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Loss of cytosolic Mg^{2+} binding sites in the *Thermotoga maritima* CorA Mg^{2+} channel is not sufficient for channel opening

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Abstract

The CorA Mg^{2+} channel is a homopentamer with five-fold symmetry. Each monomer consists of a large cytoplasmic domain and two transmembrane helices connected via a short periplasmic loop. In the *Thermotoga maritima* CorA crystal structure, a Mg^{2+} is bound between D89 of one monomer and D253 of the adjacent monomer (M1 binding site). Release of Mg^{2+} from these sites has been hypothesized to cause opening of the channel. We generated mutants to disrupt Mg^{2+} interaction with the M1 site. Crystal structures of the D89K/D253K and D89R/D253R mutants, determined to 3.05 and 3.3 Å, respectively, showed no significant structural differences with the wild type structure despite absence of Mg^{2+} at the M1 sites. Both mutants still appear to be in the closed state. All three mutant CorA proteins exhibited transport of $^{63}Ni^{2+}$, indicating functionality. Thus, absence of Mg^{2+} from the M1 sites neither causes channel opening nor prevents function. We also provide evidence that the *T. maritima* CorA is a Mg^{2+} channel and not a Co^{2+} channel.

Keywords

Tm CorA; D89K/D253K; D89R/D253R; crystal structures; closed; functional; magnesium; channel; transport

1. Introduction

Mg^{2+} is ubiquitous and plays an important role in many cellular activities. It functions as a cofactor for many enzymes and is necessary for maintenance of genomic stability. Problems in Mg^{2+} homeostasis can cause several diseases (1, 2). Thus, cytosolic concentrations of divalent cations such as Mg^{2+} must be regulated to maintain proper cell health. In about half of bacteria and archaea, the CorA Mg^{2+} channel is primarily responsible for Mg^{2+} homeostasis (3–5). The eukaryotic homolog of CorA is the Mrs2 Mg^{2+} channel of the

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Author Contributions

TK performed KK protein purification, crystallization and KK and RR structure determination. MEM performed transport experiments. TK and MEM wrote the paper.

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mitochondrion and is an essential gene (6). The CorA of all bacteria tested can also transport Co^{2+} and Ni^{2+} (3, 7).

The first X-ray structure of a divalent ion channel was that of CorA from the thermophilic Gram negative marine bacterium *Thermotoga maritima* (Tm CorA) (8). Tm CorA is a homopentamer with five-fold symmetry about a central pore. Each monomer consists of a large cytoplasmic domain, two transmembrane helices connected via a short periplasmic loop and a very short highly conserved C-terminal tail. The soluble cytoplasmic domain of each monomer consists of seven antiparallel β -strands surrounded by six α -helices. A long seventh helix termed the “stalk helix” kinks upon entering the membrane and becomes the first transmembrane domain (TM1). It contains the highly conserved “YGMNF” motif at the periplasmic end (8–10), which has been proposed to function as a selectivity filter (9, 11). Mutations of residues within that cluster block Mg^{2+} transport (10, 12–13). In most published structures, the periplasmic loop distal to the YGMNF motif is disordered, probably due to its flexibility (8–10).

Crystal structures of Tm CorA show several Mg^{2+} ions bound to the large cytosolic domain. A Mg^{2+} is located between D89 of the $\alpha 3$ helix of one monomer and D253 of the $\alpha 7$ stalk helix of the neighboring monomer in all Tm CorA structures (M1 binding site) (8–10, 14). An additional Mg^{2+} ion interaction within each monomer has been observed in vicinity of L12, E88, D175 and D253 in some structures (M2 binding site) (9–10, 14–16).

All published Tm CorA crystal structures are of the closed state of the channel (8–10). Two possibly open state Tm CorA cryo-EM structures of low resolution ($\sim 7 \text{ \AA}$) have recently been reported (14). X-ray crystallography, electrostatic analysis, molecular dynamics simulation and cryo-EM studies have proposed that Mg^{2+} binding to the M1 cytosolic binding sites is responsible for the closed form of CorA (8–10, 14–20) and may help keep the N-terminal cytosolic domains together (9). Mechanistically, it has been proposed that Mg^{2+} dissociation from the M1 site due to low intracellular Mg^{2+} concentration initiates significant conformational changes involving the long stalk helices and the first transmembrane domain leading to a subsequent opening of the pore, allowing Mg^{2+} influx (8–10, 14–20). Conversely, as intracellular Mg^{2+} increases, the five M1 sites are proposed to fill, closing the channel.

In this study, we generated both the D253K Tm CorA as well as double mutants by changing both D89 and D253 to either K or R, respectively (denoted KK-CorA and RR-CorA) to disrupt the M1 Mg^{2+} binding site. The KK-CorA and RR-CorA mutants were expressed, purified, and crystallized. Their structures were solved to 3.05 and 3.3 $\text{\AA}$ resolution, respectively. Neither the KK-CorA nor the RR-CorA crystal structures have Mg^{2+} ions bound to the M1 site.

