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Why Is the GMN Motif Conserved in the CorA/Mrs2/Alr1 Superfamily of Magnesium Transport Proteins?

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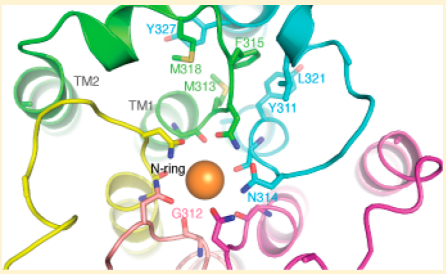
ABSTRACT: Members of the CorA/Mrs2/Alr1 superfamily of transport proteins mediate magnesium uptake in all kingdoms of life. Family members have a low degree of sequence conservation but are characterized by a conserved extracellular loop. While the degree of sequence conservation in the loop deviates to some extent between family members, the GMN family signature motif is always present. Structural and functional data imply that the loop plays a central role in magnesium selectivity, and recent biochemical data suggest it is crucial for stabilizing the pentamer in the magnesium-free (putative open) conformation. In this study, we present a detailed structure−function analysis of the extracellular loop of CorA from *Thermotoga maritima*, which provides molecular insight into how the loop mediates these two functions. The data show that loop residues outside of the GMN motif can be substituted if they support the pentameric state, but the residues of the GMN motif are intolerant to substitution. We conclude that G^{312} is absolutely required for magnesium uptake, M^{313} is absolutely required for pentamer integrity in the putative open conformation, and N^{314} plays a role in both of these functions. These observations suggest a molecular reason why the GMN motif is conserved throughout the CorA/Mrs2/Alr1 superfamily.



Members of the CorA/Mrs2/Alr1 superfamily of magnesium (Mg^{2+}) transport proteins are characterized by a large cytoplasmic N-terminal domain followed by two transmembrane helices. The transmembrane helices are separated by a conserved loop, which is partially buried in the membrane interface and partly exposed to the hydrophilic extracellular space.[1−6] Loop conservation is more extensive in prokaryotes (YGMNFxxMPELxxxxGYP), but three residues (GMN) are universally conserved in all phyla and essential for protein function[7−10] (Figure 1A).

The prokaryotic CorA has come to serve as a model for the whole superfamily. The closed conformation has been captured in a number of X-ray structures from *Thermotoga maritima* (*Tm*CorA) and *Methanocaldococcus jannaschii* (*Mj*CorA)[1−6] (Figure 1B). These structures reveal a pentameric assembly of CorA subunits arranged around a central >50 Å long pore, and a large cytoplasmic domain. At the extracellular pore entrance, the conserved GMN motif seems to play a central role in the formation of the Mg^{2+} selectivity filter (Figure 1C). Metal binding at the interface between each protomer in the cytoplasmic domain appears to be responsible for stabilizing the closed conformation (Figure 1B).[11,12]

The open conformation is still structurally uncharacterized. However, it is believed that CorA assumes the open conformation when the cytoplasmic metal binding sites are unoccupied. In line with this notion, the *Tm*CorA pentamer is stable in the absence of Mg^{2+} [11] and the isolated membrane domain from *Mycobacterium tuberculosis* CorA (consisting of TM1, the extracellular loop, and TM2) can form a pentamer.[13] Recently, we showed that the five extracellular loops of the *Tm*CorA pentamer played a prominent role in supporting this Mg^{2+}-free (putative open) conformation, as single-alanine substitutions within the loop disrupted the pentameric state and Mg uptake[2+ 11,12] (see underlined residues in the ^{311}YG-MNFxxMPELxxxxGYP^{328} sequence). The addition of Mg^{2+} caused the loop-destabilized mutants to re-form as pentamers via binding of metal to the cytoplasmic metal binding sites, suggesting that the protein had adopted the closed conformation as captured in the X-ray structures.[11]

The emerging picture is thus that the five extracellular loops of the pentamer perform two important functions. (1) They scaffold the pentamer in the Mg^{2+}-free conformation, and (2) they form the Mg^{2+} specific selectivity filter. Here we present a structure−function analysis of *Tm*CorA, that better defines how the extracellular loops mediate these distinct processes.

