The Periplasmic Loop Provides Stability to the Open State of the CorA Magnesium Channel*

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Crystal structures of the CorA Mg2+ channel have suggested that metal binding in the cytoplasmic domain stabilizes the pentamer in a closed conformation. The open “metal free” state of the channel is, however, still structurally uncharacterized. Here, we have attempted to map conformational states of CorA from Thermotoga maritima by determining which residues support the pentameric structure in the presence or absence of Mg2+. We find that when Mg2+ is present, the pentamer is stabilized by the putative gating sites (M1/M2) in the cytoplasmic domain. Strikingly however, we find that the conserved and functionally important periplasmic loop is vital for the integrity of the pentamer when Mg2+ is absent from the M1/M2 sites. Thus, although the periplasmic loops were largely disordered in the x-ray structures of the closed channel, our data suggests a prominent role for the loops in stabilizing the open conformation of the CorA channels.

Ion transport in a cell is mediated by channels and transporters that are embedded within membranes. These proteins alternate between different conformations in response to changes in substrate concentration or other regulators in the intra-/extracellular milieu (1, 2). Unfortunately, it has proven difficult to capture and study different conformational states of these proteins, particularly by x-ray crystallography. An illustrative example is the CorA/Mrs2 family of cation channels, which are responsible for magnesium (Mg2+) uptake in prokaryotic cells and mitochondria (3–5). Three separate x-ray structures of CorA from Thermotoga maritima (TmCorA)² have been solved (6–8), but they are all in a single closed conformation, and many questions about the uptake mechanism remain unanswered.

The structures of TmCorA revealed that it is a homopentamer with a centrally located ion conduction pathway and a large soluble domain in the cytoplasm (Fig. 1). The soluble domain contains two Mg2+ binding sites at the interface between each protomer (termed M1 and M2), which appear to stabilize the pentamer in a closed conformation by negating static repulsion (Fig. 1) (6–8). Subsequent biochemical characterization indicated that these sites act as “homeostasis sensors” that gate the opening and closing of the ion-conducting pore in response to changes in intracellular Mg2+ (9). An additional Mg2+ binding site was identified at the cytoplasmic pore exit and was termed the aspartate ring (Fig. 1). Unfortunately, no x-ray structure of a metal-free protein has been captured. Thus, our understanding of the role of the cytoplasmic metal-binding sites in mediating conformational transitions is limited.

Another issue that cannot be answered by the x-ray structures is the role of the periplasmic loop. This loop contains a conserved motif (YGMNFXXMPEL) that is required for Mg2+ uptake (10, 11). As the five loops in the pentamer constitute the periplasmic entrance to the pore, it was presumed that they form the ion selectivity filter. However, in the x-ray structures of the closed channel, the loops were largely disordered and, thus, poorly resolved. A subsequent study employing electron paramagnetic resonance spectroscopy and molecular dynamics simulations have helped generate a model for the conserved periplasmic loop in the closed conformation (12), but the structure of the loop in the open state is still unclear. Curiously, two conserved residues in the loop are important for the pentameric structure (9). This latter observation caught our interest, as it cannot be easily explained by the x-ray structures of the closed channel, where considerable structural stability appears to be mediated by metal coordination to the M1/M2 sites (6–9).

In this study, we have investigated the role of the periplasmic loop in stabilizing the TmCorA pentamer. Our data reveal that when Mg2+ is absent from the M1/M2 sites, conserved residues in the periplasmic loop are essential for maintaining a pentameric state. This suggests that there is a structurally uncharacterized conformation of the TmCorA pentamer in which packing interactions in the periplasmic loops are critical for structural stability. Revealingly, we found that when Mg2+ is added, the pentamer is reassembled via M1/M2-mediated interactions in

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* The abbreviations used are: TmCorA, Thermotoga maritima CorA; LB, Luria Bertani broth; DDM, dodecyl-β-d-maltoside; DSS, disuccinimidyl suberate; BN, blue native; TEV, tobacco etch virus.