Further, no major conformational changes of the cytosolic domains, stalk helices and first transmembrane domains could be observed in either structure. Both mutants represent a closed form of the channel. Moreover, the D253K Tm CorA, KK-CorA and RR-CorA channels are able to transport $^{63}\text{Ni}^{2+}$, indicating that they are functional channels. Our results indicate that loss of Mg^{2+} interaction at the M1 cytosolic binding site does not cause

significant structural conformational changes in the Tm CorA structure and does not prevent channel function. Thus, the absence of Mg^{2+} at the M1 site does not automatically cause channel opening nor is Mg^{2+} binding to the M1 site required to close an open channel.

2. Results

2.1. Overall structures.

Both KK-CorA and RR-CorA structures share the same overall fold as the wild type structures (Figures 1, 2, and Supplemental Figure 1). In both mutant structures one pentamer is present in each asymmetric unit. Superposition of KK-CorA and wild type (PDB ID 2IUB) yields an rmsd of 1.0 Å (Figure 1). When RR-CorA and wild type (PDB ID 4I0U) are superimposed, the rmsd value is 1.4 Å (Figure 2). Superposition of the mutant structures with each other gives an rmsd of 0.9 Å (Supplemental Figure 1). Superposition of either structure with other published wild type Tm CorA structures yielded similar results. Superposition of only the stalk helices, the transmembrane domains and C-termini of KK-CorA and RR-CorA with the wild type gives rmsd values of 0.7 Å and 0.8 Å, respectively. Aligning the same structural parts of KK-CorA and RR-CorA to each other yields an rmsd of 0.5 Å. Clearly, the loss of the M1 site in these mutants causes no significant structural changes in the stalk helix and membrane domain. Further, manual inspection of the entire superimposed structures revealed no significant differences. Thus, the mutant Tm CorA proteins displayed essentially the same structure as the wild type (Figures 1, 2, and Supplemental Figure 1).

In RR-CorA but not the KK-CorA structure, the periplasmic loop is resolved. There are only small conformational differences with the wild type periplasmic loop (PDB ID 4I0U). Superposition of these regions of RR-CorA, with a recently published Tm CorA cryo EM structure in its closed state (PDB ID 3JCF) also shows some small differences. Such conformational differences are not surprising as this part of the channel is very flexible (8–10, 14–15).

In both mutant structures there are some minor shifts of initial residues of the stalk helices of two monomers away from the permeation tunnel (chains A and B in KK-CorA and B and C in RR-CorA). The main chain carbons (CAs) of F251 of chains B and C of RR-CorA and wild type are located 5.1 and 5.6 Å from each other, respectively (Figure 3). Distances between main chains of the same residue of subunits A and B are 4.0 and 4.6 Å, respectively, when KK-CorA and wild type are aligned to one another (Supplemental Figure 2). However, these small movements do not propagate further to the stalk helices or the TM1 pore forming domains (Figure 3 and Supplemental Figures 2 and 3).

2.2. Mg^{2+} binding at the M1 and M2 sites in KK-CorA and RR-CorA.

The mutated M1 site residues K89 and K253 as well as M2 site amino acids (E88, D175) are well resolved in some of the subunits of the KK-CorA structure (Figure 4(A) and Supplemental Figure 4). There were no Mg^{2+} ions bound at the M1 sites. In RR-CorA, R89 and D175 side chains are well resolved in most monomers, whereas E88 and R253 side chains are disordered in all five subunits (Figure 4(B) and Supplemental Figure 5). There is

likewise no electron density for Mg^{2+} in any of the five M1 sites either. In the RR-CorA structure, three of the five M2 sites are occupied with a Mg^{2+} ion (Supplemental Figure 5). B factors for those divalent cations range from 105 to 116. In KK-CorA only one Mg^{2+} could be modelled into an M2 site (Supplemental Figure 4, B factor 103).

Alignment of M1 and M2 site residues of KK-CorA and RR-CorA with the wild type structure shows that all of those residues, except the side chains of E88, are at the same position in all three structures disregarding the mutations (Supplemental Figure 6). In KK-CorA, the side chain of E88 has moved 2 to 3 Å towards the initial N-terminal loop, depending on the subunit. In the case of RR-CorA there is no such shift except in subunit E where a motion of 2.2 Å was observed.

2.3. Other Mg^{2+} binding sites in KK-CorA and RR-CorA.

In both mutant structures, a Mg^{2+} ion was observed at the pore entry (Supplemental Figure 4(A) and 4(B), Supplemental Figure 5(A) and 5(B)). A divalent cation occurs at about the same position in other published Tm CorA structures as well (14–16). It has been proposed that this Mg^{2+} ion could be (partially) hydrated as its location is about 4 Å away from the carbonyl groups of G312 and the side chains of N314 of the YGMNF motif within the pore (15, 16). Similar distances were observed in RR-CorA. In KK-CorA, however, it was not possible to define distances as the YGMNF motif and the loop are somewhat disordered.