EXPERIMENTAL PROCEDURES

***In Vitro* Analysis of the Oligomeric State.** *Cloning.* The *corA* gene from *T. maritima* was cloned with an N-terminal six-His affinity tag and TEV protease cleavage site (MHHHHHHENLYFQGM) into pGFPi (a modified version of the pGFPe expression vector[14,15] from which the *gfp* gene has been removed). The coding region was therefore under the control of the T7 promoter. This construct is termed WT. All mutants were obtained by site-directed mutagenesis using the QuickChange Site-Directed Mutagenesis Kit or PfuUltra II

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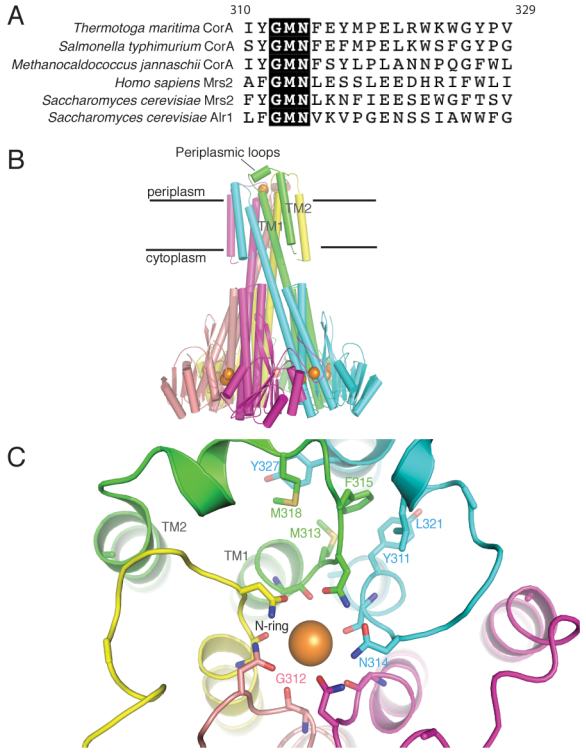

Figure 1. Loop conservation and structural organization. (A) Representative sequence alignment of the extracellular loops from CorA, Mrs2, and Alr1. The numbering is that of the *Tm*CorA sequence. (B and C) Structural organization of *Tm*CorA in the closed conformation, shown as a cartoon representation (Protein Data Bank entry 4I0U).[6] Each protomer in the pentamer is colored differently, and Mg^{2+} ions are displayed as orange spheres. (B) Side view of the *Tm*CorA pentamer in the closed conformation. Two Mg^{2+} ions occupy the cytoplasmic metal binding sites located between each of the five protomers (10 sites in total). It is believed that when Mg^{2+} levels drop in the cell, the 10 sites become unoccupied and the protein assumes the open (Mg^{2+}-free) conformation. (C) Close-up of the extracellular loops. Loop residues substituted in this study are labeled and shown as sticks in blue and green protomers. N^{314} from each protomer forms a polar N ring that, together with the ring of the carbonyl oxygen of G^{312}, is believed to function as the Mg^{2+} selectivity filter. A Mg^{2+} ion is shown inside. M^{313} together with Y^{311}, F^{315}, M^{318}, L^{321}, and Y^{327} form a hydrophobic collar that surrounds the putative Mg^{2+} filter. The packing interactions within the collar appear to be vital for holding the pentamer together in the Mg^{2+}-free conformation.

Fusion HS DNA Polymerase (Stratagene) using WT as a template. Primers were obtained from MWG. Cloning was performed in MACH1 cells. All constructs were confirmed by sequencing (MWG).