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the cytoplasmic state. Together, these data indicate that the pentameric state can be maintained either through interactions mediated by the periplasmic loops or the cytoplasmic M1/M2 sites. We propose that these two alternative stabilities reflect the molecular mechanism by which the channel stabilizes the open and closed state.

**EXPERIMENTAL PROCEDURES**

**Mutant Construction**—The corA gene from *T. maritima* was cloned with an N-terminal 6× His affinity tag and a tobacco etch virus (TEV) protease cleavage site (MHHHHHHENLY-FQGM) into pGFPi (a modified version of the pGFPe expression vector (13, 14) where the *gfp* gene has been removed). The coding region was therefore under the control of the T7 promoter. This construct is referred to as wt. All mutants were obtained by site-directed mutagenesis using the QuikChange site-directed mutagenesis kit or PfuUltra II Fusion HS DNA polymerase (Stratagene, Sweden) using *wt* as a template. The *Salmonella typhimurium* MM281 strain used for the in vivo complementation assay does not have a chromosomal T7 RNA polymerase gene. For that reason, *wt* and alanine mutants were recloned (excluding the N-terminal 6× His affinity tag and TEV protease cleavage site) into the pBAD/HisA vector (Invitrogen) downstream of an arabinose-inducible promoter. Primers were obtained from MWG (Germany). Cloning was performed in MACH1 cells. Mutations were confirmed by sequencing (MWG).

**Protein Purification**—Plasmids were transformed into the *E. coli* strain BL21(DE3) and grown at 37 °C in Luria Bertani broth (LB). Protein expression was induced for 3 h by the addition of isopropyl 1-thio-β-d-galactopyranoside (0.4 mm) when *A*₆₀₀ 0.7 was reached. Cells were harvested, washed with 1× PBS (pH 7.4) and resuspended in lysis buffer (1× PBS (pH 7.4) with EDTA-free complete protease inhibitor mixture (Roche) and deoxyribonuclease I from bovine pancreas (Sigma)). All subsequent steps were carried out at 4 °C. Cells were broken using an Emulsiflex C3 (Avestin, Canada). Unbroken cells were removed by centrifugation at 9300 × g for 20 min, and membranes were isolated by centrifugation at 210 000 × g for 2 h. Membranes were washed in 1× PBS (pH 7.4). Membrane proteins were solubilized with 1% n-dodecyl-β-d-maltopyranoside (DDM) (Affymetrix) in 7 ml of solubilization buffer (20 mM Tris-HCl (pH 8), 0.3 M NaCl, 20 mM imidazole) for 1 h. Unsolubilized material was removed by centrifugation at 142000 × g for 30 min. The supernatant containing the solubilized proteins was added to 2 ml of nickel-nitriotriacetic acid-agarose (Invitrogen) and incubated for 30 min to allow for His tag binding to the resin. The column was washed with wash buffer (20 mM Tris-HCl (pH 8), 0.3 M NaCl, 40 mM imidazole, 0.03% (w/v) DDM) before elution with 3 ml of elution buffer (20 mM Tris-HCl (pH 8), 0.1 M NaCl, 300 mM imidazole, 0.03% DDM). The elution buffer was changed to gel filtration buffer (20 mM Tris-HCl (pH8), 0.3 M NaCl, 0.03% (w/v) DDM) using prepacked PD-10 columns (GE Healthcare). Amicon Ultra Centrifugal Filters 30K (Millipore) was used to concentrate the proteins to ~5 mg/ml. The purity and integrity of the concentrated proteins was verified by denaturing PAGE (NuPAGE 4–12% Bis-Tris gels (Invitrogen)). The protein was stored at 4 °C for immediate use or snap-frozen in liquid nitrogen and stored at −80 °C.

**BN-PAGE**—The oligomeric state of the purified proteins was verified by (non-denaturing) BN-PAGE. 5 μl of purified protein was diluted with 80 μl of ACP150 buffer (750 mm n-amino caproic acid, 50 mM Bis-Tris, 0.5 mM Na₂EDTA (pH 7.2)) and 15 μl of G250 solution (5% (w/v) Coomassie G250 in ACA750 buffer) and loaded onto gels with dimensions of 14 cm × 20 cm × 1.5 mm. High molecular mass markers were obtained from Amersham Biosciences (Sweden). Buffers and gel compositions used were prepared as described previously (15). The experiment was repeated at least twice for every construct.