Within the cytosolic permeation tunnel, Mg^{2+} ions have been reported near S284 and also near D277 (9–10, 14–15). There is a Mg^{2+} inside the permeation tunnel in both the KK-CorA and the RR-CorA mutant near D277 (Supplemental Figures 4(C) and 5(C)).

2.4. Cell growth and expression assay.

A cell growth assay using the Mg^{2+} -transporter deficient *Salmonella typhimurium* strain MM281 complemented with Tm CorA wild type and the D253K-CorA, KK-CorA and RR-CorA mutants was carried out in minimal media supplemented with different concentrations of Mg^{2+} . Protein expression levels for wild type and each mutant were at similar levels (Supplemental Figure 7).

The Mg^{2+} concentration required for growth was greater for all three mutants than for wild type (Figure 5) with the D253K-CorA growing only weakly.

2.5. $^{63}\text{Ni}^{2+}$ Transport by wild type and mutant Tm CorA.

Since $^{28}\text{Mg}^{2+}$ is not available, $^{63}\text{Ni}^{2+}$ was used to measure uptake by a previously published protocol (3, 7, 12, 13). Wild type Tm CorA exhibited uptake of $^{63}\text{Ni}^{2+}$ (Figure 6). Mg^{2+} inhibition gave an apparent K_i of 300 μM , about 20-fold greater than that for the *Salmonella typhimurium* CorA. A poorer affinity for Mg^{2+} is logical for Tm CorA since it is a marine organism and lives in Mg^{2+} concentrations up to 55 mM. The D253K-CorA, KK-CorA and RR-CorA channels all exhibited substantial transport of $^{63}\text{Ni}^{2+}$, at a level comparable to wild type Tm CorA (data not shown), and all exhibited a comparable apparent K_i for Mg^{2+} of about 300 μM (Figure 6).

The selective inhibitor of other CorA channels, Co(III) hexaammine, was a relatively poor inhibitor of the Tm CorA with an apparent K_i about 100-fold greater than its K_i for *Salmonella typhimurium* CorA (Supplemental Figure 8). Similar to the *Salmonella typhimurium* CorA, Co^{2+} inhibited uptake with an apparent affinity similar to that of Mg^{2+} . Finally, again as with the *Salmonella typhimurium* CorA (7), Mn^{2+} gave incomplete, likely non-competitive inhibition (Supplemental Figure 9).

3. Discussion

Here we report two structures of *Thermotoga maritima* CorA, denoted KK-CorA and RR-CorA, where the M1 Mg^{2+} binding residues D89 and D253 were mutated to K and R, respectively. It has been proposed by several groups that Mg^{2+} at the M1 and possibly the M2 sites are responsible for the closed state of the channel. Removal of these cations is proposed to lead to conformational changes in the cytosolic domain propagating to the pore forming TM1 helices, resulting in an open channel (8–10, 14–20). Our structures, however, do not show any significant structural differences to the wild type and still seem to represent the closed, non-conductive form of the channel. In the wild type-structure, charge repulsion between the acidic residues between monomers due to loss of bound Mg^{2+} could take place and theoretically lead to conformational changes to open the channel (10).

In KK-CorA, the lysine side chains point away from each other, thus avoiding charge repulsion. In RR-CorA, the larger size of the side chains makes charge repulsion more likely as indicated by their high flexibility, particularly of the sidechain of 253R. However, in neither structure does the loss of Mg^{2+} or possible charge repulsion cause any major structural changes. The guanidinium moieties of R89 and R253 may also interact favorably and be stabilized by surrounding negatively charged residues (E88, E100, D175). However, the disordered state of the guanidinium moieties suggests that such stabilization would be very weak and contribute little to pentamer stability. Even in the absence of bound cytosolic Mg^{2+} , CorA is able to assemble into a pentamer and to fold appropriately within the membrane.

We conclude that the stability of the pentamer does not require bound Mg^{2+} at either the M1 site (KK-CorA and RR-CorA structures) or the M2 site (KK-CorA structure). Salt bridges between R89 and D256 and R253 and E88 (between two monomers) might also contribute to a weaker stabilization of the pentamer and the closed state of RR-CorA. In KK-CorA, distances between K89 and D256 and K253 and E88 are more than 4 Å, however, due to the coordinate error at that resolution, distances might be about 4 Å and therefore it is also possible that ionic interactions between those residues are formed. It was reported that the Tm CorA D253K mutant was more stable than the wild-type protein due to salt bridge formation with D89 (10). The same scenario is very likely by mutating only D89 to K which could then form a salt bond with D253 (16). While it is also possible that support for the pentameric assembly derives from crystal contacts and nonspecifically bound Mg^{2+} because both proteins were crystallized in 0.2 M Mg^{2+} , we do not think this is likely. The truncated cytosolic domain spontaneously forms a pentamer under multiple conditions (8). Likewise, the isolated membrane domain spontaneously forms a pentamer in lipid vesicles with and without Mg^{2+} (21).

