAATCGTGATGGAGCGCTATATGGCACCGCAACG T
Rev_R198E	CGTTGCGGTGCCATATAGCGCTCCATCACGATTAATTGTTTGCG
Fw_R191E_R198E	GTGGATTCCTGGCTCTGCTGGAGAAACAATTAATTGTGATGG
Rev_R191E_R198E	CCATCACAATTAATTGTTTCTCCAGCAGAGCCAGGAATCCAC
Fw_M270H	CTCGTCGTACCTATACACATTTCGTTGATGGCAATGG
Rev_M270H	CCATTGCCATCAACGAATGTGTATAGGTACGACGAG

Protein expression and membrane vesicle preparation. EcZntB and its mutant proteins were expressed in the same way. Expression of target protein was performed in a 5-l flask containing 2 l of LB medium (10 g l⁻¹ Bacto trypton, 5 g l⁻¹ Bacto yeast extract, 10 g l⁻¹ NaCl), supplemented with 50 ug ml⁻¹ kanamycin and 34 ug ml⁻¹ chloramphenicol. The *E. coli* BL-21(DE3) cells with the needed plasmid were grown at 37 °C, 200 rpm to an OD₆₀₀ of 0.8, with an induction by addition of 0.1 mM IPTG. After 3 h of expression the cells were collected by centrifugation (15 min, 7,446g, 4 °C), washed in buffer A (50 mM Tris/HCl, pH 8.0) and resuspended in the buffer B (50 mM Tris/HCl, pH 8.0, 250 mM NaCl, 10% glycerol). Membrane vesicles were either prepared immediately, or the resuspended cells were stored at -80 °C after flash freezing in liquid nitrogen.

Before membrane vesicle preparation, 1 mM MgSO₄ and 50–100 ug ml⁻¹ DNase were added to the cells. The cells were lysed by high-pressure disruption (Constant Cell Disruption System Ltd, UK, two passages at 25 kPsi for *E. coli* cells, 5 °C) and cell debris was removed by low-speed centrifugation (30 min, 12,074g, 4 °C). Membrane vesicles were collected by ultracentrifugation (120 min, 193,727g, 4 °C), and resuspended in buffer C (50 mM Tris/HCl, pH 8.0, 150 mM NaCl, 15% glycerol) to a final volume of 5 ml per 1 l of cell culture. Subsequently, the membrane vesicles were aliquoted, flash frozen in liquid nitrogen and stored at - 80 °C.

Protein purification. Membrane vesicles of EcZntB with his-tag or strep-tag were thawed rapidly and solubilized in buffer D (50 mM Tris/HCl, pH 8.0, 150 mM NaCl, 10 mM imidazole, 10% glycerol, 1% (w/v) n-dodecyl- β -D-maltopyranoside (DDM, Anatrace)) for 1 h at 4 °C, while gently rocking. Unsolubilized material was removed by centrifugation (30 min, 442,907g, 4 °C). The supernatant of EcZntB with his-tag was incubated for 1 h at 4 °C under gently rocking with Ni²⁺-sepharose resin (column volume of 0.5 ml), which had been equilibrated with 10 CV of buffer E (50 mM Tris/HCl, pH 8.0, 250 mM NaCl, 50 mM imidazole, 0.03% DDM). Subsequently, the suspension was poured into a 10-ml disposable column (Bio-Rad) and the flow through was collected. The column material was washed with 10 ml of buffer E. The target protein was eluted in three fractions of buffer F (50 mM Tris/HCl, pH 8.0, 250 mM NaCl, 500 mM imidazole, 0.03% (w/v) DDM) of 200, 750 and 500 μ l, respectively. 2 mM of EDTA was added to the second elution fraction to remove co-eluted Ni²⁺ ions and any residual divalent cations.

The supernatant of EcZntB with strep-tag was incubated for 2 h at 4 °C under gently rocking with Strep-Tactin Superflow resin (Iba-Lifesciences) with column volume of 0.5 ml, which had been equilibrated with 5 CV of buffer W (50 mM Tris/HCl, pH 8.0, 250 mM NaCl, 0.03% DDM, 1 mM EDTA). Subsequently, the suspension was poured into a 10-ml disposable column (Bio-Rad) and the flow through was collected. The column material was washed with 10 ml of buffer W. The target protein was eluted in three fractions of buffer ES (50 mM Tris/HCl, pH 8.0, 250 mM NaCl, 0.03% (w/v) DDM, 1 mM EDTA, 2.5 mM desthiobiotin) of 200, 750 and 500 μ l, respectively.

Subsequently, the second elution fraction of EcZntB with his-tag or strep-tag was purified by size-exclusion chromatography using a Superdex 200 10/300 gel filtration column (GE-Healthcare), equilibrated with buffer G (50 mM Tris/HCl, pH 8.0, 250 mM NaCl, 0.03% (w/v) DDM). After size-exclusion chromatography, the fractions containing the target protein were combined and used directly for the following experiments.

Reconstitution into proteoliposomes. Reconstitution in proteoliposomes was performed as follows (see [29] for details): polar lipids of *E. coli* and egg phosphatidylcholine (in 3:1 (w/w) ratio) were dissolved in chloroform, then dried in a rotary evaporator and subsequently resuspended in buffer containing 50 mM KPi, pH 7.5 to the concentration of 20 mg ml⁻¹. After three freeze-thaw cycles, large unilamellar vesicles (LUVs) were obtained and stored in liquid nitrogen. To prepare proteoliposomes, LUVs were extruded through a 400-nm-diameter polycarbonate filter (Avestin, 11 passages). Obtained liposomes were diluted to 4 mg ml⁻¹ in buffer H (50 mM HEPES, pH 7.5) or buffer I (50 mM HEPES, pH 6.5) and subsequently

destabilized beyond R_{sat} with Triton X-100. The target purified protein was added to the liposomes at a weight ratio of 1:250 (protein/lipid), followed by detergent removal using Bio-beads (50 mg ml⁻¹, four times after 0.5 h, 1 h, 2 h and overnight incubation). Afterwards, proteoliposomes were collected by centrifugation (25 min, 285,775×g, 4 °C) and resuspended in buffer H or buffer I to a lipid concentration of 10 mg ml⁻¹. Finally, after three freeze-thaw cycles, obtained proteoliposomes were stored in liquid nitrogen until subsequent experiments.

Fluorescent transport assays. Zinc transport was measured with the Zn²⁺-sensitive fluorophore FluoZin-1 (ThermoFisher, USA). To avoid bleaching of the fluorophore, the sample was shielded from the direct light as much as possible. FluoZin-1 (stock concentration 3 mM in H₂O) was added to a final concentration of 5 μM to the proteoliposomes. FluoZin-1 encapsulation was performed by three freeze-thaw cycles and subsequent extrusion through 0.4 μm polycarbonate filters. Extravesicular dye was removed from approximately 500 μl of liposome suspension by size exclusion chromatography on a 2 ml Sephadex G-75 column equilibrated with buffer H or I. Proteoliposomes were collected by ultracentrifugation (25 min, 285,775g, 4 °C), and the supernatant was removed. Proteoliposomes were resuspended with 10 μl buffer H or I per 2.5 mg of proteoliposomes (protein to lipid ratio 1:250). Transport assays were initiated by the addition of 10 mM stock solution of zinc acetate to the desired final concentration. For each measurement, 0.3 mg of proteoliposomes was diluted in 1 ml of desired buffer. A fluorescence time course was measured in a 1 ml cuvette with a stirrer (350 rpm) using an excitation wavelength of 490 nm and an emission wavelength of 525 nm. Experiments with empty liposomes were performed in parallel as controls.

Isothermal titration calorimetry. Nano ITC Low Volume instrument (TA Instruments) was used to perform all ITC experiments. The thermally equilibrated ITC cell was filled with 250 μ l of ZntB or its mutants in buffer G (10–15 μ M) and studied substrates (in the same buffer) were titrated into the cell. Temperature was fixed at 25 °C. Analysis of data was performed using the origin-based software provided by TA Instruments.

Cryo-EM data collection. Purified EcZntB at concentration 15 mg/ml (380 μ M) was mixed with either 5 mM CdCl₂ or 5 mM ZnCl₂ or 5mM CoCl₂ to final ration 1:10 (protein:ion) and applied to a glow-discharged Quantifoil R1.2/1.3 300-mesh copper holey carbon grid (Quantifoil, Micro Tools), blotted under 100% humidity at 4 °C for 3-5 s and plunged into liquid ethane using a Mark IV Vitrobot (FEI). Micrographs were acquired on a Titan Krios microscope (FEI) with a K2 Summit direct electron detector (Gatan) in the super-resolution counting mode, operated at 300 kV using the EPU software (FEI). The slit width of the GIF-Quantum energy filter was adjusted to 20 eV. Images were collected with the pixel size of 0.88 Å and a defocus range of –1.5 to –3.0 μ m.

Image processing and 3D reconstruction.

Motion correction, CTF estimation, template-based picking, 2D classification, Ab initio volume generation and non-uniform 3D refinement (without symmetry applied if not stated otherwise) were performed using cryoSPARC [30]. Maps were sharpened using Autosharpen Map procedure in Phenix [31]. The sharpened maps were used for the manual model placement and the models were refined using realspace refine module of

Phenix. Visualization and structure interpretation were carried out in Chimera.

Cd^{2+} bound state. After motion correction and CTF estimation 2862 micrographs were selected for the following processing. The template for particle picking was generated from 100 manually picked particles. Template-based picking identified 676582 particles. Subsequent 2D classification reduced the number of particles to 140571 and later 113374 particles left in the selected ab initio class. Final non-uniform 3D refinement resulted in a 7.5 Å map.

Zn^{2+} bound state. After motion correction and CTF estimation 1315 micrographs were selected for the following processing. The template for particle picking was generated from 100 manually picked particles. Template-based picking identified 154475 particles. Subsequent 2D classification reduced the number of particles to 25818 and later 19814 particles left in the selected ab initio class. Final non-uniform 3D refinement resulted in a 9.8 Å map.

Co^{2+} bound state. After motion correction and CTF estimation 1215 micrographs were selected for the following processing. However, after visual inspection of the images the severe aggregation of particles was detected hence this dataset was not used for any further processing.

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Chapter 4

Cation permeability in CorA family of proteins

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Abstract

CorA proteins belong to 2-TM-GxN family of membrane proteins, and play a major role in Mg^{2+} transport in prokaryotes and eukaryotic mitochondria. The selection of substrate is believed to occur via the signature motif GxN, however there is no consensus how strict this selection within the family. To answer this question, we employed fluorescence-based transport assays on three different family members, namely CorA from bacterium *Thermotoga maritima*, CorA from the archeon *Methanocaldococcus jannaschii* and ZntB from bacterium *Escherichia coli*, reconstituted into proteoliposomes. Our results show that all three proteins readily transport Mg^{2+} , Co^{2+} , Ni^{2+} and Zn^{2+} , but not Al^{3+} . Despite the similarity in cation specificity, ZntB differs from the CorA proteins, as in the former transport is stimulated by a proton gradient, but in the latter by the membrane potential, confirming the hypothesis that CorA and ZntB proteins diverged to different transport mechanisms within the same protein scaffold.

