

Recent crystal structures of the CorA Mg$^{2+}$ transport protein from Thermotoga maritima (TmCorA) revealed an unusually long ion pore putatively gated by hydrophobic residues near the intracellular end and by universally conserved asparagine residues at the periplasmic entrance. A conformational change observed in an isolated funnel domain structure also led to a proposal for the structural basis of gating. Because understanding the molecular mechanisms underlying ion channel and transporter gating remains an important challenge, we have undertaken a structure-guided engineering approach to probe structure-function relationships in TmCorA. The intracellular funnel domain is shown to constitute an allosteric regulatory module that can be engineered to promote an activated or closed state. A periplasmic gate centered about a proline-induced kink of the pore-lining helix is described where “helix-straightening” mutations produce a dramatic gain-of-function. Mutation to the narrowest constriction along the pore demonstrates that a hydrophobic gate is operational within this Mg$^{2+}$-selective transport protein and likely forms an energetic barrier to ion flux. We also provide evidence that highly conserved acidic residues found in the short periplasmic loop are not essential for TmCorA function or Mg$^{2+}$ selectivity but may be required for proper protein folding and stability. This work extends our gating model for the CorA-Alr1-Mrs2 superfamily and reveals features that are characteristic of an ion channel. Aspects of these results that have broader implications for a range of channel and transporter families are highlighted.

The magnesium ion is essential for life and performs crucial roles in biology. As the major intracellular divalent cation, Mg$^{2+}$ is a required cofactor in hundreds of enzymes and is indispensable for maintaining genomic stability. The deregulation of Mg$^{2+}$ homeostasis has been implicated in numerous pathological states, including cardiovascular disease and tumorigenesis (1, 2). Of the four major biological cations, Mg$^{2+}$ transport and homeostasis remains the most poorly understood. These processes have been best characterized in prokaryotes, where detailed molecular insights have recently been obtained through structure determinations of the CorA and MgtE Mg$^{2+}$ transport systems (3–6), as well as those of the PhoQ and “M-box” regulatory components (7, 8).

CorA is the primary Mg$^{2+}$ uptake system of the Bacteria and Archaea domains (9); it can also functionally substitute for the distantly related eukaryotic Alr1 and Mrs2 proteins (10, 11). Alr1 and Mrs2 are the major Mg$^{2+}$ transporters found in the plasma and inner mitochondrial membranes of yeast, plants, and mammals. Thus, CorA represents a ubiquitous and highly relevant model Mg$^{2+}$ transport system. Three crystal structures of CorA from Thermotoga maritima (TmCorA) have been reported in an apparently closed conformation (3–5), establishing TmCorA as a representative member of the so-called 2-TM-GxN metal ion transporter superfamily (12, 13). TmCorA revealed a pentameric assembly with a large intracellular N-terminal funnel domain (FD) linked to a membrane-embedded ion pore. In each subunit, two transmembrane (TM) helices are connected by a short periplasmic loop, with the universally conserved GMN motif found at the C-terminal end of the pore-lining helix, TM1. Two cation binding sites, M1 and M2, have been identified by crystallography at the protomer-protomer interface within the FD (4, 5) and have been characterized as a “divalent cation sensor (DCS)” using a protease protection assay (5). A conformational change that was observed in the crystal structure of an isolated FD from Archaeoglobus fulgidus CorA (AfCorA) led us to propose a model for the structural basis of gating (Fig. 1A). These results implicated the DCS as part of a Mg$^{2+}$-sensing molecular switch that may coordinate CorA gating with the regulation of cellular activities and ion homeostasis (5).

The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–6.

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3. The abbreviations used are: TmCorA, Thermotoga maritima CorA; AfCorA, Archaeoglobus fulgidus CorA; TM, transmembrane; FD, funnel domain; DCS, divalent cation sensor; LOF, loss-of-function; GOF, gain-of-function; ACR, acetylcholine receptor; DDM, n-dodecyl-$\beta$-d-maltoside; TCEP, Tris(2-carboxyethyl)phosphine hydrochloride; IPTG, isopropyl-$\beta$-d-thiogalactopyranoside; NMDG-Cl, N-methyl-$\beta$-d-glucamine chloride; WT, wild type.
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Mg$_2^+$ levels (5); they also suggest that CorA might function as a ligand-gated ion channel.

Evidence indicates that the eukaryotic Alr1 and Mrs2 proteins operate as high conductance Mg$_2^+$-selective channels (14–16); however, a coupled transport mechanism for CorA cannot yet be ruled out. For instance, certain CorA-dependent microbes thrive in environments that may require magnesium to be concentrated against its chemical potential (17), and at least one CorA homologue has been characterized as a metal ion efflux system (18). Although TmCorA has been characterized structurally, its function has not yet been demonstrated. Some authors suggest that TmCorA is a metal ion effluxer (4, 19), whereas others have indicated a Mg$_2^+$ import system (3, 5, 13). Definitive assignment of the CorA proteins as channels or transporters will require further investigation, and an understanding of their gating mechanisms will aid in future analyses.