In the RR-CorA structure, in three of the five M2 sites, Mg^{2+} ions were modelled (Supplemental Figure 5), whereas in KK-CorA only one Mg^{2+} could be modelled at an M2 site (Supplemental Figure 4). Moreover, it is hard to distinguish between waters and Mg^{2+} ions at those positions in the difference maps. Finally, there is no electron density for either water or Mg^{2+} at three M2 sites (between monomers A-D, B-A and E-C) in KK-CorA and at one M2 site (between monomers B-A) in RR-CorA. Thus, it is very likely, that Mg^{2+} at the M2 sites do not replace the role of Mg^{2+} at the M1 site.

Although the structures of both mutants are closed, both are nonetheless capable of opening to form a functional Mg^{2+} channel as shown by significant levels of cation transport, measured using $^{63}Ni^{2+}$. Moreover, the apparent affinity of Mg^{2+} for Tm CorA is unchanged in the mutants.

This is reasonable since apparent affinity would be dominantly determined by the initial interaction of Mg^{2+} with the extracellular pore (13) and/or with the conserved YGMNF motif. The structure of these elements of CorA is unchanged in our structures of KK-CorA and RR-CorA.

A recently published model for channel opening suggests removal of a Mg^{2+} from an M1 site leads to a disruption of contacts between two monomers of the pentamer when the Mg^{2+} concentration inside the cell decreases. This leads to a further sequential removal of Mg^{2+} ions between the remaining protomers and consequently to their disruption to create an open conformation of the CorA channel (14). The complete absence of Mg^{2+} ions in the M1 sites of KK-CorA and RR-CorA structures does not support this model.

An alternative proposed mechanism involves a concerted iris-like movement of the monomers, causing a larger pore diameter as a result of inward movements of the $\alpha 7$ helices that propagate to the TM1 domains when Mg^{2+} is absent from the M1 site (17,19). More complex movements such as radial tilts and lateral motions have also been proposed (15, 16). That none of those movements could be observed in KK-CorA or RR-CorA structures implies that the closed conformation of the channel is stable even in the absence of bound Mg^{2+} .

Molecular dynamics simulation studies proposed that upon removal of Mg^{2+} from the M1 site, structural changes of the YGMNF motif occur, loosening the binding of hexahydrated Mg^{2+} to its second shell ligands G312 and N314 of that highly conserved region. A complete loss of divalent cation binding to the GMN could occur (20).

However in both KK-CorA and RR-CorA, a Mg^{2+} ion is still bound adjacent to the GMN motif (Supplemental Figures 4 and 5), even in the complete absence of M1 site bound Mg^{2+} . We conclude that both the KK-CorA and RR-CorA structures represent a closed state of the channel.

Our transport data clearly show transport for the various Tm CorA mutants. Therefore, binding of Mg^{2+} to the cytosolic binding sites is not required for channel opening or closure. Moreover, the apparent affinity of D253K-CorA, KK-CorA and RR-CorA for external Mg^{2+}

is unchanged compared to wild type, indicating that presence or absence of Mg^{2+} at the M1 site does not affect apparent affinity.

Finally, it has been argued, based on indirect growth inhibition data in *S. typhimurium* expressing Tm CorA, that Tm CorA is a Co^{2+} channel rather than a Mg^{2+} channel (16, 20). This interpretation is problematic for multiple reasons. First, the extracellular concentration of Co^{2+} required to induce growth inhibition is the result of a multitude of intracellular interactions of transported Co^{2+} , only one of which is its apparent affinity for Tm CorA. A direct comparison of the concentrations of Mg^{2+} and Co^{2+} that alter growth cannot be used to infer affinity for CorA. Second, it is unclear why *T. maritima* would need a specialized Co^{2+} uptake system, since each cell would only require a miniscule amount of Co^{2+} . This amount could easily be supplied by “leakage” through the CorA channel. Third, the apparent affinity of Co^{2+} for actual transport by Tm CorA is approximately equal to that of Mg^{2+} , indicating no preference of Tm CorA for either cation (Figure 6 and Supplemental Figure 9). Given the affinity of Co^{2+} for TmCorA, should environmental Co^{2+} be sufficiently high to enter through TmCorA, it would be immediately toxic to the cell.