Labeling of Proteins with [^{35}S]Methionine in Vivo. Labeling of proteins *in vivo* with [^{35}S]methionine was conducted as described in ref 16, with a few minor modifications. In short, plasmids were transformed into *Escherichia coli* strain BL21-(DE3). A single colony was inoculated into a 2 mL tube containing 1 mL of LB medium with kanamycin (50 μg/mL) and incubated with vigorous shaking at 37 °C for 16 h. The culture was back-diluted 1:20 and grown until the OD_{600} reached ≈0.4. Cells were harvested by centrifugation at 850g for 5 min, and the cell pellet was resuspended in 1 mL of M9 minimal medium, supplemented with thiamine (10 mM) all amino acids except methionine. The cells were then incubated at 37 °C for 90 min to starve them of methionine. Synthesis of the plasmid-encoded proteins was initiated by incubation of the cells with 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 5 min. To suppress the production of native *E. coli* proteins, genomic transcription was suppressed by the addition of 0.2 mg/mL rifampicin for 10 min. To label newly synthesized protein, 15 mCi of [^{35}S]methionine was added to the sample. Following the pulse-labeling reaction, the cells were resuspended in 1 mL of LB medium with antibiotics and chased for 30 min, to give the radiolabeled proteins a chance to assemble. Expression was confirmed by analyzing 50 μL of the culture via sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

BN-PAGE. Whole cells from 500 μL of culture were resuspended in 1 mL of H_2O supplemented with 0.4 mg/mL lysozyme and incubated at 30 °C for 45 min. Crude membranes were collected by centrifugation at 264000*g* for 30 min at 4 °C and resuspended in 85 μL of ACA_{750} buffer [750 mM *n*-aminocaproic acid, 50 mM Bis-Tris, and 0.5 mM Na_2EDTA (pH 7.0)]. Membrane proteins were then solubilized by the addition of 0.5% (w/v) *n*-dodecyl β-D-maltoside (DDM) and incubated on ice for >1 h. Unsolubilized material was removed by centrifugation at 264000*g* for 30 min at 4 °C. The supernatant was added to 15 μL of G250 solution [5% (w/v) Coomassie G250 in ACA_{750} buffer] and analyzed by BN-PAGE on 14 cm × 20 cm × 1.5 mm gels. High-molecular mass markers were obtained from Amersham Biosciences. Buffers and gels were prepared as described previously.[17] Gels were dried and exposed to a BAS-IP MS2040 plate for 24 h prior to detection with a Fuji FLA-3000 phosphorimager (Fuji, Tokyo, Japan). The analysis, including rifampicin expression, was repeated at least three times for each construct.

***In Vivo* Analysis of Mg^{2+} Import Function.** *Cloning.* WT and loop mutants in pGFPi (described above) were recloned without the His affinity tag or TEV protease cleavage site into the pBAD/HisA vector (Invitrogen), downstream of an arabinose-inducible promotor. Primers were obtained from MWG. Cloning was performed in MACH1 cells. Constructs were confirmed by sequencing (MWG). pBADa contructs were introduced into the restrictionless *Salmonella typhimurium* MM1242 strain (also known as JR501) for plasmid preparation.[18,19]

In Vivo MM281 Complementation Assay. The *in vivo* complementation assay was conducted essentially as described in ref 11. Mg^{2+} import deficient *S. typhimurium* strain MM281 was transformed with MM1242 (JR501)-derived pBADa plasmids and grown in LB medium supplemented with 100 mM $MgCl_2$, 100 μg/mL carbencillin, 50 μg/mL kanamycin, and 30 μg/mL chloramphenicol in 24-well growth plates at 37 °C while being vigorously shaken (1200 rpm) for 16 h. The cultures were back-diluted 1:100 into 4 mL of fresh LB medium with the same composition. When cells reached an OD_{600} of ≈0.8, they were induced with 0.02% arabinose for 1 h, before being harvested. To remove Mg^{2+}, the pellet was washed with 1 mL of buffer A [20 mM Tris-HCl (pH 8), 0.3 M NaCl, and 20 mM imidazole] and resuspended in 4 mL of the same buffer (1:1). To verify protein expression, 5 μL of cell culture was used for Western blotting with a rabbit anti-*Tm*CorA antiserum (Innovagen AB, Lund, Sweden). For functional analysis of the mutants, a dilution series with 1:2 increments and an end dilution of 1:64 was performed. From each dilution step, 4 μL of cell culture was spotted on a dried LB agar plate supplemented with the appropriate antibiotics and 0.02% arabinose, but without Mg^{2+}. As a positive control, the cultures were also spotted on a plate containing 20 mM $MgCl_2$.