Traditionally, “gating” refers to the opening or closing of an ion channel in response to a physiological stimulus. Recent studies have established that gating events can range from slight backbone or side-chain movements (20, 21) to larger scale conformational and packing rearrangements (22–25). Providing these insights remains a prominent challenge, and it is unknown whether universal principles might underlie the fine details of ion channel or transporter function. We have recently proposed the following CorA gating model. As intracellular Mg$_2^+$ levels decline, loss of bound cations at the M1 and M2 sites in the DCS should lead to a charge repulsion at this interface, which ultimately destabilizes helix-packing interactions within the FD. A possible structural rearrangement between α5 and α6 (relative to α7) has been indicated based on the available crystal structures of TmCorA and AfCorA (Fig. 1A). These conformational transitions would impinge on intracellular and periplasmic gating residues through helix rotations and/or translations. Upon the withdrawal of positively charged residues found on α6, an intracellular “electrostatic sink” created within the FD interior may help drive ion permeation through the CorA pore. Although this model postulates extensive conformational changes (5), direct structural and/or functional evidence on the full-length protein is still lacking. Therefore, we set out to investigate critical components of this gating model and probe other structure-function relationships in the TmCorA metal ion transport protein.

Experimental Procedures

Protein Purification—TmCorA was cloned into pET-15b (26), and site-directed mutants were generated using the QuikChange protocol (Stratagene). All plasmids were confirmed by DNA sequencing. Proteins were expressed in _Escherichia coli_ BL21-CodonPlus(DE3)-RIL (Stratagene) and purified as described previously (26). In brief, TmCorA was solubilized in _n_-dodecyl-β-D-maltoside (DDM, Anatrace) and purified over nickel-nitrilotriacetic acid-agarose (Qiagen). The N-terminal His$_6^+$ tag was removed by thrombin digestion, and proteins were passed over a Superdex 200 column (Amersham Biosciences). Peak fractions containing TmCorA were passed over a second nickel column, and the flow-through was collected and concentrated using a centrifugal filter device (Millipore). The final protein buffer contained 20 mM Tris, pH 8.0, 100 mM NaCl, 0.026% DDM, 1 mM TCEP.

TmCorA Reconstitution and Fluorescence Assay—Reconstitution buffer containing 150 mM KCl, 8 mM HEPES, pH 7.2, was added to dried phosphatidylcholine (PC, Avanti Polar Lipids) to produce a final 10 mg/ml liposome preparation by sonication. Freshly purified TmCorA was mixed with an excess of phosphatidylcholine liposomes to produce a lipid:protein ratio of 10:1, and the protein/liposome mix was dialyzed against 4 liters of reconstitution buffer overnight. Membrane-impermeable Mag-Fura-2 (Molecular Probes) was dissolved in water to a final concentration of 10 μM, and liposomes with (or without) incorporated TmCorA were loaded with Mag-Fura-2 by sonication on ice. Mag-Fura-2-loaded liposomes were subsequently applied to a Sephadex G-50 column with potassium-free buffer (150 mM N-methyl-D-glucamine chloride (NMDG-Cl), 8 mM HEPES, pH 7.2) in order to remove extraliposomal potassium and generate an outward potassium gradient. Magnesium influx was initiated by adding 1–10 mM MgCl$_2$ and 10 μM vancomycin (where specified) to the liposome suspensions. Measurements made at 25 °C in 3 ml cuvettes containing 2 ml of liposome suspensions with stirring were conducted five times for each sample. All studies were performed on a set of TmCorA proteins that were purified and reconstituted at the same time, with the wild-type protein included in each set. Continuous recording of fluorescence emission intensity of Mag-Fura-2 at 509 nm was used to monitor the rate of magnesium influx. The rate of fluorescence change was determined by fitting the initial 50 s with a linear function (Prism software). The rate of fluorescence change was converted to an uptake rate on the basis of a calibration curve generated by preloading liposomes with EDTA and MgCl$_2$ in concentrations known to lead to free Mg$_2^+$ concentrations ranging from 10 μM to 10 mM. Uptake rates obtained for each set of proteins were compared using one-way analysis of variance (Prism software) with a secondary analysis between pairs (the Tukey test). p values less than 0.05 were considered statistically significant. To measure the Ca$_2^+$ permeability of TmCorA, 1–10 mM extraliposomal CaCl$_2$ and 10 μM vancomycin were added to proteoliposomes loaded with Mag-Fura-2, and measurements were performed as described above.