Finally, there is the simple matter of basic physiology. *Thermotoga maritima*, as the name implies, is a marine organism. It lives in seawater which normally contains 55 mM Mg^{2+} although this may be lower in effluent from deep sea vents (22). For Co^{2+} to effectively compete with Mg^{2+} for CorA, a Co^{2+} concentration of at least molar would be required. This is not the case, since Co^{2+} concentrations in deep sea vents are in the nanomolar range (22).

Although the KK-CorA and RR-CorA channels transport quite well, neither functions normally in the intact cell. Growth of the Mg^{2+} channel deficient *S. typhimurium* strain MM281 could only be initiated at higher Mg^{2+} concentrations when complemented with these Tm CorA mutants. Thus, Mg^{2+} binding to the M1 site still plays some important role in controlling cellular Mg^{2+} homeostasis. Detailed electrophysiological characterization of CorA will be required to elucidate the role of the cytosolic Mg^{2+} sites.

4. Materials and Methods

4.1. Cloning, site directed mutagenesis, expression and purification of *T. maritima* CorA D89K/D253K and D89R/D253R mutants.

The coding sequence of *T. maritima* CorA was cloned into a modified version of the T7 polymerase expression vector pET-15b (Novagen, Madison, WI, USA) containing an N-terminal six-histidine tag and a TEV protease cleavage site using BamHI and NdeI as restriction sites. Site directed mutagenesis was performed using the QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA) with forward and reverse primers shown in Supplemental Table 1. Mutant proteins were expressed in *E. coli* C41 (DE3) (Lucigen, Middleton, WI, USA) grown in 0.75 L LB media containing ampicillin at a concentration of 100 μ g/mL. Cultures were grown at 200 rpm at 37°C until an OD₆₀₀ of about 0.6, then IPTG (Roche) was added to a final concentration of 0.5 mM and expression continued for about 15 hours at 28°C. After harvesting, cells were resuspended in lysis buffer (50 mL per 3.5 g wet cell paste; 50 mM HEPES, pH 7.5, 350 mM NaCl, 10 mM

imidazole, 0.5 mM DTT) containing 10 mM MgSO₄, 12.5 mg lysozyme, 0.5 mg DNase and one cOmplete EDTA free Protease Inhibitor Cocktail tablet (Roche, Pleasanton, CA, USA) per 50 mL. Cells were lysed by two passes through a microfluidizer (Microfluidics M-110Y Microfluidizer, Westwood, MA, USA).

The crude lysate was spun at 3,000 rpm at 4°C for 20 min. The supernatant was collected and an additional centrifugation step at 36,000 rpm at 4°C for 20 min was performed. The pellet was resuspended in lysis buffer containing 1% n-dodecyl- β -D-maltoside (DDM, Anatrace, Maumee, OH, USA) and gently stirred for about 15 hours at 4°C. After centrifugation at 36,000 rpm at 4°C for 20 min, the supernatant was applied by gravity onto a column containing 2.5 mL Ni-NTA agarose (Qiagen, Germantown, MD, USA) pre-equilibrated in lysis buffer. After an extensive wash step with buffer 2 (50 mM HEPES, pH 7.5, 350 mM NaCl, 40 mM imidazole, 0.5 M DTT, 0.025% DDM), mutant proteins were eluted in buffer 3 (50 mM HEPES, pH 7.5, 350 mM NaCl, 200 mM imidazole, 0.5 M DTT, 0.025% DDM).

The N-terminal six-histidine tag was cleaved by adding 1mg TEV protease per 20 mg target protein and dialyzing in buffer 4 (50 mM HEPES, pH 7.5, 100 mM NaCl, 0.5 mM DTT) for about 15 hours at 4°C. After concentrating using a centrifugal filter device (Millipore, 100 kDa molecular weight cutoff, Billerica, MA, USA) proteins were applied onto a HiLoad™ 16/600 Superdex™ 200 pg size exclusion column (GE Healthcare, Cleveland, OH, USA) and eluted in buffer 5 (50 mM HEPES, pH 7.5, 100 mM NaCl, 0.5 mM DTT, 0.025% DDM). Peak fractions were pooled and concentrated to 4–8 mg/mL using a centrifugal filter device. Purified concentrated protein was aliquoted into microcentrifuge tubes, flash frozen in liquid nitrogen and stored at –80°C until further use.

4.2. Crystallization, structure solution and refinement.

Initial crystallization trials of Tm CorA D89K/D253K and D89R/D253R in the presence of many different divalent cations (Supplemental Table 2) yielded either no crystals or crystals of poor quality.

However, after extensive optimization, well diffracting crystals of KK-CorA and RR-CorA were obtained within 5–7 days by vapor diffusion (2 μ L protein + 2 μ L precipitant (hanging drop; KK-CorA) or 1 μ L protein + 0.5 μ L Silver Bullets optimization screen (Hampton Research, Aliso Viejo, CA, USA) + 0.5 μ L precipitant (sitting drop; RR-CorA) against a reservoir containing 500 μ L precipitant (0.2 M Mg(HCO₂)₂, 0.1 M MES, pH 6.0, 25% PEG 400 (KK-CorA); 0.2 M MgCl₂, 0.1 M MES pH 6.0, 25% PEG 400 (RR-CorA)).