**Size Exclusion Chromatography**—Purified proteins were subjected to analytical size exclusion chromatography (aSEC) on a Superdex™200 5/150 GL column (GE Healthcare) in the presence and absence of Mg²⁺. Protein concentration was adjusted to 0.5 mg/ml in gel filtration buffer (20 mM Tris-HCl (pH8), 0.3 M NaCl, 0.03% (w/v) DDM ± 100 mM MgCl₂) and incubated for a minimum of 1 h at 4 °C prior to analyses. To estimate the fraction of pentamer and lower oligomers, QtiPlot version 0.9.7. was used to fit two Gaussians to the elution profile. To allow for a qualitative comparison between elution profiles ± Mg²⁺, each absorption curve was divided by its peak value.

**Chemical Cross-linking**—The oligomeric state of the purified proteins was verified by chemical cross-linking using disuccinimidyl suberate (DSS) (Thermo Scientific). Purified protein was adjusted to 0.2 mg/ml in cross-linking buffer (20 mM Na-phosphate buffer (pH 7.5), 0.3 M NaCl, 0.03% (w/v) DDM). The reaction was allowed to equilibrate for 30 min at room temperature. Freshly prepared DSS cross-linker was dissolved in 100% dimethyl sulfoxide to a concentration of 0.1 M and then added to the reactions to a final concentration of 4 mM. Samples were incubated at 37 °C for 30 min, followed by quenching with 100 mM Tris-HCl (pH 8) for 5 min. Samples were mixed with 4×
sample buffer and then loaded onto denaturing gels (NuPAGE 4–12% Bis-Tris gels (Invitrogen)). The experiment was repeated at least twice for every protein. The cross-linking experiment entailing Mg\(^{2+}\) was carried out essentially the same way, except that increasing concentrations (0.25–9 mM) of MgCl\(_2\) (titration) or 5 mM MgCl\(_2\) (all loop mutants) was included in the cross-linking buffer. The fraction pentamer and lower oligomers were quantified from three independent repetitions using Bio-Rad Image Lab software (version 4.0.1).

**In Vivo Complementation Assay—**pBADa constructs were first introduced into *Escherichia* strain JR501 for plasmid preparation. The Mg\(^{2+}\)-import deficient *S. typhimurium* strain MM281 was transformed with JR501-derived pBADa plasmids and grown in LB medium supplemented with 100 mM MgCl\(_2\), 100 \(\mu\)g/ml carbencillin, 100 \(\mu\)g/ml ampicillin, 50 \(\mu\)g/ml kanamycin, 30 \(\mu\)g/ml chloramphenicol in 24-well growth plates at 37 °C with vigorous shaking (1200 rpm) for 16 h. The cultures were back-diluted 1:100 into 4 ml of fresh LB media with the same composition. When cells reached \(A_{600}\) \(\approx 0.8\), they were induced with 0.02% arabinose for 1 h before harvest. To remove Mg\(^{2+}\), the pellet was washed with 1 ml of buffer A (20 mM Tris·HCl (pH 8), 0.3 M NaCl, 20 mM imidazole) and resuspended in 4 ml of the same buffer (1:1). To verify protein expression, 5 \(\mu\)l of cell culture was used for Western blotting using rabbit anti-CorA antisera (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 \(\mu\)l was spotted on a dried LB agar plate supplemented with the appropriate antibiotics and 0.02% arabinose but no Mg\(^{2+}\). As a control, the cultures were also spotted on a plate containing 100 mM MgCl\(_2\). Following overnight incubation at 37 °C, the plates were photographed, and growth density was quantified as described previously (16) using ImageGauge V4.23. 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 mutant = (growth mutant – growth empty vector)/(growth WT – growth empty vector). At least three independent growth measurements were made for each construct.