Introduction

Magnesium is one of the essential metal ions, which is invariantly required for every cell, as it is involved in numerous metabolic reactions and also plays additional roles, for example as a stabilizer of highly charged adenosine triphosphate and lipidic bilayer, where it compensates the negative charge of phosphate groups¹. Since magnesium is normally present in biological systems in its ionic form as Mg^{2+} , it cannot readily cross the biological membrane, thus it is channeled via membrane-embedded proteins². In prokaryotes, this is often done via MgtE and CorA families of proteins^{2,3}. The latter are homo- or hetero- pentamers^{2,4-7}, possess large cytoplasmic domains, which are believed to play a regulatory function^{8,9} and the transmembrane part, which consists of two α -helices per protomer, arranged as an inner and an outer pentamer, while the loops connecting them bear the signature motif GxN, which is at the same time is the selectivity filter^{6,10,11} (Figure 1).

Figure 1. The general organization of CorA family of proteins as exemplified by TmCorA (pdb code 4I0U). **(a)** side view, each protomer is color-coded; the position of membrane is indicated with black lines; **(b)** view from the extracellular part, transmembrane helices are numbered with the numerals, those with ' indicate the outer transmembrane helix of each protomer. The asparagine side chains of GxN motif are shown as sticks; **(c)** the sequence alignment of TmCorA, MjCorA and EcZntB. Essentially conserved amino acids are in red. The turquoise bars show the position of the long helices

(numbered in panel **b**) forming the channel and of the periphery helices (indicated with ' in panel **b**). The signature motif / selectivity filter is indicated with *.

Most of the efforts in the structural characterization on CorA family of proteins have been focused on CorAs from *Thermotoga maritima* (TmCorA) and *Methanocoldococcus jannaschii* (MjCorA)^{4,6,8,9,12-15}. The initial functional characterization of the family was done with orthologous CorA from *Salmonella typhimurium*^{16,17} which was later extended by the vast amount of data on TmCorA generated by different groups in an attempt to understand its transport mechanism. This led to a situation that only TmCorA is characterized in a great detail by various techniques (including but not limited to transport assays, electrophysiology, Molecular Dynamics simulations, mutagenesis) but for other members such characterization is rather scarce. With the goal to extend such a functional characterization on other members of CorA family we performed the extensive *in vitro* functional characterization of CorA from *M. jannaschii* and of homologous zinc transporter ZntB¹⁸ in comparison with CorA from *T. maritima*. Our results show that similarly to TmCorA, both MjCorA and EcZntB are not highly selective, and support the hypothesis that CorA and ZntB proteins might utilize different transport mechanisms.

Results

Purified CorA proteins from *T. maritima* and *M. jannaschii* and ZntB from *E.coli* (Supplementary Fig. 1) were reconstituted into proteoliposomes and their transport activity was assayed as described previously¹⁸. Both TmCorA and MjCorA readily transported Zn^{2+} , Cd^{2+} , Co^{2+} and Ni^{2+} similarly to EcZntB as seen in the experiments with the FluoZin-1 dye (Fig.2a-b); all three proteins readily transport Mg^{2+} as registered with FluoZin-3 dye

(Fig. 2c), but are not capable to transport Al^{3+} as seen in the experiments with morin dye (Fig. 2d).

Figure 2. Transport of different cations assayed by the fluorophores trapped inside the proteoliposomes. Dequenching of FluoZin-1 dye fluorescence with **a** TmCorA and **b** MjCorA (added at 1 min and color-coded: black—25 μM Zn^{2+} , red—25 μM Cd^{2+} , blue—100 μM Ni^{2+} , magenta—empty liposomes with 25 μM Zn^{2+}). **c** Dequenching of FluoZin-3 fluorescence during transport of Mg^{2+} via red—ZntB, blue—TmCorA, black—MjCorA, magenta—empty liposomes. 100 μM Mg^{2+} was added at 1 min. **d** Incapability of these proteins to transport Al^{3+} as registered by morin dye (the same coloring as in **c**, 200 μM Al^{3+} was added at 1 min). Error bars represent s.e.m. from three or more technical replicates of independent batches of proteoliposomes.

Surprisingly the transport activity of TmCorA and MjCorA was not efficiently inhibited by Hexaminecobalt(III) chloride, the known CorA inhibitor^{19,20} (Fig. 3).

Figure 3. The incapability of hexaminecobalt(III) chloride (CoHex) to inhibit the transport of Zn²⁺ by proteoliposomes with (a) TmCorA, (b) MjCorA and (c) ZntB. Black—25 μM Zn²⁺ with no added CoHex, red— 25 μM Zn²⁺ + 1 μM CoHex, blue—25 μM Zn²⁺ + 10 μM CoHex, green—25 μM Zn²⁺ + 50 μM CoHex, magenta—empty liposomes with 25 μM Zn²⁺ + 50 μM CoHex. All substrates were simultaneously added at 1 min. Higher concentrations of CoHex led to the collapse of proteoliposomes (see Supplementary Fig. 2). Error bars represent s.e.m. from three or more technical replicates of independent batches of proteoliposomes.

To test whether either of CorA has a preference for Co²⁺ over Mg²⁺ we performed competition assay experiments. We compared the transport of Co²⁺ by TmCorA and MjCorA in the absence of Mg²⁺ and in the presence of 100-1000 μM of Mg²⁺ (Fig. 4). Whereas transport of Co²⁺ via MjCorA was significantly affected already by addition of 100 μM of Mg²⁺ (Fig. 4a) transport of Co²⁺ via TmCorA was not much affected even in the presence of 1 mM of Mg²⁺ (Fig.4b). This result supports the previous hypothesis that TmCorA might be a Co²⁺-selective channel²¹.

Figure 4. transport competition assays with Co^{2+} and Mg^{2+} in the proteoliposomes, loaded with FluoZin-1, with **a** MjCorA and **b** TmCorA, (black—100 μM CoSO_4 with no added MgSO_4 , red—100 μM CoSO_4 + 100 μM MgSO_4 , blue —100 μM CoSO_4 + 200 μM MgSO_4 , green—100 μM CoSO_4 + 500 μM MgSO_4 , light blue—100 μM CoSO_4 + 1000 μM MgSO_4 , magenta—empty liposomes with 100 μM CoSO_4 + 1000 μM MgSO_4). All substrates were simultaneously added at 1 min. Error bars represent s.e.m. from three or more technical replicates of independent batches of proteoliposomes.

In stark contrast with ZntB, both TmCorA and MjCorA do not seem to transport protons as seen in the experiments with ACMA dye (Fig. 5), supporting our previous hypothesis that ZntB and CorA proteins evolved to use different transport mechanisms despite the same general architecture.

Figure 5. Quenching of the pH-dependent fluorophore ACMA at different Zn^{2+} concentrations in proteoliposomes with **a** EcZntB **b** TmCorA and **c** MjCorA (addition of Zn^{2+} after baseline stabilization, black—50 μM Zn^{2+} , red—25 μM Zn^{2+} , blue—5 μM Zn^{2+} , green—1 μM Zn^{2+} , magenta—empty liposomes with 50 μM Zn^{2+}). Error bars represent s.e.m. from three or more technical replicates of independent batches of proteoliposomes.

This is further corroborated by the fact that ZntB and CorA respond differently to the presence of membrane potential: a creation of membrane potential of -116 mV by addition of valinomycin to proteoliposomes leads to the enhanced transport via CorA but not ZntB proteins (Fig. 6).

Figure 6. Effect of membrane potential on the transport of Zn^{2+} (added after 1 min) assayed by the fluorophore FluoZin-1 trapped inside the proteoliposomes with EcZntB (black), TmCorA (red) and MjcorA (blue) and empty liposomes (magenta). 1 μM of valinomycin were added after 5 min. Error bars represent s.e.m. from three or more technical replicates of independent batches of proteoliposomes.

The rates of transport, as well as K_m values of 9.5 μM and 9.9 μM for TmCorA and MjCorA respectively (Fig. 7), are similar to the previously reported K_m value of 7.5 μM for EcZntB¹⁸.

Figure 7. Rate of transport dependence on Zn²⁺ concentration in **a** TmCorA **b** MjCorA. The solid lines represent the fit to the Michaelis–Menten equation (based on FluoZin-1 experiments). Error bars represent s.e.m. from three or more technical replicates of independent measurements.

Discussion

CorA proteins are the most characterized representatives of 2-TM-GxN family of transporters /channels, which additionally includes ZntB, Alr, Mrs2 and other proteins^{22–26}. However there is an obvious knowledge imbalance within CorA subfamily itself, where basically only TmCorA is extensively characterized both functionally^{17,27} and structurally^{4,8,9,12,14}. In an attempt to mend it we performed the extensive *in vitro* functional characterization using fluorescence-based transport assays on two other representatives of 2-TM-GxN family, for which the full-length structures are available, namely on archaeal MjCorA⁶ and bacterial EcZntB¹⁸ in parallel with TmCorA. Using these fluorescence-based transport assays on proteins reconstituted into proteoliposomes we tried to answer whether there is a high substrate specificity within subfamilies – a controversial issue originating from an array of different experiments, including whole cell uptakes, isothermal titration calorimetry and patch clamp experiments and structural models of CorA and ZntB proteins which do not always agree^{4,7,18–21,28,29,30}.

Our results show that despite there is a strict selection of divalent over trivalent cations (Fig. 2) for all studied members, the uptake of divalent ions is rather promiscuous. For example, it was assumed that ZntB proteins are strictly selective for Zn^{2+} over Mg^{2+} and *vice versa* for CorA. To monitor the intraliposomal accumulation of Mg^{2+} we used FluoZin-3 dye and not Magfura 2 dye as we found the performance of the latter not satisfactory in our experimental setup. The transport of Mg^{2+} via ZntB is comparable with one via TmCorA and MjCorA (Fig. 2c). Similarly, both TmCorA and MjCorA readily transport Zn^{2+} (Fig. 2a-b). Furthermore, for TmCorA it has been shown that in absence of any Mg^{2+} (which is rather a non-physiological) it becomes rather a non-selective divalent channel³⁰. Taking into account similarity among studied cations it is feasible to assume that the single main recognition pattern is indeed the hydration radius (close to 2.1 Å) and octahedral arrangement of water molecules in the first hydration shell of a cation as it was proposed earlier^{18,31,32}, however that fails to explain the fact why TmCorA prefers Co^{2+} as its substrate even in the presence of 1 mM Mg^{2+} (Fig. 4b). To this moment the most plausible explanation is the presence of additional recognition patterns – such as threonine residues inside the pore⁸, however more systematic study involving more members of both subgroup A (supposedly Co^{2+} -selective channels) and B (supposedly true Mg^{2+} -channels)³³ is necessary. This result also disfavors the explanation that since TmCorA reside in water habitat it should just prefer Mg^{2+} (ref¹⁵), as the exact elemental pattern in its preferential dwelling (hot springs and hydrothermal vents) might be very different from the normal water composition³⁴. If this is correct then basically the preferred substrate will be dictated by the environmental milieu – organisms exposed to high Co^{2+} and low Mg^{2+} evolved to scavenge Co^{2+} more efficiently; the observation that some

enzymes of *T. maritima* are cobalt-dependent^{35,36} supports this hypothesis. Furthermore, the results of complementation assay of TmCorA in the salmonella strain devoid of all Mg²⁺ transporters¹⁹, thermostability assays and competition studies²¹ all indicate that Mg²⁺ is not the most preferred substrate for TmCorA and electrophysiological measurements on TmCorA expressed in oocytes revealed that its affinity for Co²⁺ is ~10 times higher than for Mg²⁺ (ref³⁷).