These crystals were also soaked extensively with a variety of organic and inorganic di- and trivalent anions in an attempt to form a salt bridge between the lysine or arginine residues substituted for D89 and D253. In no case were we able to detect anions between the mutated residues.

Diffraction data were collected at 100K at a wavelength of 0.9792 Å on beamlines 24-ID- (RR-CorA) and 24-ID-E (KK-CorA) at the APS, Argonne, IL, USA. Data from one KK-CorA and one RR-CorA crystal were collected to 3.05 and 3.3 Å, respectively. Indexing and

merging of the data was carried out using XDSGUI as a front end to XDS/XSCALE/XDS CONV (23). Both structures were solved using Phaser (24). A pentamer of an already solved Tm CorA structure (PDB IDs 2IUB for KK-CorA and 4I0U for RR-CorA) was used as a search model with all ligands and water molecules removed (1,10). In both cases a solution was found for one pentamer in the asymmetric unit. Both structures were refined using REFMAC (25). Isotropic B-factors, local NCS restraints and TLS parameters were applied throughout refinement.

Manual adjustments including adding water molecules and Mg^{2+} ions were performed using COOT (26). The final models were checked and validated using MOLPROBITY (27). Both structures were deposited to the RCSB Protein Data Bank (PDB IDs 5JTG (KK-CorA) and 5JRW (RR-CorA). Data and refinement statistics are shown in Supplemental Table 3. Figures were prepared using Pymol (28).

4.3. Other assays.

Growth assays and uptake of $^{63}Ni^{2+}$ were performed as previously described (7, 12, 13, 29–31). Uptake of $^{63}Ni^{2+}$ was linear for between 15 and 20 min (Supplemental Figure 10). To ensure first order kinetics, uptake was measured after 10 min for all dose response curves.

Antibodies to Tm CorA were raised in rabbits by injection of purified Tm CorA (MP Biomedicals, Solon, OH, USA). Immunoblotting was performed at a dilution of 1:10,000 with protein visualized by enhanced chemiluminescence (ECL, Amersham, GE Healthcare, Cleveland, OH, USA).

Oligonucleotides were obtained from Sigma, St. Louis, MO, USA. Supplemented N-minimal medium (N-Min) (32) contained 0.1% casamino acids and 0.1 mM arabinose. $MgSO_4$ was used when medium was supplemented with Mg^{2+} . The concentrations of antibiotics used were 50 $\mu g/ml$ ampicillin, 20 $\mu g/ml$ chloramphenicol, and 50 $\mu g/ml$ kanamycin.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Tm CorA	Thermotoga maritima CorA
KK-CorA	D89K/D253K Tm CorA
RR-CorA	D89R/D253R Tm CorA