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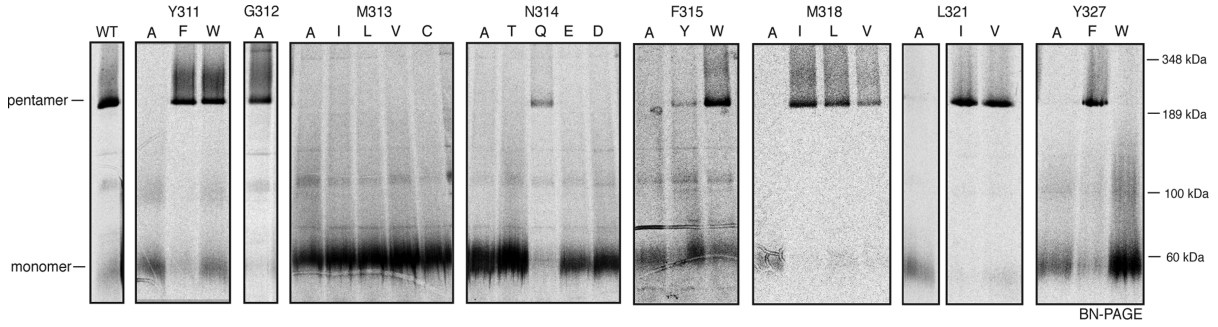

Figure 2. Pentamer integrity analyzed by BN-PAGE. WT and variants with an amino acid substitution in the extracellular loop were radiolabeled with ^{35}S methionine and analyzed by nondenaturing BN-PAGE in the absence of Mg^{2+}. Pentamers and monomers are indicated to the left and molecular mass markers to the right. M^{313} of the GMN motif is required for the pentameric architecture, as no substitution was tolerated at this position. For all other positions, we identified at least one amino acid substitution that supported a pentameric state. The alanine-substituted mutants were presented in a previous study.[11] The data are summarized in Table 1.

Following overnight incubation at 37 °C, the plates were photographed and growth density was quantified as described previously,[20] using ImageGauge version 4.23. The growth density of each spot was plotted with respect to dilution factor. The line of best fit was calculated, and the growth of each mutant was quantified as the integral beneath the best-fit line. Growth was normalized using the following equation: normalized growth of the mutant = (growth of the mutant − growth of the empty vector)/(growth of WT − growth of the empty vector). At least three independent growth measurements were made for each loop mutant.

RESULTS

The aim of this study was to better define how the extracellular loop contributes to the pentameric architecture and Mg^{2+} uptake in *Tm*CorA. We focused on seven conserved amino acids in the extracellular loop (see the underlined residues in the ^{311}YGMNFEYMPELRWKWGYP^{328} sequence), which a previous alanine scan had revealed to be critical for pentamer integrity in Mg^{2+}-free buffer.[11,12]