Another evidence for possible extra selectivity features is the metallothionein of *Cupriavidus metallidurans*, which encodes three different CorA proteins (CorA₁-CorA₃, with sequence identity below 7%) as well as ZntB protein^{7,38}. Interestingly, CmCorA₁ and CmCorA₂ are involved in Ni²⁺ import, whereas all three forms can transport Zn²⁺ and CmCorA₁₂₃ heterotrimer is responsible for the import of Co²⁺ (ref⁷). Clearly, the structural and functional *in vitro* characterization of *C. metallidurans* transporters will be essential to pinpoint residues responsible for such substrate specificity.

A puzzling observation we made is that hexamminecobalt(III) chloride (CoHex) is not a potent inhibitor (Fig. 3) of CorA and ZntB proteins at least under our experimental conditions. The previously reported IC₅₀ values of 0.5-1 μM for StCorA obtained with the radioactive ⁶³Ni²⁺ whole cell uptakes²⁰ were taken as a reference, however CoHex concentrations up to 50 μM showed little impact on the transport uptake by TmCorA and MjCorA (Fig. 3). We noticed that for the homologous yeast Mrs2 channel³⁹ and for TmCorA reconstituted in liposomes¹⁹ the inhibition of transport was observed with the cobalt hexamine concentration of 1mM. Furthermore, two-electrode voltage clamp experiments revealed the similar value of K_m of 0.9±0.4 mM³⁰. We tried performing our uptake experiments in the presence of 0.2-1 mM range of CoHex, however at such high concentrations it led to the collapse of

proteoliposomes (Supplementary Fig. 2). We can only speculate why it did not work in our case (different lipid composition, reconstitution ratio, etc.) but it might be as well as in the published work with TmCorA in the presence of 1 mM CoHex the authors observed the collapse of proteoliposomes and not the inhibition effect. Furthermore, in the aforementioned work on Mrs2 complete inhibition was not observed³⁹. Interestingly, thermal shift assays studies on MjCorA revealed that CoHex exerts the stabilization effect on the protein (stabilization from 76.7 to ~85°C), albeit it is considerably lower than Co²⁺ ion itself (stabilization up to 95°C)⁴⁰. Altogether this might indicate that CoHex indeed binds to CorA proteins but either not with the high affinity or not exactly at the selectivity filter.

Our results also provide further evidence that CorA and ZntB proteins diverged to use different transport mechanisms. First of all, CorA proteins seem not to utilize any proton gradient in contrast with ZntB (Fig. 5). In EcZntB proteoliposomes under conditions of equal pH inside and outside, 9-amino-6-chloro-2-methoxyacridine (ACMA) dye senses the buildup of pH gradient upon Zn²⁺ transport and the fluorescence is quenched (Fig.5a). In case of TmCorA and MjCorA apparently there is no co-transport of H⁺ thus the fluorescence is more or less at the same level (Fig.5b-c).

In line with the previous reports^{19,41} the influx of Mg²⁺ via CorA proteins is driven by the membrane potential (Fig. 6). Addition of valinomycin to CorA proteoliposomes loaded with 25 mM potassium chloride, leads to the fast escape of potassium ions and build-up of membrane potential to the -116 mV enhancing the transport of divalent cations. The similar behavior of TmCorA was shown before in the experiments with Magfura 2 dye¹⁹, however the enhancement of transport was less pronounced. This discrepancy could be caused by difference in the experimental setup and

liposome preparation and / or fluorescent properties of different dyes. In the yeast mitochondria expressing Mrs2 (in KCl buffer), the influx of Mg^{2+} was significantly reduced upon addition of valinomycin, which dissipated mitochondrial membrane potential³⁹.

The emerging picture is that in the 2-TM-GxN family the homopentameric fold evolved for recognition of similar divalent cations - such as Mg^{2+} , Co^{2+} , Ni^{2+} , Zn^{2+} . However, in the particular milieu, where a certain cation is prevailing, specificity might have evolved. Furthermore, for not yet discovered reasons, some members, such as CorA and Mrs2 evolved to be highly-conductive magnesium channels^{24,30}, whereas others such as ZntB and Alr proteins became proton-coupled symporters^{18,22}. Clearly there are still open questions, such as what is the actual mode of CoHex binding to CorA proteins, and how some members can be involved in the transport of significantly different Al^{3+} cation (1.9 Å first hydration shell radius vs ~2.1 Å for aforementioned cations). The elucidation of structures as well as thorough functional characterization of other members of 2-TM-GxN is necessary to answer such questions and to fully understand the transport of ions in this family of proteins.

Methods

Cloning. TmCorA and MjCorA were cloned into pNIC28-Bsa4 vector encoding an N-terminal 6xHis-tag and a tobacco etch virus protease cleavage site. The full-length CorA genes were amplified from genomic DNAs of *Thermotoga maritima* and *Methanocaldococcus jannaschii* (DSMZ, Germany). The expression vector was constructed using ligation independent cloning with primers for TmCorA (forward 5'-TACTTCCAATCCATGGAGGAAAAGAGGCTGTCTGC-3' and reverse 5'-TATCCACCTTTACTGTACAGCCACTTCTTTTCTTG-3') and

MjCorA (forward 5'-TACTTCCAATCCATGATTACGGTAATTGCTATAGC-3' and reverse 5'-TATCCACCTTTACTGCTAAATCCATCCTGACCTTC-3').

Protein expression and membrane vesicle preparation. TmCorA, MjCorA and EcZntB proteins were expressed in the same way according to the previously established protocol¹⁸. Expression of target protein was performed in a 5-l flask containing 2 l of LB medium (10 g l⁻¹ Bacto trypton, 5 g l⁻¹ Bacto yeast extract, 10 g l⁻¹ NaCl), supplemented with 50 ug ml⁻¹ kanamycin and 34 ug ml⁻¹ chloramphenicol. The *E. coli* BL-21(DE3) cells with the needed plasmid were grown at 37 °C, 200 rpm to an OD₆₀₀ of 0.8, with an induction by addition of 0.1 mM IPTG. After 3 h of expression the cells were collected by centrifugation (15 min, 7,446g, 4 °C), washed in buffer A (50 mM Tris/HCl, pH 8.0) and resuspended in the buffer B (50 mM Tris/HCl, pH 8.0, 250 mM NaCl, 10% glycerol). Membrane vesicles were either prepared immediately, or the resuspended cells were stored at -80 °C after flash freezing in liquid nitrogen. Before membrane vesicle preparation, 1 mM MgSO₄ and 50–100 ug ml⁻¹ DNase were added to the cells. The cells were lysed by high-pressure disruption (Constant Cell Disruption System Ltd, UK, two passages at 25 kPsi for *E. coli* cells, 5 °C) and cell debris was removed by low-speed centrifugation (30 min, 12,074g, 4 °C). Membrane vesicles were collected by ultracentrifugation (120 min, 193,727g, 4 °C), and resuspended in buffer C (50 mM Tris/HCl, pH 8.0, 150 mM NaCl, 15% glycerol) to a final volume of 5 ml per 1 l of cell culture. Subsequently, the membrane vesicles were aliquoted, flash frozen in liquid nitrogen and stored at - 80 °C.

Protein purification. Protein purification was done as described previously¹⁸. Membrane vesicles were thawed rapidly and solubilized in

buffer D (50 mM Tris/HCl, pH 8.0, 150 mM NaCl, 10 mM imidazole, 10% glycerol, 1% (w/v) n-dodecyl- β -D-maltopyranoside (DDM, Anatrace)) for 1 h at 4 °C, while gently rocking. Unsolubilized material was removed by centrifugation (30 min, 442,907g, 4 °C). The supernatant was incubated for 1 h at 4 °C under gently rocking with Ni²⁺-sepharose resin (column volume of 0.5 ml), which had been equilibrated with 10 CV of buffer E (50 mM Tris/HCl, pH 8.0, 250 mM NaCl, 50 mM imidazole, 0.03% DDM). Subsequently, the suspension was poured into a 10-ml disposable column (Bio-Rad) and the flow through was collected. The column material was washed with 10 ml of buffer E. The target protein was eluted in three fractions of buffer F (50 mM Tris/HCl, pH 8.0, 250 mM NaCl, 500 mM imidazole, 0.03% (w/v) DDM) of 200, 750 and 500 μ l, respectively. 2 mM of EDTA was added to the second elution fraction to remove co-eluted Ni²⁺ ions and any residual divalent cations. Subsequently, the second elution fraction was purified by size-exclusion chromatography using a Superdex 200 10/300 gel filtration column (GE-Healthcare), equilibrated with buffer G (50 mM Tris/HCl, pH 8.0, 250 mM NaCl, 0.03% (w/v) DDM). After size-exclusion chromatography, the fractions containing the target protein were combined and used directly for proteoliposome reconstitution.

Reconstitution into proteoliposomes. Reconstitution in proteoliposomes was performed as described previously¹⁸: polar lipids of *E. coli* and egg phosphatidylcholine (in 3:1 (w/w) ratio) were dissolved in chloroform, then dried in a rotary evaporator and subsequently resuspended in buffer containing 50 mM KPi, pH 7.5 to the concentration of 20 mg ml⁻¹. After three freeze-thaw cycles, large unilamellar vesicles (LUVs) were obtained and stored in liquid nitrogen. To prepare proteoliposomes, LUVs were extruded through a 400-nm-diameter polycarbonate filter (Avestin, 11

passages). Obtained liposomes were diluted to 4 mg ml^{-1} in buffer H (50 mM HEPES, pH 7.5) or buffer I (50 mM HEPES, pH 6.5) and subsequently destabilized beyond R_{sat} with Triton X-100. The target purified protein was added to the liposomes at a weight ratio of 1:250 (protein/lipid), followed by detergent removal using Bio-beads (50 mg ml^{-1} , four times after 0.5 h, 1 h, 2 h and overnight incubation). Afterwards, proteoliposomes were collected by centrifugation (25 min, $285,775g$, 4°C) and resuspended in buffer H or buffer I to a lipid concentration of 10 mg ml^{-1} . Finally, after three freeze-thaw cycles, obtained proteoliposomes were stored in liquid nitrogen until subsequent experiments.