**RESULTS**

**The Periplasmic Loop Is Important for Pentamer Integrity in Low Mg\(^{2+}\)**—It has been reported previously that two conserved amino acids in the periplasmic loop of *Tm* CorA (Met-313 and Asn-314) are vital for the isolation of stable pentameric species (9). This observation suggested that the conserved periplasmic loop plays an important structural role. To investigate this issue further, we performed an alanine scan of the loop region (including the flanks of the transmembrane helices) and then purified the resultant 20 proteins (each carrying a single alanine substitution).

To determine whether the loop is important for pentamer integrity when the cytoplasmic metal binding sites are unoccupied, we analyzed the oligomeric state of the 20 mutant proteins in the absence of Mg\(^{2+}\). Two non-denaturing methods were employed: blue native (BN) PAGE (Fig. 2A) and aSEC (B and C). To substantiate our observations we used a third approach that entailed adding DSS (a chemical cross-linker) to the purified protein and analyzing the oligomeric state by denaturing PAGE (Fig. 2D). This latter method is non-reversible, thus allowing us to fix protomer interactions in their closest conformation.

From the data reported in Fig. 2, we conclude that the wild-type *Tm* CorA and 12 of the alanine-substituted proteins were pentamers. Intriguingly however, eight of the loop mutants were predominantly detected as lower oligomers (Fig. 2, A–D, I310A, Y311A, M313A, N314A, F315A, M318A, L321A, and Y327A). This observation suggests that each of the alanine-substituted proteins has lost important interprotomer interactions that are normally mediated by the native side chain. Note that the oligomeric state of N314A was influenced by the method. In the BN-PAGE it was only detected as lower oligomers (Fig. 2A). However, when analyzed by aSEC and DSS cross-linking, it was detected in two populations, a high molecular mass population and a low molecular mass population (Fig. 2, B and D, and Ref. 9).

From this analysis we conclude that eight residues in the periplasmic loop of *Tm* CorA are important for the integrity of the pentamer when Mg\(^{2+}\) is absent. Remarkably, seven of these residues are part of either the CorA-family signature motif (YGMNFXXMPEL) or another conserved loop motif (GYP) present in many prokaryotic family members (see underlined residues, \(_{310}\)YGMNFEYMPEL\(_{312}\) and \(_{329}\)GWKYGYP\(_{329}\) (10, 11, 17). This latter observation suggests that our findings are not just relevant for *Tm* CorA but that the loop mediates pentamer packing interactions in all CorA family members. Such a loop scaffold, or nexus, could potentially be important for ion conduction in an open state of the CorA channels, perhaps by ensuring a relatively defined fold for Mg\(^{2+}\) selectivity.

**Interactions Mediated by the Periplasmic Loop Are Vital for Channel Function—**If the eight positions in the loop are important for the integrity of the *Tm* CorA pentamer when cytoplasmic Mg\(^{2+}\) levels are low (*i.e.* a putative open conformation), their substitution to alanine should affect channel function *in vivo*. To test this hypothesis, we expressed the 20 loop mutants in the *S. typhimurium* strain MM281. This strain is devoid of all Mg\(^{2+}\) uptake systems and can only grow in LB medium when complemented with a functional *Tm* CorA variant or when supplemented with 100 mM Mg\(^{2+}\) (Fig. 3A) (18). In agreement with our *in vitro* analysis, we found that seven of the mutants that were detected as lower oligomers and were part of the family signature motifs (Y311A, M313A, N314A, F315A, M318A, L321A, and Y327A) were either unable to complement MM281 or displayed a significantly reduced growth phenotype compared with wild-type *Tm* CorA (Fig. 3, A and B). Thus, there is a clear correlation between pentamer integrity and channel function. Surprisingly, the growth of the cells expressing the I310A mutant exceeded that of the cells expressing the wild-type *Tm* CorA, even though I310A was detected as a lower oligomer *in vitro* (Fig. 3, A and B). Further work is required to explain this observation. We note, however, that I310 is not a conserved residue in the CorA family (*i.e.* it is not part of the CorA loop motifs). The apparent requirement for the seven conserved residues to scaffold the *Tm* CorA pentamer provides a plausible...
explanation to why the equivalent residues have been found important for channel function in other CorA family members (11). In agreement with Payandeh et al. (9), we found that the cells expressing the G312A mutant could not complement the MM281 strain. Thus, the G312A mutant is a non-functional pentamer. We also confirm that the two negatively charged
loop-residues, Glu-316 and Glu-320, are not essential for channel function (9, 19).