M^{313} of the GMN Motif Is Essential for Pentamer Integrity. First, we tested if a conserved substitution could support the pentameric state at positions where an alanine substitution could not. The purpose of this exercise was to investigate the overall tolerance to variations in loop composition with respect to pentamer integrity. To determine if the mutants were pentameric, they were radiolabeled in *E. coli*, solubilized in *n*-dodecyl *β*-D-maltoside (DDM), and separated by nondenaturing blue-native polyacrylamide gel electrophoresis (BN-PAGE).[16,21,22] Mg^{2+} was not added in the BN-PAGE experiment to ensure that we were monitoring the structural contribution of the loop and not the cytoplasmic metal binding sites.[11] For six of the seven positions tested, we identified at least one conserved amino acid substitution that maintained the pentameric state [i.e., Y^{311}F/W, N^{314}Q, F^{315}Y/W, M^{318}I/L/V, L^{321}I/V, and Y^{327}F (Figure 2 and Table 1)]. Similarly, it has been noted that a G^{312}A mutant is pentameric (Figure 2 and Table 1).[11,12] Strikingly, however, none of the residues that replaced M^{313} supported the pentameric state (Figure 2 and Table 1). When comparing these data with recent structural data of *Tm*CorA, we find that M^{313} together with Y^{311}, F^{315}, M^{318}, L^{321}, and Y^{327} form a hydrophobic "collar" around the hydrophilic pore entrance (Figure 1C).[10] In addition to positioning of the putative Mg^{2+} selectivity filter, our observations suggest that packing interactions within the

Table 1. Summary of Experimental Data for All Mutants[a]

Position	Mutation	Pentamer	Activity (%)	st error (%)
WT	-	-	100	0
Y311	A	-	1	1
	F	+	100	13
	W	+	52	5
G312	A	+	0	0
M313	A	-	2	1
	I	-	2	1
	L	-	2	1
	V	-	0	0
	C	-	0	0
N314	A	-	0	0
	T	-	0	0
	Q	+	0	0
	E	-	0	0
	D	-	1	0
F315	A	-	0	0
	Y	+	58	8
	W	+	221	15
M318	A	-	21	3
	I	+	50	7
	L	+	31	4
	V	+	36	14
L321	A	-	30	6
	I	+	47	12
	V	+	94	17
Y327	A	-	16	5
	F	+	156	24
	W	-	60	5

[a]Pentamer (+) or no pentamer (−) was deduced from the *in vitro* BN-PAGE assay (see Figure 2 and the text for details). Activity measurements depict normalized growth values relative to that of wild-type *Tm*CorA (WT) obtained from the *in vivo* MM281 complementation assay (see Figure 3 and the text for details). The alanine-substituted mutants were presented in a previous study.[11]

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collar are vital for scaffolding the pentamer in the Mg^{2+}-free conformation. The integrity of the collar is not compromised by subtle variations in amino acid composition, as the majority of contributing residues tolerate conserved amino acid substitutions. Importantly, however, it appears that M^{313} functions as the "keystone" of CorA architecture, as it is absolutely required for pentamer integrity.

Only Pentameric Mutants Mediate Mg^{2+} Uptake *in Vivo*. Next, we tested if the mutant proteins were functional, using an *in vivo* complementation assay. Mutant proteins were expressed in MM281, a *S. typhimurium* strain devoid of all Mg^{2+} uptake systems that grows only in the presence of a functional version of CorA or a high concentration of Mg^{2+}.[23] We found that the mutants that were unable to maintain the pentameric state in the *in vitro* BN-PAGE assay either were nonfunctional or displayed the lowest activity of all mutants tested. This observation strongly suggests that the loop-mediated interactions identified in our *in vitro* BN-PAGE assay are also important in the membrane environment. In contrast, the majority of mutant proteins that formed pentamers could, at least to some extent, complement the growth of MM281 (i.e., $Y^{311}F/W$, $F^{315}Y/W$, $M^{318}I/L/V$, $L^{321}I/V$, and $Y^{327}F$) (Figure 3 and Table 1). We noted one instance in which a mutant protein could complement growth but failed to maintain a pentameric state in the *in vitro* BN-PAGE assay ($Y^{327}W$), suggesting that this mutant is destabilized when it is solubilized in DDM.