References

1. Kolte D, Vijayaraghavan K, Khera S, Sica DA, Frishman WH (2014) Role of magnesium in cardiovascular diseases. *Cardiology in Review* 22:182–192. [PubMed: 24896250]
2. de Baaij JH, Hoenderop JG, Bindels RJ (2015) Magnesium in man: implications for health and disease. *Physiological Reviews* 95:1–46. [PubMed: 25540137]
3. Hmiel SP, Snavely MD, Florer JB, Maguire ME, and Miller CG (1989) Magnesium transport in *Salmonella typhimurium*: genetic characterization and cloning of three magnesium transport loci. *Journal of Bacteriology* 171:4742–4751. [PubMed: 2548998]
4. Papp-Wallace KM, and Maguire ME (2007) Bacterial homologs of eukaryotic membrane proteins: the 2-TM-GxN family of Mg^{2+} transporters. *Molecular Membrane Biology* 24:351–356. [PubMed: 17710639]
5. Knoop V, Groth-Malonek M, Gebert M, Eifler K, and Weyand K (2005) Transport of magnesium and other divalent cations: evolution of the 2-TM-GxN proteins in the MIT superfamily. *Molecular Genetics and Genomics* 274:205–216. [PubMed: 16179994]
6. Piskacek M, Zotova L, Zsurka G, and Schweyen RJ. (2009) Conditional knockdown of hMRS2 results in loss of mitochondrial Mg^{2+} uptake and cell death. *J. Cell Mol. Med* 13:693–700. [PubMed: 18384665]
7. Snavely MD, Florer JB, Miller CG, and Maguire ME (1989) Magnesium transport in *Salmonella typhimurium*: $^{28}Mg^{2+}$ transport by the CorA, MgtA, and MgtB systems. *Journal of Bacteriology* 171:4761–4766. [PubMed: 2670893]
8. Lunin VV, Dobrovetsky E, Khutoreskaya G, Zhang R, Joachimiak A, Doyle DA, Bochkarev A, Maguire ME, Edwards AM, and Koth CM (2006) Crystal structure of the CorA Mg^{2+} transporter. *Nature* 440:833–837. [PubMed: 16598263]
9. Eshaghi S, Niegowski D, Kohl A, Martinez Molina D, Lesley SA, and Nordlund P (2006) Crystal structure of a divalent metal ion transporter CorA at 2.9 angstrom resolution. *Science* 313:354–357. [PubMed: 16857941]
10. Payandeh J, and Pai EF (2006) A structural basis for Mg^{2+} homeostasis and the CorA translocation cycle. *EMBO Journal* 25:3762–3773. [PubMed: 16902408]
11. Palombo I, Daley DO, and Rapp M (2012) The periplasmic loop provides stability to the open state of the CorA magnesium channel. *Journal of Biological Chemistry* 287:27547–27555. [PubMed: 22722933]
12. Szegegy MA, and Maguire ME (1999) The CorA Mg^{2+} transport protein of *Salmonella typhimurium*. Mutagenesis of conserved residues in the second membrane domain. *Journal of Biological Chemistry* 274:36973–36979. [PubMed: 10601252]
13. Moomaw AS, and Maguire ME (2010) Cation selectivity by the CorA Mg^{2+} channel requires a fully hydrated cation. *Biochemistry* 49:5998–6008. [PubMed: 20568735]
14. Matthies D, Dalmas O, Borgnia MJ, Dominik PK, Merk A, Rao P, Reddy BG, Islam S, Bartesaghi A, Perozo E, and Subramaniam S (2016) Cryo-EM Structures of the Magnesium Channel CorA Reveal Symmetry Break upon Gating. *Cell* 164:747–756. [PubMed: 26871634]
15. Pfoh R, Li A, Chakrabarti N, Payandeh J, Pomes R, and Pai EF (2012) Structural asymmetry in the magnesium channel CorA points to sequential allosteric regulation. *Proceedings of the National Academy of Sciences U.S.A.* 109:18809–18814.
16. Nordin N, Guskov A, Phua T, Sahaf N, Xia Y, Lu S, Eshaghi H, and Eshaghi S (2013) Exploring the structure and function of *Thermotoga maritima* CorA reveals the mechanism of gating and ion selectivity in Co^{2+}/Mg^{2+} transport. *The Biochemical Journal* 451:365–374. [PubMed: 23425532]
17. Chakrabarti N, Neale C, Payandeh J, Pai EF, and Pomes R (2010) An iris-like mechanism of pore dilation in the CorA magnesium transport system. *Biophysical journal* 98:784–792. [PubMed: 20197031]
18. Dalmas O, Cuello LG, Jogini V, Cortes DM, Roux B, and Perozo E (2010) Structural dynamics of the magnesium-bound conformation of CorA in a lipid bilayer. *Structure* 18:868–878. [PubMed: 20637423]
19. Dalmas O, Sompornpisut P, Bezanilla F, and Perozo E (2014) Molecular mechanism of Mg^{2+} -dependent gating in CorA. *Nature Communications* 5:3590.