Taken together, the data show that the five periplasmic loops in the pentamer play at least two roles: they stabilize the pentamer in low Mg\textsuperscript{2+} conditions (when the cytoplasmic Mg\textsuperscript{2+} binding sites are presumably unoccupied), and they are important for Mg\textsuperscript{2+} uptake.

Mg\textsuperscript{2+} Induces a Structural Response—The X-ray structures of TmCorA and subsequent biochemical studies suggested that bound Mg\textsuperscript{2+} in the cytoplasmic domains stabilize the pentamer (9). Therefore, we hypothesized that addition of Mg\textsuperscript{2+} might restabilize the eight destabilized loop mutants (i.e. to a pentameric state). To explore this possibility, we added 100 mM Mg\textsuperscript{2+} to the purified proteins (i.e. a similar concentration to that used in the crystallization experiments (6–8)) and then reanalyzed the oligomeric state by aSEC. BN-PAGE could not be employed because Mg\textsuperscript{2+} perturbed running of the gels. As expected, the addition of Mg\textsuperscript{2+} to the wild-type TmCorA made no difference to the oligomeric state, which remained pentameric (Fig. 4A). Revealingly, the addition of Mg\textsuperscript{2+} to the eight destabilized loop mutants caused them to transit from lower oligomers toward pentamers (Fig. 4, B and C). Predictably, we found that the Mg\textsuperscript{2+}-dependent transition from lower oligomers to pentamer was reversible. When we washed the samples with buffer containing EDTA (or buffer that simply lacked Mg\textsuperscript{2+}) during aSEC analysis, the mutants were again detected as lower oligomeric species (Fig. 4B, inset).

Taken together, we conclude that mutations in the periplasmic loop do not affect pentamer stability when sufficient concentrations of Mg\textsuperscript{2+} are present. In accordance with the observation that Mg\textsuperscript{2+} stabilizes the closed channel conformation (9), we conclude that the addition of Mg\textsuperscript{2+} drives the destabilized loop mutants to adopt a structural arrangement similar to that captured in the crystal structures (i.e. a closed conformation) where the cytoplasmic domains pack together and the structural contribution of the periplasmic loops is negligible.

The M1/M2 Sites Drive the Mg\textsuperscript{2+}-dependent Pentamerization—How does Mg\textsuperscript{2+} influence the stability of TmCorA? Is the pentamerization effect brought about by Mg\textsuperscript{2+}-binding to the M1/M2 sites (i.e. the putative homeostasis sensors), the aspartate ring (at the pore exit), or perhaps another unidentified binding site? To address these questions, we picked one protein with a destabilizing alanine substitution in the periplasmic loop, Y311A, and then incorporated a second alanine substitution that should prevent Mg\textsuperscript{2+} binding to either the aspartate ring (Y311A:D277A) or the M1/M2 sites (Y311A:D253A) (Fig. 5). The reaction was Mg\textsuperscript{2+}-specific, as when EDTA was present, the fraction of pentameric species was low for all mutant proteins. We therefore conclude that association of Mg\textsuperscript{2+} to the M1/M2 sites can drive pentamerization of the loop destabilized Y311A mutant. This observation is in complete agreement with previous studies suggesting that interactions within the M1/M2 sites stabilize the TmCorA protein in a closed conformation (6, 9). It thus appears that by disrupting the loop scaffold, we have isolated the M1/M2 homeostasis sensor/gating machinery.
Periplasmic Loop Stabilizes CorA