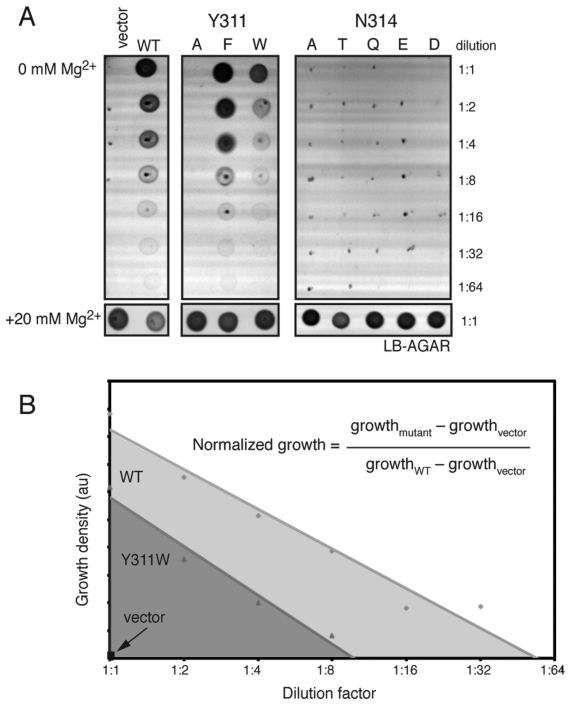

Figure 3. Functional analysis using Mg^{2+} transport deficient strain MM281. (A) The top panels show growth of serial dilutions of the MM281 strain expressing WT and indicated constructs on LB agar plates devoid of Mg^{2+}. $Y^{311}F/W$ are viable, while $Y^{311}A$ and all substitutions at N^{314} failed to complement growth. The bottom panels show that all cells were viable when LB plates were supplemented with 20 mM Mg^{2+}. The alanine-substituted mutants were presented in a previous study.[11] Data of all mutants are summarized in Table 1. (B) The growth of a particular construct was normalized by calculating the ratio of the area under its growth dilution curve relative to that obtained for wild-type *Tm*CorA (see Experimental Procedures).

N^{314} of the GMN Motif Is Essential for Mg^{2+} Uptake and Important for Pentamer Integrity. In the crystal structures of the closed transport protein, N^{314} of the GMN motif forms an N ring that is believed to function as the Mg^{2+} selectivity filtertogether with the G_{312} carbonyl oxygen ring (Figure 1C).[4,6] Intriguingly, our functional analysis revealed that even though $N^{314}Q$ was pentameric, it could not mediate Mg^{2+} uptake (Figures 2 and 3). Importantly, this shows that the side chain of N^{314} (the N ring) contributes unique geometrical properties that are essential for Mg^{2+} uptake. We conclude N^{314} is subjected to both structural and functional constraints: A larger polar residue (Q) promotes the pentameric state but impairs Mg^{2+} import, while a charged or smaller polar residue (E, D, or T) prevents the formation of stable pentamers. Similarly, it has previously been noted that the pentameric $G^{312}A$ mutant fails to mediate Mg^{2+} influx.[11,12]

DISCUSSION

Studies of the prototypic CorA protein suggest the conserved extracellular loop serves both structural and functional purposes. The five loops of the pentamer assemble into a hydrophobic collar that surrounds the hydrophilic pore entrance. Packing interactions within the hydrophobic collar seem to be vital for maintaining the pentameric arrangement when cytoplasmic metal binding sites are unoccupied (the putative open conformation). Analysis of *Tm*CorA reveals seven residues of the loop motif as being particularly important for packing (underlined residues in the ^{311}YGMNFxxMPELxxxxGYP^{328} sequence), as the pentameric state becomes disrupted by single-alanine substitutions at these positions.[11] In addition, loop residues mediate Mg^{2+} uptake through the centrally located ion conduction pore. Here, the asparagine (N) and glycine (G) residues of the universally conserved GMN motif are believed to form a cation selectivity filter. Still, however, many fundamental questions remain about the molecular construction of these types of transport proteins. Notably, what side chain qualities are fundamental for sustaining pentameric packing interactions in the open conformation? What side chain properties are required for Mg^{2+} import? We have probed these questions by systematically testing how conserved substitutions at seven positions in the loop of *Tm*CorA affect Mg^{2+} uptake properties and pentamer integrity at low Mg^{2+} concentrations.