Fluorescent transport assays. Transport of metals was measured according to the previously established protocol¹⁸. Zinc transport was measured with the Zn^{2+} -sensitive fluorophore FluoZin-1 (ThermoFisher, USA). To avoid bleaching of the fluorophore, the sample was shielded from the direct light as much as possible. FluoZin-1 (stock concentration 3 mM in H_2O) was added to a final concentration of 5 μM to the proteoliposomes. FluoZin-1 encapsulation was performed by three freeze-thaw cycles and subsequent extrusion through 0.4 μm polycarbonate filters. Extravesicular dye was removed from approximately 500 μl of liposome suspension by size exclusion chromatography on a 2 ml Sephadex G-75 column equilibrated with buffer H or I. Proteoliposomes were collected by ultracentrifugation (25 min, $285,775g$, 4°C), and the supernatant was removed. Proteoliposomes were resuspended with 10 μl buffer H or I per 2.5 mg of proteoliposomes (protein to lipid ratio 1:250). Transport assays were initiated by the addition of 10 mM stock solution of zinc acetate to the desired final concentration. For each measurement, 0.3 mg of proteoliposomes was diluted in 1 ml of desired buffer. A fluorescence time course was measured in a 1-ml cuvette with a

stirrer (350 rpm) using an excitation wavelength of 490 nm and an emission wavelength of 525 nm. Experiments with empty liposomes were performed in parallel as controls. Initial transport rates ($\Delta F \text{ s}^{-1}$) were calculated by performing a linear regression on the transport data between 1 and 10 s after addition of zinc acetate. The resulting data was fitted to a Michaelis-Menten equation. All measurements were at least triplicated.

To investigate the inhibition effect of hexamminecobalt (III) chloride (CoHex), the proteoliposomes loaded with FluoZin-1 were preincubated with various concentrations of CoHex from 1 μM to 1 mM for 3 minutes, after that 25 μM zinc acetate was added. All other steps were performed in the similar way as described above. Experiments with empty liposomes were performed in parallel as controls. All measurements were triplicated. To check the ability of target proteins to transport Al^{3+} , the proteoliposomes were prepared the same way as for FluoZin-1, but instead loaded with 3 μM morin (Sigma-Aldrich). A fluorescence time course was measured in a 1-ml cuvette with a stirrer using an excitation wavelength of 420 nm and an emission wavelength of 500 nm; 50 μM AlCl_3 was added after 1 minute of equilibration time. Experiments with empty liposomes were performed in parallel as controls. All measurements were triplicated. Magnesium transport was measured by FluoZin-3 (ThermoFisher, USA). All preparations of the proteoliposomes were the same as with FluoZin-1 except FluoZin-3 (stock concentration 1 mM in H_2O) was added to a final concentration of 5 μM to the proteoliposomes. A fluorescence time course was measured in a 1-ml cuvette with a stirrer using an excitation wavelength of 494 nm and an emission wavelength of 516 nm. After 3 minute of the baseline's stabilisation 100 μM MgSO_4 was added. Experiments with empty liposomes were performed in parallel as controls. All measurements were triplicated. H^+ transport assays were performed as

described previously¹⁸: the luminal buffer of the proteoliposomes was exchanged for buffer J (5 mM HEPES pH 6.7) by resuspension of the liposomes in this buffer followed by three freeze-thaw cycles and extrusion through 0.4 μ m polycarbonate filters. Proteoliposomes were collected by ultracentrifugation (25 min, 285,775g, 4 °C), and the supernatant was removed. Proteoliposomes were resuspended with 10 μ l buffer J per 2.5 mg of proteoliposomes (protein to lipid ratio 1:250). For each measurement, 0.3 mg of proteoliposomes was diluted in 1 ml of buffer K (5mM HEPES, pH 6.7, 150 nM ACMA). A fluorescence time course was measured in a 1-ml cuvette with a stirrer using an excitation wavelength of 419 nm and an emission wavelength of 483 nm; zinc acetate was added after 3 minutes of equilibration time. Experiments with empty liposomes were performed in parallel as controls. All measurements were triplicated.

Data analysis. The structural figures were produced with an open source version of Pymol (<https://github.com/schrodinger/pymol-open-source>). The sequence alignment was produced with T-coffee⁴² (<http://tcoffee.crg.cat/apps/tcoffee/index.html>) and annotated with Esript 3.0 (ref⁴³) (<http://esript.ibcp.fr>). The statistical analysis was performed in Excel (Microsoft Corp.) and the final graphs were produced in Origin Pro 7 (OriginLab Corp.)

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Author contributions

A.S. performed all experiments. A.S. and A.G. analyzed the data and wrote the manuscript.

Competing interests

Authors declare no competing interests.

Data availability

All data reported in this research are available from the corresponding author on reasonable request.

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Supplementary

Supplementary Figure 1. Collapse of proteoliposomes with (a) TmCorA (b) MjCorA under addition of $> 200 \mu\text{M}$ Hexaminecobalt(III) chloride as seen from the instability of the fluorescence time course.

Supplementary Figure 2. Protein purification. Size-exclusion chromatography profiles of (a) EcZntB, (b) TmCorA and (c) MjCorA.

Chapter 5

An overview of the top ten detergents used for membrane protein crystallization

Artem Stetsenko, Albert Guskov. *Crystals* 7(7), 197 (2017).

Abstract.

To study integral membrane proteins, one has to extract them from the membrane—the step that is typically achieved by the application of detergents. In this mini-review, we summarize the top 10 detergents used for the structural analysis of membrane proteins based on the published results. The aim of this study is to provide the reader with an overview of the main properties of available detergents (critical micelle concentration (CMC) value, micelle size, etc.) and provide an idea of what detergents to may merit further study. Furthermore, we briefly discuss alternative solubilization and stabilization agents, such as polymers.

1. Introduction

Every cell is encircled by the semipermeable membrane (often termed as the lipid bilayer), not only to protect the cell content from the environment, but also to differentiate it externally from the other cells, and internally to form the dedicated organelles within the cell. However, this forced evolution to design a myriad of membrane-embedded and associated proteins, which are essential for the transport of charged and large chemicals in and out of the cell (since they either cannot pass or diffuse too slowly across the membrane) and also for communication between adjacent cells. Not surprisingly, malfunction of these proteins can be extremely detrimental, therefore there is a constantly growing interest to study these proteins in order to decipher the molecular basis of diseases. However they are also attractive from a more fundamental point of view—since many crucial processes occur in the membrane or associated with it, for example photosynthesis [1] or G-protein-coupled receptor (GPCR)-signalling [2] to name just a few. Unfortunately, membrane proteins turned out to be rather difficult to study, since one needs to extract a protein of interest from the membrane, but more importantly extracted protein requires a special environment mimicking the membrane to keep it stable. Historically, the most widely used agents for membrane protein extraction and stabilization are detergents, the amphiphatic molecules, bearing a hydrophilic headgroup (typically polar, sometimes charged) and a hydrophobic (apolar) tail. Due to this nature, detergents are capable of inserting their hydrophobic tails into the lipidic membrane, thus disrupting the latter and eventually (with an increasing concentration of detergent) extracting membrane-embedded proteins (Figure 1). Since the other part of detergent molecule is polar, detergent molecules spontaneously form micelles (pseudo-spherical assemblies) as soon as critical micelle concentration

(CMC) is achieved and under condition that the sample is above critical micellar temperature (CMT). Below CMC value, detergent molecules are mostly present as monomers. Due to the micelle formation, a membrane protein becomes a part of the detergent (lipid)-protein complex, sometimes with the complete loss of surrounding lipids. In many cases, the importance of lipids for the function and/or getting crystals of membrane proteins has been highlighted, e.g., for photosynthetic proteins [3,4], transporters and channels [5–8], and GPCRs [9]. Furthermore, even the partial removal of lipids sometimes can be destabilizing, possibly due to the decrease in lateral pressure [10] or increase in hydrophobic mismatch [11]. Also, it is important not to forget that the formed detergent-protein complex is dynamic, thus there is a constant exchange of the detergents molecules between the complex and the free detergent pool. This implies a strict (and rather costly) condition to maintain the concentration of detergent above its CMC value during all the steps of protein study. Regardless of all these and other limitations, detergents still to remain the first choice agents in studies on membrane proteins. Despite all of the chemical variety of detergents, all are typically characterized by several parameters. In addition to the aforementioned CMC value, the aggregation number N , and the micelle size (in terms of M_w) are commonly reported. The latter value is extremely helpful during the concentration step of purified protein-detergent complex—the right cut-off value of a concentrator will prevent the overconcentration of a detergent (in free micelles), a potentially harmful event for crystallization [12]. The higher concentration of a detergent, the higher the chance of phase separation, which might ultimately lead to protein denaturation. Parameters such as temperature, salt, and precipitant also influence the detergent phase behavior [13,14]. However, the phase separation can be also used in a beneficial way,

for example as a simple and cheap purification technique (reviewed in [15,16]) or to design crystallization experiments [17].

To estimate the amount of bound detergent to the protein of interest, several techniques can be used. The application of matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) is extremely powerful, since it allows both quantification and identification of bound detergents/lipids [18,19] and even analysis of (membrane) protein–ligand interactions [20]. In cases where the identity of the bound substance is known, one can also use multi-angle laser light scattering coupled with size-exclusion chromatography (SEC-MALLS) to quantify the amounts of bound material [21,22].

The most general classification of detergents divides them into three major classes: non-ionic, ionic and zwitter-ionic. Non-ionic detergents are the most commonly used for cases when native state of protein is critical. These detergents are considered mild—a qualitative indicator of the impact of detergent onto protein stability. Non-ionic detergents typically disrupt protein-lipid (and lipid-lipid) but not protein-protein interactions, thus maintaining (or better to phrase—not interfering with) the native state of a protein. On the contrary, ionic detergents are typically harsh, since they disrupt additionally protein–protein interactions, thus often bringing proteins into the denatured state. Ionic detergents have head groups charged either positively (cationic) or negatively (anionic). Due to this fact, the properties of these detergents (most importantly their CMC values) are affected by the ionic strength, thus special care should be taken during experiments. Zwitter-ionic detergents, as their name implies, have both charges present, rendering an electro-neutral molecule. These detergents are typically milder than the ionic ones, but somewhat harsher than non-ionic detergents. There are

numerous comprehensive reviews on different aspects of detergents available [23–25], to which we would like to point out to the readers' attention for more detailed information.

Apart from detergents, there are several other agents capable of extraction and stabilization of membrane proteins. In the past few years, the styrene maleic acid (SMA) polymer has gained much attention (see refs. [26,27] for recent reviews). There are a few benefits of SMA—it is a very cheap material, it extracts membrane proteins with the accompanying lipids (both intrinsic and annular), and also keeps them stable as a SMA-lipid-protein (SMALP) complex (Figure 1). Since SMA does not form micelles, there is no need to maintain the pool of freely available SMA molecules in buffers as in case of detergents (see above). Clearly, there are also some limitations of using SMAs, preventing them from the ubiquitous application, and those are a rather defined size of SMALPs of ~10 nm [28], thus large proteins will probably not fit in; pH sensitivity (at pH 6.5 and below SMA starts precipitating [29]), and limited compatibility with the crystallogensis. However, very recently, the group of Oliver Ernst reported the first crystal structure of bacteriorhodopsin extracted and purified in SMA and crystallized in the lipidic cubic phase [30]. Additionally, SMAs might find their niche in the cryo-Electron Microscopy (Cryo-EM) field [31,32] and with the future generations of SMAs, (e.g., with the various lengths and ratios of polymers [33]) they might become a very useful tool for studies on membrane proteins.