20. Kitjaruwankul S, Wapeesittipan P, Boonamnaj P, and Sompornpisut P (2016) Inner and Outer Coordination Shells of Mg^{2+} in CorA Selectivity Filter from Molecular Dynamics Simulations. *The Journal of Physical Chemistry B* 120:406–417. [PubMed: 26727882]
21. Hu J, Sharma M, Qin H, Gao FP, Cross TA (2009) Ligand Binding in the Conserved Interhelical Loop of CorA, a Magnesium Transporter from *Mycobacterium tuberculosis*. *Journal of Biological Chemistry* 284:15619–15628. [PubMed: 19346249]
22. Seyfried WE, Jr., Seewald JS, Berndt ME, Kang Ding, Foustoukos DI (2003) Chemistry of hydrothermal vent fluids from the Main Endeavour Field, northern Juan de Fuca Ridge: Geochemical controls in the aftermath of June 1999 seismic events. *Journal of Geophysical Research* 108:1–23.
23. Kabsch W (2010) Xds. *Acta Crystallographica. Section D, Biological crystallography* 66:125–132. [PubMed: 20124692]
24. McCoy AJ, Grosse-Kunstleve RW, Adams PD, Winn MD, Storoni LC, and Read RJ (2007) Phaser crystallographic software. *Journal of Applied Crystallography* 40:658–674. [PubMed: 19461840]
25. Murshudov GN, Vagin AA, Lebedev A, Wilson KS, Dodson EJ (1999) Efficient anisotropic refinement of macromolecular structures using FFT. *Acta Crystallographica. Section D, Biological crystallography* 55:247–255. [PubMed: 10089417]
26. Emsley P, and Cowtan K (2004) Coot: model-building tools for molecular graphics. *Acta Crystallographica. Section D, Biological crystallography* 60:2126–2132. [PubMed: 15572765]
27. Davis IW, Leaver-Fay A, Chen VB, Block JN, Kapral GJ, Wang X, Murray LW, Arendall WB, 3rd, Snoeyink J, Richardson JS, and Richardson DC (2007) MolProbity: all-atom contacts and structure validation for proteins and nucleic acids. *Nucleic Acids Research* 35:W375–383. [PubMed: 17452350]
28. DeLano W (2007) The Pymol Molecular Graphics System DeLano Scientific, Palo Alto, CA, USA.
29. Hmiel SP, Snively MD, Miller CG, Maguire ME (1986) Magnesium Transport in *Salmonella typhimurium*: Characterization of Magnesium Influx and Cloning of a Transport Gene. *Journal of Bacteriology* 168:1444–1450. [PubMed: 3536881]
30. Snively MD, Florer JB, Miller CG, Maguire ME (1989) Mg^{2+} Transport in *Salmonella typhimurium*: Expression of Cloned Genes for Three Distinct Mg^{2+} Transport Systems. *Journal of Bacteriology* 171:4752–4760. [PubMed: 2548999]
31. Gibson MM, Bagga DA, Miller CG, Maguire ME (1991) Mg^{2+} Transport in *Salmonella typhimurium*: Influence of New Mutations Conferring Cobalt Resistance on the CorA Mg^{2+} Transport System. *Molecular Microbiology* 5:2753–2762. [PubMed: 1779764]
32. Nelson DL, and Kennedy EP (1971) Magnesium transport in *Escherichia coli*. Inhibition by cobaltous ion. *Journal of Biological Chemistry* 246:3042–3049. [PubMed: 4928897]

Highlights

- Crystal structures of *Tm* CorA M1 Mg²⁺ binding sites mutants (D89K/D253K; D89R/D253R)
- Release of Mg²⁺ from M1 sites hypothesized to cause opening of channel
- Both mutant structures still in closed conformation despite absence of Mg²⁺ at M1 sites
- Mutant CorA proteins still show transport of ⁶³Ni²⁺, indicating functionality
- Absence of Mg²⁺ from M1 sites does not cause channel opening or prevent function

Figure 1.
Comparison of KK-CorA with wild type CorA. Cartoon superposition of KK-CorA (cyan) with chains A-E of wild type (orange) Tm CorA pentamer (PDB ID 2IUB). (A) Side view. (B) Cytoplasmic view. (C) Periplasmic view. The rmsd between the two structures is 1.0 Å (1316 to 1316 atoms).

Figure 2.
Comparison of RR-CorA with wild type CorA. Cartoon superposition of the RR-CorA (cyan) with chains A-E of wild type (orange) Tm CorA pentamer (PDB ID 4I0U). (A) Side view. Cytoplasmic view. (C) Periplasmic view. The rmsd between both structures is 1.4 Å (1452 to 1452 atoms).

Figure 3.

Comparison of stalk helices and membrane domain of RR-CorA with wild type CorA. Cartoon superposition of stalk helices (residues 251–312), transmembrane helices 1 and 2 (residues 326–345) and C-termini (residues 346–349) of the RR-CorA (cyan) with wild type (pink) Tm CorA (PDB ID 4I0U). (A) Side view. (B) Cytoplasmic view.. (C) Periplasmic view. The first stalk helix residue F251, the stalk helix M1 site residue 253 and residue 269 at the end of the stalk helix region are shown as sticks. The rmsd between both structures is 0.8 Å (407 to 407 atoms).

Figure 4. Residues of M1 and M2 cytosolic binding sites involved in interaction with Mg^{2+} in CorA. Stick representation of binding sites between monomers A (green) and D (yellow) in KK-CorA (A) and RR-CorA (B). Carbons are shown in green and yellow, respectively, oxygens in red and nitrogens in blue. The water in (B) is shown as red sphere. The $2F_o - F_c$ map around residues and water is shown as blue mesh and contoured at 1σ .

Figure 5. Cell growth of wild type and mutant Tm CorA strains. Strains were inoculated in 5 ml of N-minimal medium at an approximate OD600 of 0.04 and grown overnight at 37°C with shaking at the indicated Mg²⁺ concentration. This is a single experiment representative of several similar experiments.

Figure 6. Inhibition of $^{63}\text{Ni}^{2+}$ transport by Mg^{2+} . Transport was performed at a total Ni^{2+} concentration of $200 \mu\text{M}$ as previously described (7,12,13). This is a representative experiment from several similar experiments. Error bars are $\pm$ SEM. Where no error bars appear, the error was less than the size of the data point.