Our most striking observation is that M^{313} of the GMN motif is absolutely required for maintaining the pentameric state in the putative open conformation. Evidently, the nonbranched sulfur-containing methionine side chain provides a unique property that not even the most conserved substitutions (I, L, V, or C) can surrogate. In sharp contrast, other residues in the hydrophobic collar were structurally replaceable. For each position, we identified at least one conserved substitution that supported the pentameric state (i.e., $Y^{311}F/W$, $F^{315}Y/W$, $M^{318}I/L/V$, $L^{321}I/V$, and $Y^{327}F$), suggesting that the replacement residues preserved packing interactions within the hydrophobic collar. In line with this observation, the *in vivo* Mg^{2+} complementation assays showed that the majority of pentameric mutants could mediate Mg^{2+} uptake.

What side chain properties are required for Mg^{2+} uptake? The X-ray structures indicate that the hydroxyl side chains of N^{314} and the carbonyl oxygen from G^{312} form two consecutive rings at the extracellular pore entrance (the N ring and carbonyl ring) (Figure 1C).[5−10] These rings are thought to govern the cation selectivity process by coordinating a partly hydrated

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Mg^{2+}, thus shedding the second water shell.[4−6] In addition, G^{312} appears to create a hinge that breaks the pore-lining helix and initiates the loop structure. Intriguingly, we find that an $N^{314}Q$ substitution preserves the pentameric state of *Tm*CorA but abolishes Mg^{2+} uptake. This observation indicates that a Q ring, which is extended by one methyl group but retains the polar side chain chemistry, cannot functionally substitute for the native N ring. In light of the current structural data available for CorA, we presume it is because the resulting spatial organization of the N ring is perfectly suited to coordinate a partly hydrated Mg^{2+}. In addition, our data indicate that N^{314} is involved in supporting the pentameric state. When it was substituted with a nonpolar (A), smaller polar (T), or negatively charged residue (D or E), the oligomeric state was lost. Our analysis thus reveals both structural and functional reasons why N^{314} has been conserved in the GMN motif. Similarly, a $G^{312}A$ substitution results in a nonfunctional pentamer.[11,12] The deleterious effect of the alanine substitution on Mg^{2+} uptake is not obvious, as it does not preclude formation of a carbonyl ring or detrimentally affect pentamer stability. However, we speculate that the introduction of a larger, less flexible residue in a hinge region could give rise to local structural distortions at the mouth of the pore. The distortions could cause inappropriate positioning of the alanine carbonyls or shift the positioning of the neighboring N ring, thereby causing disruption of cation selectivity or uptake.

The analysis presented here and that in a previous study[11] provide novel insights into how the extracellular loop contributes to function and architecture in the CorA/Mrs2/Alr1 superfamily. We find that all loop residues outside the GMN motif (17 of 20 amino acids) can be substituted without losing transport function, if the pentameric state is preserved. This observation provides an explanation for naturally occurring motif variations outside of the GMN motif.[9] It also presents a new perspective to other studies on the loop, in which mutants have been assayed for transport activity but where the oligomeric state is unknown.[7,24] In stark contrast, we find the three amino acids in the GMN motif were irreplaceable. This is in perfect agreement with studies of other CorA/Mrs2/Alr1 family members showing the GMN motif is essential for function.[7,8,12,25] Our data suggest G^{312} is essential for Mg^{2+} uptake, M^{313} is required for pentamer integrity in the putative open conformation, and N^{314} plays a role in both of these functions. We propose that these highly restrictive structural and functional restraints explain why the GMN motif is conserved throughout the CorA/Mrs2/Alr1 superfamily.

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Notes

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ABBREVIATIONS

Tm, *T. maritima*; *Mj*, *M. jannaschii*; IPTG, isopropyl β-D-thiogalactopyranoside; DDM, *n*-dodecyl β-D-maltoside; BN-PAGE, blue-native polyacrylamide gel electrophoresis.

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