**FIGURE 4.** Destabilized TmCorA loop mutants shifts from lower oligomers to pentamers in the presence of Mg\(^{2+}\). A, analyses by aSEC in the presence or absence of Mg\(^{2+}\) show that wild-type TmCorA is a stable pentamer in buffer containing low and high concentrations of Mg\(^{2+}\). B, the Y311A mutant protein incubated in Mg\(^{2+}\)-free buffer (gray trace) shifts from lower oligomer to pentamer when washed in buffer containing high concentrations of Mg\(^{2+}\) (black trace). Inset, the Mg\(^{2+}\)-dependent pentamerization reaction is reversible. The Y311A mutant protein shifts from pentamers in buffer containing high concentrations of Mg\(^{2+}\) (black trace) to lower oligomers when washed in Mg\(^{2+}\)-free buffer (gray trace). Pentamers and lower oligomers are indicated. C, bar graph showing fraction pentamers for the eight destabilized loop mutants as quantified from aSEC analysis in high and low Mg\(^{2+}\) (n = 3).

M1/M2-mediated Pentamerization Is Induced at Physiologically Relevant Mg\(^{2+}\) Levels—At what concentration is Mg\(^{2+}\) sensed by the putative M1/M2-gating machinery? To address this question, we analyzed the oligomeric state of the purified Y311A loop mutant (which has intact M1/M2 sites) in increasing concentrations of Mg\(^{2+}\). To prevent disassembly during analysis, the oligomeric state was verified by adding the cross-linker DSS prior to analysis by denaturing PAGE. The titration revealed a strong increase in the amount of pentamer between 0.25–5 mM Mg\(^{2+}\) (Fig. 6, A and B). When the concentration of Mg\(^{2+}\) exceeded 5 mM, the reaction appeared saturated (fraction of cross-linked pentamers around ∼0.4). As expected for wild-type TmCorA, the fraction of cross-linked pentamers remained relatively constant in this assay (∼0.5), regardless of surrounding Mg\(^{2+}\) concentration (Fig. 6B). 5 mM Mg\(^{2+}\) was also effective in causing the other loop mutants to transit from lower oligomers to pentamers (Fig. 6C). From this analysis we conclude that the M1/M2-driven pentamerization response is induced at physiologically relevant concentration of Mg\(^{2+}\) (0.25–5 mM).

Taken together, the data indicate that Mg\(^{2+}\) sensing by the M1/M2 sites can be triggered at concentrations below 5 mM. However, a higher concentration of Mg\(^{2+}\) is required to keep all M1/M2 sites saturated (i.e. constantly occupied) in solution (Fig. 6 versus Fig. 4B).

**DISCUSSION**

The x-ray structures of TmCorA were obtained in the presence of high concentrations of divalent ions, and they provided an important framework for understanding how Mg\(^{2+}\) regulation may be accomplished (6–8). Unfortunately, all structures were locked in a single closed conformation because of Mg\(^{2+}\) binding in the cytoplasmic domain, leaving many fundamental aspects of Mg\(^{2+}\) uptake unanswered. Notably, when cytoplasmic Mg\(^{2+}\) levels are low, what does the open conformation look like? And how is the transition (gating) between an open and closed conformation sensed by the putative M1/M2-gating machinery? To address this question, we analyzed the oligomeric state of the purified wild-type TmCorA, regardless of whether 2 mM EDTA or 100 mM Mg\(^{2+}\) was present (Fig. 5D). This experiment shows that when the periplasmic loop is intact, TmCorA is not dependent on Mg\(^{2+}\) binding to the M1/M2 sites to remain pentameric. It also implies that the putative open loop-mediated conformation is stable in both high and low Mg\(^{2+}\).
closed channel achieved? We have probed these questions by performing biochemical analysis on the $Tm$ CorA pentamer in the absence as well as in the presence of Mg$^{2+}$.