Another polymer widely used in membrane protein research are amphipols (see ref. [34] for the detailed review). Amphipols are amphipathic (bearing both hydrophobic and hydrophilic units) and highly soluble, thus capable to wind around a membrane protein, shielding its hydrophobic patches away and providing the necessary solubility of the formed protein—

amphipol complex (Figure 1). The key difference from the aforementioned SMA polymers is that amphipols cannot be used for membrane-protein extraction and can be only applied to the detergent-solubilized sample. However the amphipol–detergent exchange is simple and quick, and many proteins are more stable in the presence of amphipols (see ref. [35] and references therein). The most commonly used of the amphipols is A8-35, comprised of ~35 acrylate units with Mw of ~4.3 kDa. On its own, amphipols can form micelle-like spherical particles (with ~3.15 nm radius) at the concentrations exceeding their ‘CMC’ value (0.002% (w/v) for A8-35 [36]). Despite this, there is an exchange between the protein–amphipol complex and amphipol particles (there are almost no free amphipol molecules in solution); amphipols stay bound to membrane proteins strongly even in the amphipol-free buffers. However amphipols can be very easily washed away by detergents, thus upon an amphipol exchange, care should be taken to not expose a sample to detergent-containing buffers. Currently, amphipols gained significant popularity in the Cryo-EM field [37–39] but also in solution NMR studies [40,41]. For the X-ray crystallography field, it has been shown that amphipols are compatible with lipidic cubic phases [42], thus currently amphipols can only be used for *in meso* crystallization [43], similarly to SMALPs.

Figure 1. (A) Extraction of a membrane protein (cyan) from the lipid bilayer (lipid heads as circles, tails as lines and detergent molecules as red squares). (From top to bottom) the concentration of detergent is increased, until the protein is extracted in the form of the complex with detergent (and some residual lipids), surrounded by free detergent-lipid micelles. Note that detergent/lipid molecules are omitted in the plane towards a viewer. (B) A membrane protein encircled with the styrene maleic acid (SMA) belt (the chemical structure is shown in the insert). Note that SMA cuts out a fraction of membrane with the protein, thus maintaining the endogenous lipids. (C) A membrane protein encircled with the amphipol belt (the chemical structure of the most commonly used amphipol A8-35 is shown in the insert). Since amphipols are applied only after extraction with a detergent, there are far fewer lipid molecules bound to the protein.

There are several other approaches developed (and being actively developed) for the stabilization of membrane proteins, including nanodiscs [44], calixarens [45], fluorinated surfactants [46] and others, but they are beyond the scope of this mini-review.

2. Results

We have analyzed all the entries in the “Membrane Proteins of Known Structure” database (up to 31 December 2016) and manually extracted information regarding what kind of detergent was used for protein purification and crystallization. It is obvious that the increased interest in membrane proteins during the past 30 years caused not only the cumulative growth in use of detergents but also forced the development of novel detergents. The first membrane protein structure of the photosynthetic reaction center solubilized using zwitter-ionic detergent Lauryldimethylamine-N-oxide (LDAO) was solved in 1985 [47] and since then LDAO application has grown steadily. Another zwitter-ionic detergent 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) (and its hydroxylated form 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate (CHAPSO)) started gaining popularity in the early 2000s with an increasing application in Cryo-EM. But in fact more than a half of proteins during these years were investigated using alkyl maltosides and glycosides. The most commonly used detergent in both categories (purification and crystallization) is n-Dodecyl- β -D-Maltopyranoside (DDM) (40.6% and 36.3% respectively), followed by n-Decyl- β -D-Maltopyranoside (DM) (13.9% and 12.1%), and n-Octyl- β -D-Glucopyranoside / n-Nonyl- β -D-Glucopyranoside (OG/NG) (11.0% and 11.9%/4.5% and 5.4% respectively) (Figure 2). The other detergents that scored more than 1% are LDAO (4.8% and 5.2%), C₁₂E₈ (4.6% and 5.3%), n-Undecyl- β -D-maltopyranoside (UDM) (3.1% and 3.5%), Lauryl maltose neopentyl glycol (LMNG) (3.0% and 3.5%), Triton X-100 (1.9% and 0.3%), Thesit (1.8% and 1.6%), Digitonin (1.6% and 1.3%), Cymal-5 (1.4% and 1.4%) and Cymal-6 (1.5% and 1.8%),

and CHAPS (1.0% and 2.6%). In only about 50% cases the same detergent was used for both purification and crystallization.

Interestingly, if we take into account only the past decade, the results do not differ much (Figure. 2) – DDM, DM, and OG dominate, both for purification and crystallization.

Figure 2. The bar representation (in %) of different detergents used for (A) purification and (B) crystallization. Panels (C) and (D) include data only for the time period of 2006–2016.

Remarkably, in the field of Nuclear magnetic resonance (NMR) spectroscopy, the distribution is totally different. DDM is used very rarely, since it forms large micelles that tumble slowly, causing the broadening of

protein signal in NMR spectra, rendering this detergent not very suitable for NMR spectroscopy. However anionic detergents, such as sodium dodecyl sulfate (SDS) and different variants of phosphocholine (e.g., *n*-Dodecylphosphocholine (FC-12), 1,2-Dimyristoyl-*sn*-Glycero-3-Phosphocholine (DMPC), 1,2-Dioleoyl-*sn*-Glycero-3-Phosphocholine (DOPC)), considered very harsh for macromolecular crystallography studies, are widely used in NMR. The overview of detergents (and alternative membrane mimetics) explicitly used in NMR spectroscopy can be found in [48].

Below we provide the short descriptions of the detergents that are currently used the most in structural biology of membrane proteins.

2.1. *n*-Dodecyl- β -D-Maltopyranoside

Also known as Layrul maltoside or most commonly, DDM, *n*-Dodecyl- β -D-maltopyranoside is currently the most used detergent (see above). It belongs to the class of alkyl maltosides, thus it has a hydrophilic maltose headgroup and a hydrophobic alkyl chain (Figure 3).

Figure 3. Structure of Layrul maltoside (DDM) (chemical formula is $C_{24}H_{46}O_{11}$).

Its popularity can be explained by several factors: it was developed as a cheaper alternative to OG detergent basically at the dawn of membrane protein (structural) studies, in 1980 [49]. Furthermore it has a low CMC value

(~0.0087%/0.17 mM [50]) that allows reducing the amount of detergent needed (though rendering it more difficult to remove). Thus both economically and historically, as well as partly due to ‘herd behavior’, it acquired the leading position for structural studies on membrane proteins. Nevertheless, it is also quite an efficient detergent for protein extraction and since it is non-ionic, it is mild enough to maintain the stable native state of many proteins. The main drawback of DDM is that the micelle it forms is relatively large (Mw ~65–70 kDa [51,52]) and it forms a substantial and rather mobile belt around proteins that can be detrimental during crystallogenesis. DDM seems to be a truly universal detergent (however with the limited application in NMR spectroscopy), since it has proved to be successful for virtually all the classes of α -helical proteins.

2.2. *n-Decyl- β -D-Maltopyranoside*

Also known as Decyl maltoside or DM, *n-Decyl- β -D-maltopyranoside* is a shortened version of DDM (two carbons less in the hydrophobic tail) (Figure 4). Due to this shortening, it forms considerably smaller micelles (Mw ~ 40 kDa [51]) but at the expense of 10 times higher CMC (0.087%/1.8 mM [50]). This higher CMC value might partially explain why this detergent is less popular than DDM (at least in economical reasons), but in general detergents with the shorter alkyl chains seem to be less stabilizing.

Figure 4. Structure of Decyl maltoside (DM) (chemical formula is $C_{22}H_{44}O_{11}$).

2.3. *n*-Octyl- β -D-Glucopyranoside

Also known as Octyl glycoside or OG, *n*-Octyl- β -D-glucopyranoside is the one of the two most used glucoside-based detergents. It has one sugar moiety less in its head group compared to maltosides (Figure 5), but similarly to them, OG is a non-ionic, albeit less milder detergent. It has a very high CMC of 0.53%/20 mM [53] with the compact micelle ($M_w \sim 25$ kDa [53]).

Figure 5. Structure of Octyl glycoside (OG) (chemical formula is $C_{14}H_{28}O_6$). OG has gained its popularity for studies on (bacterio-) rhodopsins, photosynthetic complexes and aquaporins.

2.4. *n*-Nonyl β -D-Glucopyranoside

Also known as Nonyl glucoside or NG, *n*-Nonyl- β -D-glucopyranoside is an extended (one extra alkyl unit) version of OG (Figure 6). Such an extension rendered it to have smaller CMC (0.20%/6.5 mM) but much larger micelle ($M_w \sim 90$ kDa, this work).

Figure 6. Structure of Nonyl glucoside (NG) (chemical formula is $C_{15}H_{30}O_6$).

Application of NG correlates well with the proteins studied with OG, with an addition of the intramembrane proteases family, where NG dominates among other detergents, and the Energy coupling factor (ECF) family of ATP-binding cassette (ABC) transporters, where NG was used in combination with (D)DM.

2.5. *Lauryldimethylamine-N-Oxide*

Lauryldimethylamine-N-oxide (LDAO), also known as dodecyldimethylamine oxide (DDAO), is a zwitter-ionic detergent, bearing two opposite charges in its head group and a long hydrophobic tail (Figure 7). Though considered rather a harsh detergent due to the charged head group, nevertheless it has landed on the fifth place of the most used detergents for membrane protein research. This might be explained by the evidence that proteins stable in LDAO often produce well diffracting crystals [8,54,55]. The latter is probably dictated by the compact micelle size ($M_w \sim 21.5$ kDa [52]) that might promote better packing within crystals. Interestingly LDAO was shown to form rather elongated and not the spherical micelles [56,57]. It has CMC value of 0.023% / 1–2 mM.

Figure 7. Structure of dodecyldimethylamine oxide (LDAO) (chemical formula is $C_{14}H_{31}NO$).

2.6. Polyoxyethylene 8 (9) dodecyl Ether

Polyoxyethylene 8 (9) dodecyl ether, or $C_{12}E_8$ ($C_{12}E_9$), are non-ionic detergents that belong to the family of alkyl polyoxyethelenes with the general formula C_xE_y , where y denotes oxyethelene units in the head group, and x describes the length of the alkyl tail (Figure 8). $C_{12}E_9$ is also named Thesit or polydocanol.

$C_{12}E_8$ has a very low CMC value of 0.005% (0.09 mM) [58] and the micelle size of ~66 kDa [59]. $C_{12}E_9$ has even lower CMC value of 0.003% (0.05 mM) [60], but considerably larger micelle (Mw of ~ 83 kDa [52]).