We first wanted to understand which interprotomeric interactions are important to stabilize the $Tm$ CorA pentamer when the cytoplasmic Mg$^{2+}$/H$^{+}$ binding sites are unoccupied (i.e. a putative open conformation). A curious discrepancy directed our attention toward the conserved and functionally important periplasmic loop. Although the x-ray structures revealed that the five periplasmic loops in the pentamer were largely disordered (suggesting only a minor structural contribution), Pai and co-workers (9) reported that isolation of stable pentameric species was prevented when two residues in the loop were substituted to alanine (M313A and N314A). To determine whether the loop had a structural role when cytoplasmic Mg$^{2+}$/H$^{+}$ levels are low (as opposed to the high Mg$^{2+}$ conditions employed in the crystallization trials), we performed a systematic alanine scan of the entire loop region. Analysis of the 20 purified mutant proteins (each carrying a single alanine point mutation in the loop) revealed eight positions to be important for the integrity of the pentamer when Mg$^{2+}$ was absent, as they were primarily detected as lower oligomers. Correspondingly, we found a strong correlation between channel function and pentamer integrity, as revealed by an in vivo complementation assay.

Taken together, our in vitro and in vivo data strongly indicate that when Mg$^{2+}$/H$^{+}$ is absent from the cytoplasmic binding sites, there is a structurally uncharacterized conformation of $Tm$ CorA in which the periplasmic loops stabilize the pentamer. Intriguingly, we noted that seven scaffolding positions belonged to conserved CorA family signature loop motifs (see underlined residues $X_{310}XYGM\text{M}\text{N}E\text{X}X\text{M}P\text{E}L\text{L}X\text{G}Y\text{P}X_{329}$). Given the high evolutionary retention of these residues, it seems reasonable to assume that the structural role of the loop determined in this study is a general property in the entire CorA family. Although the structural details of the putative loop scaffold have not been resolved, we envision that its ultimate pur-
pose is to ensure an open pore and precise positioning of metal-coordinating residues.

We next wanted to determine how Mg$$^{2+}$$ affects TmCorA stability. Revealingly, by simply adding Mg$$^{2+}$$, the loop-destabilized mutants could transit from lower oligomers to pentamers. Importantly, this shows that Mg$$^{2+}$$ modulates how the TmCorA pentamer is stabilized and that the periplasmic loop is no longer critical for pentamer stability when Mg$$^{2+}$$ is present.

Closer inspection indicated that the Mg$$^{2+}$$-dependent pentamerization was concentration-dependent, reversible, and

FIGURE 6. Biologically relevant concentrations of Mg$$^{2+}$$ trigger M1/M2 mediated pentamer assembly. A, the purified Y311A loop-mutant was incubated in increasing concentrations of Mg$$^{2+}$$ and then cross-linked with DSS and analyzed by denaturing PAGE. B, plot showing fraction of pentamers as a function of Mg$$^{2+}$$ concentration (mM) for Y311A and wild-type TmCorA. A minimum of three repetitions was performed for each increment. C, the 20 alanine loop mutants were incubated in 5 mM Mg$$^{2+}$$ and then cross-linked with DSS and analyzed by denaturing PAGE.
relied on interprotomeric contacts supplied by the M1/M2 sites in the cytoplasmic domains. In light of the x-ray structures and biochemical and biophysical analysis (6–9, 12), our conclusion is that Mg\textsuperscript{2+} stabilized the loop mutants by inducing the closed channel conformation. Thus, if interpreted in terms of M1/M2-mediated gating and Mg\textsuperscript{2+} homeostasis, our data suggest that low concentrations of Mg\textsuperscript{2+} (< 5 mM) are sensed by TmCorA and can induce a transition between an open and closed conformation. This is in good agreement with other studies that have shown low concentrations of Mg\textsuperscript{2+} to have a stabilizing effect on the TmCorA protein (6). On the other hand, we find that high concentrations (> 100 mM) of Mg\textsuperscript{2+} are required to saturate the M1/M2 sites. This suggests that a high local concentration of Mg\textsuperscript{2+} is required to stabilize the closed conformation of the channel, perhaps explaining why a high concentration of divalent cations was reported to be a prerequisite for crystal growth.

In short, our data suggest that intracellular levels of Mg\textsuperscript{2+} determine whether the periplasmic loops or M1/M2 sites scaffold the pentamer and that this dictates if the ion conduction pore is opened or closed.

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