Figure 8. Structure of $C_{12}E_8$ and $C_{12}E_9$ (chemical formula is $(C_2H_4O)_n C_{12}H_{26}O$, $n \sim 8$ or 9).

$C_{12}E_8$ has been successfully used for purification and crystallization of several ABC-transporters and is the number one detergent for studies on P-type ATPases, whereas $C_{12}E_9$ has proven to be successful in the studies on the electron-transfer chain complex II.

2.7. *n*-Undecyl- β -D-Maltopyranoside

On the next position is yet another maltoside—n-Undecyl- β -D-maltopyranoside, also known as UM or UDM. It is an intermediate between DDM and DM in terms of the length of the hydrophobic tail: 11 carbon atoms for UDM (Figure 9) versus 12 and 10 for DDM and DM respectively. Its CMC value is about 0.029%/0.59 mM with the micelle size of about 50 kDa [51].

Figure 9. Structure of n-Undecyl- β -D-maltopyranoside (UDM) (chemical formula is $C_{23}H_{44}O_{11}$).

UDM has been used in the studies of many different membrane protein families, with the highest occurrence in the studies of cytochrome bc1 and cytochrome b6f.

2.8. Lauryl Maltose Neopentyl Glycol

Lauryl maltose neopentyl glycol (LMNG or MNG-3) is a representative of the novel, recently developed maltose neopentyl glycol detergents [50]. The main feature of this class of detergents is the presence of quaternary carbon atom that allows incorporation of two hydrophilic head groups and two hydrophobic tails (Figure 10).

Figure 10. Structure of Lauryl maltose neopentyl glycol (LMNG) (chemical formula is $C_{47}H_{88}O_{26}$).

LMNG has a low CMC value (0.001%/0.01 mM [33]), with the size of micelle of Mw ~ 91 kDa (this work), which is in contrast with the recent result of ~ 393 kDa obtained with MALDI-TOF MS [19]. It has been reported to be efficient both at protein extraction and stabilization of several delicate membrane proteins [37,61,62]. So far it has been rather extensively used for studies on GPCRs and was also successful for the family of transient receptor potential (TRP) channels and the N-methyl-D-aspartate (NMDA) receptors among others.

2.9. Triton X-100

Triton X-100, or octyl phenol ethoxylate (Figure 11) is one of the oldest classical non-ionic detergents still in use. It has CMC value of 0.01%/0.2 mM, with the micelle size of 60 to 90 kDa (temperature-dependent) [63,64].

Figure 11. Structure of Triton X-100 (chemical formula is $C_{14}H_{22}O(C_2H_4O)_n$ where $n = 9, 10$).

The main disadvantage of Triton X-100 is the presence of the aromatic ring that absorbs strongly in the UV region of the spectrum, thus interfering with the protein quantification. Furthermore, since Triton X-100 is obtained during the polymerization reaction of octylphenol with ethylene oxide, the final product is heterogeneous, containing on average 9.5 units of ethylene oxide. To aggravate that, polyethelene glycol is a typical by-product, and additionally peroxides were reported to be present in the detergent preparations as the result of aging [65]. This explains the significant difference between the number of cases, where Triton X-100 was used for extraction/purification (1.8%) and for crystallization (0.3%), where typically homogeneity and purity play a crucial role in crystallogenesis [66].

2.10. Digitonin

Digitonin is a truly natural (albeit toxic) detergent obtained from the purple foxglove plant *Digitalis purpurea*. It has a steroid-like saposin structure (Figure 12).

Figure 12. Structure of digitonin (chemical formula is $C_{56}H_{92}O_{29}$).

Digitonin has a CMC value of 0.02%–0.03% / 0.25–0.5 mM [67,68] with the micelle size of ~ 70 –75 kDa [69]. Since it is not synthesized but extracted from the plant, there might be considerable batch-to-batch variations; therefore the alternative glyco-diosgenin (GDN) has been recently proposed as a suitable non-toxic replacement [70]. Nevertheless, during the past few years digitonin gained a considerable popularity for the structural studies on eukaryotic membrane proteins with Cryo-EM [71,72].

2.11. Cymal-5 (Cymal-6)

These detergents are the special case of maltoside detergents with the cyclohexyl aliphatic tail (Figure 13).

Figure 13. Structure of Cymal-5 (chemical formula is $C_{23}H_{42}O_{11}$; Cymal-6 has an extra carbon atom in its tail with formula $C_{24}H_{44}O_{11}$).

Cymal-5 has a very high CMC value of ~0.12% (~2.5 mM [73]) and the micelle size of ~ 23 kDa [74], whereas slightly longer Cymal-6 has a lowered CMC value of ~0.028% (0.56 mM) and a larger micelle size of ~32 kDa.

Cymal-5 and -6 were used for a broad range of channels and transporters, and in many cases as a second detergent or an additive.

2.12. CHAPS (CHAPSO)

CHAPS (CHAPSO), also known as 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate and 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate respectively, are other representatives of zwitter-ionic detergents derived from the bile salts and having cholesterol-like structures (Figure 14). Both CHAPS and CHAPSO have a very high CMC value of ~0.5% (8–10 mM) with the very small micelles of just 6 and 7 kDa respectively. Such a combination of high CMC and small micelle size makes it extremely easy to remove CHAPS and CHAPSO by dialysis. Both detergents have been used in the structural studies on a variety of transporters, with the recently increased interest in their application for Cryo-EM.

Figure 14. Structure of CHAPS and CHAPSO (chemical formula is $C_{32}H_{58}N_2O_7S$ / $C_{32}H_{58}N_2O_8S$).

For convenience, we compiled Table 1 to summarize the main characteristics of the aforementioned detergents.

Table 1. The properties of the most commonly used detergents.

Detergent	CMC (%/mM)	Aggregation Number, N ¹	Micelle		Mw (Da)
			Size (kDa)	Mw	
DDM	0.0087/0.17	80–150	65–70		510.6
DM	0.087/1.8	69	40		482.6
OG	0.53/20	30–100	25		292.4
NG	0.20/6.5	133	85		306.4
LDAO	0.023/1-2	76	21.5		229.4
C ₁₂ E ₈	0.005/0.09	90–120	66		538.7
C ₁₂ E ₉	0.003/0.05	90 ²	83		582.8
UDM	0.029/0.59	71	50		496.6
LMNG	0.001/0.01	~400 ³	91–393		1069.2
Triton X-100	0.01/0.2	75–165	60–90		624.8 (av.)
Digitonin	0.002/0.5	60 ⁴	70		1229.3
Cymal-5	0.12/2.5	47	23		494.6
Cymal-6	0.028/0.56	91	32		508.6
CHAPS	0.5/8–10	10	6		614.9
CHAPSO	0.5/8–10	11	7		630.9

¹ Data taken from Anatrace website, unless specified; ² [75]; ³ [19]; ⁴ From Sigma Aldrich website.

3. Discussion and Outlook

Looking at the results of the performed analysis, one can confidently say that the sugar-based detergents rule the field of structural studies on membrane proteins. Just two maltoside-based detergents, DDM and DM, are reported as the detergents of choice for more than a half of deposited structures. With an addition of UDM, cyclic maltosides Cymal-5 and Cymal-6, and glycoside-based detergents OG and NG, the total fraction of sugar-based detergents is around 75%. Does it mean that sugar-based detergents are the best? Not necessarily so, but the fact that they are the most common helps them to maintain their popularity over the years. Clearly, any new detergent faces a problem of how to reach the market – to make it commercially available and successful, first it must prove to be useful for a broad range of targeted proteins. Furthermore, many scientists tend to utilize proven techniques and reagents, and not only because of reproducibility of results, but quite often also for economical reasons, since detergents are rather expensive chemicals. In this respect it is interesting and exciting to witness the extension of a current toolbox with cheaper alternatives, such as SMA polymer. Clearly it has numerous limitations, but with further improvements in the design of this polymer, coupled with the recent promising results in application of SMA in crystallization and Cryo-EM studies, it is feasible to say it will gradually gain more and more attention. Amphipols are also becoming more popular, again more in the field of Cryo-EM. They are easy to apply and provide a good stability; in addition, amphipols do not form large micelles like detergents do.

In the case of a detergent solubilized protein, one can face the situation that the most of the protein is obscured by large detergent belt, therefore impeding the analysis of images, whereas amphipols form a relatively small

belt, thus not interfering with classification of images. Furthermore, other alternatives exist, such as bicelles for crystallization [76]—commercial kits available (in the form of lipid-detergent mixture, typically DMPC with CHAPSO); fluorinated surfactants [46] and nanodiscs (a patch of the lipid bilayer encircled by membrane scaffolding proteins) [44] for protein stabilization. Fluorinated surfactants have not gained much attention so far, probably due to its nature—it has a hydrophobic tail with the incorporation of several fluorine atoms, rendering it lipophobic and preventing effective interactions with a membrane protein, typically causing aggregation of the latter. The insertion of a hydrogenated tip reduced lipophobicity [77] and improved protein stability, but nevertheless these agents are still far from being widely used. In contrast, reconstitution in nanodiscs has become one of the standard methods, especially for functional studies on membrane proteins. However, it was recently found that they also were useful for structural studies using Cryo-EM [78]. Recently, more tunable and size-controlled covalently circularized nanodiscs with enhanced stability have been engineered [79].

Nevertheless, there is also considerable research being conducted in the design and development of novel detergents. One of the very successful examples are neopentyl glycol maltoside and glucoside detergents [50]. Introduced less than a decade ago, it has confidently landed in top 10 of the most used detergents in our analysis. The further design of this class of detergents is still ongoing; very recently the same group presented so-called tandem-neopentyl glycol maltosides [80]. Another example is the development of calixarenes [45]—this novel class of detergents is built on a rigid calixarene scaffold to which hydrophobic tail and hydrophilic and charged headgroups are attached. The charge present on their headgroups

promotes salt-bridge interactions with the patches of basic residues readily available on surfaces of membrane proteins at the cytosol-membrane interface [81]. Furthermore the steroid-based facial amphiphiles (FA) are another type of solubilization/stabilization agents being actively developed. They utilize the cholate scaffold, to which (typically) sugar headgroups are attached [82,83]. Such design proved to be useful to not only to stabilize membrane proteins (via tight interactions between a membrane proteins and FA owing to the large hydrophobic surface of the latter), but to also improve their crystallization (via the formation of more compact protein–detergent complexes, which can pack more tightly in a crystal, owing to the ability of FA to mediate intermolecular contacts between molecules of membrane proteins) [84]. Additionally, the longer tandem version of FA is available, where two steroid moieties are connected via a linker of varying length to match the width of a lipid bilayer [85].

Taking into account a large collection of classical detergents and continuously appearing new ones, the valid question of any researcher dealing with the membrane proteins is which detergent to use. Unfortunately, there is no magic single detergent that would work for all of the proteins, therefore usually some screening is required. In cases where the high throughput is not supreme, the small-scale solubilization and purification tests can be run with about three to five detergents from the aforementioned list (in our lab we generally test DDM, NG, OG, LMNG, and LDAO) to get the initial idea about protein stability. The screening can be optimized via the introduction of GFP-fusion for a rapid assessment of detergent solubilization efficiency [86] and protein stability in a certain detergent [86,87]. In cases where the high throughput is desirable, other protocols such as BMSS (Biotinylated Membranes Solubilization & Separation) [88] or fully-automated detergent

screening [89] can be applied for fast-screening of 96 different detergents at once.

From a very naive point of view, it might appear that alternative solubilization and stabilization agents will gradually seize the Cryo-EM branch of structural biology, whereas detergents will dominate in the X-ray crystallography field. Whether it is correct or not, time will tell. However, it is exciting to witness the current developments both in the design of new detergents and further improvements in the alternative agents. Coupled with the technological improvements in their structural biology, it is feasible to say that the amount of new membrane proteins deposited in the protein data bank will steadily grow in coming years thus producing a larger array of data to analyze preferences of membrane proteins for different detergents.

4. Materials and Methods

We used a public database (Membrane Proteins of Known Structure) maintained by Dr. Stephen White's laboratory (UC Irvine, USA).

The determination of micelles size formed by NG and LMNG was performed using size-exclusion chromatography coupled with multi-angle laser light scattering (SEC-MALLS) as described in [51].

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List of publications

1. Gati, C.†, **Stetsenko, A.** †, Slotboom, D.J., Scheres, S. H.W., Guskov, A. The structural basis of proton driven zinc transport by ZntB. Nat Commun 8, 1313 (2017) doi:10.1038/s41467-017-01483-7
2. **Stetsenko, A.**, Guskov, A. An Overview of the Top Ten Detergents Used for Membrane Protein Crystallization. Crystal, (2017) 1;7(7):1-16. 197. doi:10.3390/cryst7070197
3. **Stetsenko, A.**, Guskov, A. Cation permeability in CorA family of proteins. Sci Rep. The manuscript is accepted.
4. Singh, R. †, **Stetsenko, A.**†, Jaehme, M., Guskov, A., Slotboom, D.J. Structural and functional characterization of NadR from *Lactococcus lactis*. The manuscript is in preparation.

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Summary

Nederlandse samenvatting

Выводы

Summary

Zinc is essential for all branches of life and is the second most abundant transition metal divalent cation in living organisms after iron. Since metal ions cannot be synthesized, all living organisms rely on membrane proteins to take up metal cations from the environment. However, in many cases an excess of metals is toxic, thus there must be also membrane proteins capable to expel unnecessary ions. To fully understand these processes of metal transport in and out the cell, it is essential to solve structures of the membrane proteins in the presence and absence of their substrates, and to characterize them biochemically to propose and validate their transport mechanism. This thesis contributes in the understanding on the transport of zinc, and several other metal ions, via ZntB and other members of the 2-TM-GxN family, which are present in every kingdom of life. In **Chapter 1** I have summarized structural and biochemical data about the 2-TM-GxN family and its role in the maintaining magnesium, zinc, nickel and cobalt homeostasis in all organisms.

In **Chapter 2** I report the full-length structure of the ZntB transporter in the absence of substrate. The transport via ZntB protein was characterized via ligand binding and transport assays that demonstrated that ZntB indeed is a zinc transporting protein. By combining all the available data obtained during this PhD research project and by other groups, the conclusion was made that ZntB is a zinc importer that is driven by a proton gradient. Its transport mechanism differs from that known of homologous CorA Mg^{2+} channels. Additionally, we observed that ZntB does not collapse into a highly asymmetrical state upon depletion of divalent cations, unlike CorA in similar conditions.

In **Chapter 3**, I report two ZntB structures in the presence of its substrate – highly similar zinc and cadmium ions. Due to the low resolution of both structures it is impossible to use them to elaborate on ZntB transport mechanism. However, in this chapter I present a strong piece of evidence that the residue D253 is most probably used for coupling of Zn^{2+} and H^+ transport, as mutation introduced at this position leads to the decoupling of $\text{H}^+/\text{Zn}^{2+}$ symport and loss of sensitivity to the proton gradient. I also studied conserved arginines 191 and 198 and their roles in the transport mechanism, but it is difficult to interpret the results of these mutations without additional experiments.

In **Chapter 4** I provide additional evidence about the differences in the transport mechanism between CorA and ZntB proteins. The first difference is that CorA proteins are insensitive to a proton gradient in contrast to ZntB. In EcZntB proteoliposomes under conditions of equal pH inside and outside, the ACMA dye senses the build-up of pH gradient upon Zn^{2+} transport and the fluorescence is quenched. In case of TmCorA and MjCorA apparently there is no co-transport of H^+ , because the fluorescence stays at the same level as the control experiment. If CorA is a channel, then Mg^{2+} transport via CorA proteins should be driven by the membrane potential. In our set up, addition of valinomycin to CorA proteoliposomes loaded with potassium chloride, leads to the fast escape of potassium ions and build-up of membrane potential with enhancing the transport of divalent cations. The proteoliposomes with EcZntB did not show any increase in the zinc uptake, that adds another strong indication that ZntB is a transporter. Summarizing all these findings my proposal is that CorA and ZntB have different transport mechanisms.

Chapter 5 is a review, mainly aimed at researchers, who just entering the structural biology field. In this work I review the main properties of the most commonly used detergents in structural studies, their critical micelle concentration (CMC) value, micelle size, aggregation number and offer an idea which detergents are good starting points for membrane protein structural biology. Furthermore, I briefly discuss alternative solubilization and stabilization agents and provide statistics on the actual usage of various detergents based on the deposited structures.

Perspective

Although this thesis provides the first full-length structure of ZntB and new details about its transport mechanism, there are still many open questions. First of all, the transport mechanism of ZntB is still elusive and unclear. More studies are needed to fully understand ZntB's role in the zinc homeostasis in bacteria. Higher resolution structures of ZntB with the bound substrate are essential to understand the mechanism. Also, the roles of amino acids V289 and R191, R198 remain unclear. There is no doubt that they are playing the important role in the transport, but it is hard to interpret their roles based on the current data.

If we go one step further, on the family level, there are even more open questions. Some members, such as CorA and Mrs2 evolved to magnesium channels, whereas others such as ZntB and probably Alr proteins became transporters, but the physiological consequences of these differences in transport mechanism are unclear. Despite of the fact that CorA is the most studied member of the 2-TM-GxN family, its transport mechanism is also still elusive and controversial. Based on our data, it is a valid question if CorA can play a role in zinc transport *in vivo*, as its K_m for zinc is similar to K_m for zinc

of ZntB. Another intriguing question for CorA is the actual mode of CoHex binding to these proteins. There are a few experiments demonstrating that CoHex is a strong inhibitor for CorA, but in our experimental set up we do not see any significant inhibition up to hundred-fold higher concentration than the published K_m for CoHex. Therefore, a structure of CorA with CoHex is highly desirable to resolve this conundrum. In the end, the elucidation of high-resolution structures as well as careful biochemical characterization of ZntB, CorA and other members of 2-TM-GxN is necessary to answer these questions and to fully understand the transport of ions in this family of proteins.

This thesis describes fundamental research on bacterial and archaeal ZntB and CorA proteins, however, investigation of their transport mechanisms may also improve our knowledge about their eukaryotic homologs – Mrs2 and Alr proteins. There is data about a role for Mrs2 in the promotion of multidrug resistance in gastric cancer cells, thus Mrs2 can be a potential target for reversal therapy. Alr proteins were shown to be essential for development and virulence of fungal pathogens, so they can be potential targets for development of new antifungal agents. Therefore, members of the 2-TM-GxN family can be potential targets for new drugs development.

Nederlandse Samenvatting

Zink is essentieel voor alle domeinen van het leven en is, na ijzer, het op één na meest voorkomende divalente overgangs-metaalion in levende organismen. Omdat metaalionen niet kunnen worden gesynthetiseerd, zijn levende organismen afhankelijk van membraaneiwwitten die er voor zorgen dat deze metaalionen worden opgenomen vanuit hun omgeving. Echter, in veel gevallen is een overmaat aan metalen giftig; er zijn dus ook membraaneiwwitten nodig die een overschot kunnen uitstoten. Om de transportprocessen (in- en uitvoer) van metaalionen volledig te kunnen begrijpen, is het essentieel de atomaire structuren van deze membraaneiwwitten te bepalen in afwezigheid en aanwezigheid van hun substraten en om ze biochemisch te karakteriseren, teneinde de onderliggende transportmechanismen voor te stellen en te valideren. Dit proefschrift draagt bij aan het begrijpen van het transport van zink en andere metaalionen door het membraaneiwit ZntB en andere leden van de 2-TM-GxN familie, een groep membraaneiwwitten die in alle domeinen van leven voorkomt. In **Hoofdstuk 1** geef ik een overzicht van de structuur- en biochemische data over de 2-TM-GxN familie en de rol die deze eiwitfamilie speelt in het handhaven van homeostase voor magnesium-, zink-, nikkel- en kobaltionen in alle organismen.

In **Hoofdstuk 2** rapporteer ik de volledige structuur van het transporteiwit ZntB, in afwezigheid van substraat. ZntB werd gekarakteriseerd door middel van bindings- en transport experimenten, die laten zien dat ZntB inderdaad een zink-transporteiwit is. Door deze resultaten te combineren met die van andere onderzoeksgroepen concluderen we dat ZntB een zink-importerend eiwit is waarbij een proton gradient de drijvende

kracht vormt. Dit transportmechanisme verschilt van dat van homologe CorA Mg^{2+} eiwitten. Bovendien vonden we dat ZntB geen zeer asymmetrische conformatie aanneemt wanneer divalente kationen worden weggenomen, iets wat bij CorA onder dergelijke omstandigheden wel het geval is.

In **Hoofdstuk 3** beschrijf ik twee ZntB structuren in aanwezigheid van soortgelijke substraten, namelijk zink en cadmium ionen. Door de lage resolutie van beide structuren is het niet mogelijk hiermee het transportmechanisme van ZntB te ontrafelen. Echter in dit hoofdstuk geef ik sterke aanwijzingen voor een rol van residu D253 in het koppelen van Zn^{2+} en H^+ transport; een mutatie op deze positie leidt namelijk tot het ontkoppelen van H^+/Zn^{2+} transport en tot een verlies in gevoeligheid voor de proton gradient. Ook de rol van de geconserveerde arginine residuen op posities 191 en 198 ten aanzien van het transport mechanisme werden bestudeerd, maar de resultaten hiervan bleken moeilijk te interpreteren.

In **Hoofdstuk 4** geef ik aanvullend bewijs voor de verschillende transportmechanismes tussen CorA en ZntB eiwitten. Het eerste verschil betreft de gevoeligheid van transport voor een proton gradiënt, die wel aanwezig is bij ZntB maar niet bij CorA. In EcZntB proteoliposomen, onder omstandigheden waarbij de interne en externe pH gelijk zijn en gebruikmakend van de pH-gevoelige fluorescente kleurstof ACMA, zien we een pH-gradiënt ontstaan tijdens Zn^{2+} transport (waargenomen als uitdoving van ACMA fluorescentie). In het geval van proteoliposomen met TmCorA of MjCorA wordt geen verandering van fluorescentie waargenomen (t.o.v. een controle experiment), wat aangeeft dat er geen co-transport is van H^+ . Dus, als CorA slechts een kanaal-eiwit is en niet wordt gedreven door H^+ co-transport, dan zou Mg^{2+} transport door dit type eiwit gedreven moeten worden door de membraanpotentiaal. In onze experimenten leidt het toevoegen van

valinomycine aan CorA proteoliposomen, geladen met kaliumchloride, tot een snelle uitstoot van kalium ionen en een toename van de membraan potentiaal, en een toename in het transport van divalente kationen. In eenzelfde setup met EcZntB proteoliposomen werd geen toename in zink opname waargenomen – een verdere sterke aanwijzing dat ZntB een transporteiwit is. Samenvattend: CorA en ZntB hebben verschillende transport mechanismes.

Hoofdstuk 5 is een review, voornamelijk gericht op onderzoekers voor wie structurele biologie een nieuw veld is. Ik beschouw de belangrijkste eigenschappen van de meest gebruikte detergentia in structuuronderzoek aan membraaneiwwitten - hun kritische micel concentratie (CMC), micel grootte en aggregatie nummer - en geef ideeën voor het kiezen van een goed eerste detergens in dit soort studies. Ook bespreek ik kort alternatieve agentia voor het solubiliseren en stabiliseren van membraaneiwwitten, en geef ik statistieken voor het actuele gebruik van verschillende detergentia die bij gepubliceerde studies zijn toegepast.

Perspectieven

Dit proefschrift beschrijft voor het eerst de volledige structuur van ZntB en toont nieuwe details over het transport mechanisme van dit membraaneiwit; toch blijven er nog veel onbeantwoorde vragen. Ten eerste is het precieze transport mechanisme nog onduidelijk. Er is meer onderzoek nodig om de rol van ZntB in zink homeostase in bacteriën volledig te begrijpen. Hoge resolutie structuren van ZntB met gebonden substraat zijn hierbij essentieel. De rol van aminozuur residuen V289, R191 en R198 zijn nog onduidelijk; hoewel het zeker is dat ze een belangrijke rol bij het transport spelen, is het met de huidige kennis moeilijk te interpreteren wat die rol precies is.

Op het niveau van de eiwitfamilie zijn er nog meer onbeantwoorde vragen. Sommige leden, zoals CorA en Mrs2 zijn geëvolueerd tot magnesium kanalen, terwijl anderen zoals ZntB en waarschijnlijk ook Alr transport-eiwitten werden; de fysiologische consequenties van deze verschillen zijn onduidelijk. Hoewel CorA het meest bestudeerde lid van de 2-TM-GxN familie is, is ook het transportmechanisme van dit eiwit nog niet duidelijk. Ook met onze data blijft de vraag of CorA *in vivo* een rol in zink transport speelt, aangezien de K_m voor zink vergelijkbaar is met die van ZntB. Een andere intrigerende vraag omtrent CorA betreft de wijze van binding van CoHex aan deze eiwitten. Enkele experimenten laten zien dat CoHex een sterke remmer is voor CorA, maar in onze experimentele setup zien we geen significante remming, zelfs bij een concentratie die het honderdvoudige is van de K_m waarde. Een structuur van CorA met CoHex gebonden is dan ook zeer gewenst om hierover uitsluitsel te geven. Uiteindelijk zijn hoge resolutie structuren en gedegen biochemische karakterisatie van ZntB, CorA en andere leden van de 2-TM-GxN familie nodig om deze vragen te beantwoorden, en het transport van ionen volledig te kunnen begrijpen.

Dit proefschrift beschrijft fundamenteel onderzoek aan bacteriële en archaeële ZntB en CorA eiwitten; het ontrafelen van hun transport mechanismen kan echter ook bijdragen aan kennis over hun eukaryote homologen – Mrs2 en Alr eiwitten. Er zijn aanwijzingen voor een rol van Mrs2 in het verhogen van multidrug-resistentie in maagkankercellen; Mrs2 zou dus een doel-eiwit kunnen zijn voor het ontwikkelen van een therapie tegen maagkanker. In het geval van Alr eiwitten is aangetoond dat ze essentieel zijn voor de ontwikkeling van virulentie van schimmel-ziekteverwekkers; deze eiwitten kunnen dus potentiële doelen zijn voor het

ontwikkelen van anti-schimmel middelen. Kortom, leden van de 2-TM-GxN familie bieden mogelijkheden voor het ontwikkelen van nieuwe medicijnen.

Выводы

Цинк необходим для всех форм жизни и является вторым по распространенности двухвалентным катионом переходных металлов в живых организмах после железа. Поскольку ионы металлов не могут быть синтезированы, все живые организмы обладают набором мембранных белков для транспорта катионов металлов из окружающей среды. Однако во многих случаях избыток металлов токсичен, поэтому есть строгая необходимость в мембранных белках, способных выводить ненужные ионы. Чтобы полностью понять эти процессы транспорта металлов в клетку и из неё, важно решить структуры мембранных белков в присутствии и отсутствии субстратов, а так же охарактеризовать их биохимически, чтобы понять механизмы транспорта металлов. Эта диссертация расширяет наше понимание транспорта цинка и некоторых других ионов металлов через белок ZntB и других белков семейства 2-TM-GxN, которые присутствуют в каждом домене жизни. В **Главе 1** я обобщил структурные и биохимические данные о семействе 2-TM-GxN и её роли в поддержании гомеостаза магния, цинка, никеля и кобальта во всех организмах.

В **Главе 2** я описываю структуру транспортера ZntB в отсутствие субстрата. Транспорт ZntB был охарактеризован с помощью анализа связывания лиганда и транспортного анализа в протеолипосомах, который продемонстрировал, что ZntB действительно способен транспортировать цинк. Суммируя все имеющиеся данные, полученные во время работы над этой диссертацией, а так же другими группами, был сделан вывод, что ZntB является импортером цинка, который может быть простимулирован градиентом протонов. Его транспортный

механизм отличается от известного гомологичного магниевого канала CorA. Кроме того, мы показали, что ZntB не принимает асимметричную форму в отсутствии субстрата, в отличие от CorA в аналогичных условиях.

В Главе 3 я описываю две структуры ZntB в присутствии его субстратов - цинка и кадмия. Из-за низкого разрешения обеих структур их невозможно использовать для интерпретации транспортного механизма ZntB. Однако в этой главе я привожу убедительные доказательства того, что аминокислота D253, скорее всего, участвует в связывании транспорта Zn^{2+} и H^+ , поскольку мутация, введенная в этой позиции, приводит к разъединению симпорта (парного транспорта) H^+ / Zn^{2+} и потере чувствительности к градиенту протонов. Я также изучил влияние консервативных аргининов 191 и 198 в транспортном механизме, но их роль трудно интерпретировать на основе полученных результатов.

В Главе 4 я привожу дополнительные доказательства различий в транспортном механизме между белками CorA и ZntB. Первое отличие состоит в том, что белки CorA нечувствительны к градиенту протонов в отличие от ZntB. В протеолипосомах EcZntB в условиях одинакового pH внутри и снаружи краситель АСМА реагирует на аккумуляцию протонов при транспорте Zn^{2+} , и флуоресценция гасится. В случае TmCorA и MjCorA совместного транспорта H^+ по-видимому нет, потому что флуоресценция остается на том же уровне, что и в контрольном эксперименте. Если CorA является каналом, то транспорт Mg^{2+} через белки CorA должен управляться мембранным потенциалом. В нашем эксперименте добавление валиномицина к протеолипосомам CorA в присутствии хлорида калия, приводит к быстрому выходу ионов калия

наружу и наращиванию мембранного потенциала с усилением транспорта двухвалентных катионов. Протеолипосомы с EcZntB не показали какого-либо увеличения транспорта цинка, что добавляет еще один убедительный довод, что ZntB является транспортером. Обобщая все эти выводы, я предлагаю гипотезу, что CorA и ZntB имеют разные транспортные механизмы.

Глава 5 представляет собой обзор, в основном предназначенный для исследователей, которые только начинают свои исследования в области структурной биологии. В этой работе я рассматриваю основные свойства наиболее часто используемых детергентов в структурных исследованиях, их значение критической концентрации мицелл (СМС), размер мицелл, число агрегации и даю совет, какие детергенты являются хорошим начальным выбором для структурной биологии мембранных белков. Кроме того, я кратко обсуждаю альтернативные солюбилизирующие и стабилизирующие агенты и предоставляю статистические данные о фактическом использовании различных детергентов на основе решенных структур.

Перспективы

Хотя эта диссертация описывает первую полную структуру ZntB и новые подробности о его транспортном механизме, много вопросов остаются открытыми. Прежде всего, транспортный механизм ZntB все еще неясен до конца. Необходимы дополнительные исследования, чтобы полностью понять роль ZntB в гомеостазе цинка в бактериях. Структуры ZntB с более высоким разрешением со связанным субстратом необходимы для полного понимания механизма. Также роли аминокислот V289 и R191, R198 остаются неясными. Нет сомнений в

том, что они играют важную роль в транспорте, но трудно интерпретировать их роль на основе имеющихся данных.

Если мы пойдем дальше, на уровень семейства, у нас появляется еще больше открытых вопросов. Некоторые представители, такие как CofA и Mrs2, эволюционировали в магниевые каналы, тогда как другие, такие как ZntB и, вероятно, Alr белки, стали транспортерами, но физиологические причины этих различий в механизме неясны. Несмотря на то, что CofA является наиболее изученным членом семейства 2-TM-GxN, его транспортный механизм также все еще спорен. Основываясь на наших данных, появляется вопрос, может ли CofA участвовать в транспорте цинка *in vivo*, так как его K_m для цинка схож с K_m для цинка ZntB. Другим интригующим вопросом для CofA является вопрос каким же образом с ним связывается CoHex. Есть несколько экспериментов, демонстрирующих, что CoHex является сильным ингибитором для CofA, но в наших экспериментах мы не видим существенного ингибирования вплоть до в сто раз более высокой концентрации, чем опубликованная величина K_m для CoHex. Следовательно, структура CofA с CoHex крайне желательна для решения этого вопроса. Решение структур с высоким разрешением, а также тщательная биохимическая характеристика ZntB, CofA и других членов 2-TM-GxN необходимы, чтобы ответить на все эти вопросы и полностью понять транспорт ионов в этом семействе белков.

Эта диссертация описывает фундаментальные исследования белков ZntB и CofA в бактериях и археях, однако изучение их транспортных механизмов может также улучшить наши знания об их эукариотических гомологах - белках Mrs2 и Alr. Имеются данные о роли Mrs2 в стимулировании множественной лекарственной устойчивости в

раковых клетках желудка, поэтому Mrs2 может быть потенциальной мишенью для реверсивной терапии. Было показано, что белки Alr необходимы для развития и вирулентности грибковых патогенов, поэтому они могут быть потенциальными мишенями для разработки новых противогрибковых агентов. Следовательно, члены семейства 2-TM-GxN могут быть потенциальными мишенями для разработки новых лекарств.

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Artem