Cellular Complementation Assay—Salmonella typhimurium MM281, a strain devoid of all genomic Mg$_2^+$ transport systems (9), was transformed with the indicated TmCorA expression plasmids and plated on LB agar supplemented with 100 mM MgCl$_2$ and 50 μg/ml carbenicillin. Single colonies were grown in 2.5 ml of LB supplemented with 100 mM MgCl$_2$ and 50 μg/ml carbenicillin at 37 °C, 200 rpm. At an A$_600$ of ~2.0, IPTG was added to a final concentration of 0.1 mM, and cells were grown for another 2–3 h (A$_600$ ~ 3.5). Cell cultures were diluted 1:100 with fresh LB medium supplemented with 0.1 mM IPTG and 50 μg/ml carbenicillin, but without added MgCl$_2$, and serially diluted across a 96-well plate using the same “MgCl$_2$-free” LB medium. With a multi-blot pin tool (V&P Scientific), ~3 μl of the diluted cultures were plated in duplicate onto LB agar plates supplemented with 0.1 mM IPTG, 50 μg/ml carbenicillin, and the specified concentration of MgCl$_2$ (0.1–10 mM). Plates were...
incubated at 37 °C and imaged after 20 h. All mutant proteins were assayed on at least three separate occasions.

TmCorA protein expression levels in MM281 were extremely low owing to the lack of T7 polymerase in this Salmonella strain. To confirm membrane association for these proteins, 40-ml cultures were grown as described above. Cells were resuspended in 6 ml of lysis buffer (26) and sonicated in the presence of protease inhibitors. After low speed centrifugation (15,000 × g, 30 min), supernatant and membrane fractions were separated by ultracentrifugation (100,000 × g, 1 h) in a bench-top Airfuge (Beckman). Membrane and supernatant fractions were prepared in 10× SDS-PAGE sample buffer. Histagged proteins were detected by Western blot analysis using primary anti-His antibodies (Qiagen) following SDS-PAGE.

Circular Dichroism Spectroscopy—Proteins were diluted to 1 mg/ml in 20 mM Tris, pH 8.0, 100 mM NaCl, 0.026% DDM, 1 mM TCEP, and 2 mM EDTA (or 2 mM MgCl2, where specified). Thermal denaturation data were acquired on a circular dichroism spectrometer model 62A DS (Aviv) as described previously (5).

Analytical Gel Filtration Chromatography—For our standard analysis, proteins were diluted to 1 mg/ml in 20 mM Tris, pH 8.0, 100 mM NaCl, 0.026% DDM, 1 mM TCEP. 500 µl of sample was loaded into a 200-µl loop and passed over an analytical Superdex 200 (10/300 GL) gel filtration column at a flow-rate of 0.5 ml/min on an ÄKTA FPLC (Amersham Biosciences).

Protease Digestion Assay—For a 25-µl reaction volume, stock solutions were prepared to produce the final concentrations desired upon dilution. The reactions contained 19.5 µl of TmCorA protein (1.32 mg/ml), 5 µl of MgCl2 (0–10 mM), and 0.5 µl of trypsin (10 mg/ml, Sigma). Protein and MgCl2 solutions were mixed and equilibrated at 4 °C for 20 min; trypsin was then added, and reactions were incubated at 4 °C for 15 h. At 15 h, 25 µl of SDS-PAGE sample buffer was added, and samples were run immediately on 15% SDS-polyacrylamide gels.

Isothermal Titration Calorimetry—Experiments were performed using a VP-ITC calorimeter (MicroCal) at 25 °C. A 500 µM solution of wild-type (WT) TmCorA was placed in the sample cell and titrated with 1 mM MgCl2 prepared in the same buffer (20 mM Tris, pH 8.0, 100 mM NaCl, 0.026% DDM, 1 mM TCEP). Twenty-four injections were performed; the first injection was 0.5 µl followed by eight 2-µl, three 6-µl, five 10-µl, and seven 20-µl injections. When complete, the 1 mM MgCl2 solution was replaced by a 20 mM MgCl2 solution, and 18 further injections were performed into the same protein solution; one 0.5-µl injection followed by five 5-µl, four 10-µl, three 15-µl, and five 30-µl injections. The titration of mutant proteins was performed as described for the WT. Raw data were analyzed using the Origin 7.0 program and corrected for the ligand dilution heat by subtracting a control. Data sets representing the titration of each protein by 1 or 20 mM MgCl2 were fitted separately to estimate binding parameters. Data for the WT and L294V mutant were fit to the “two binding sites” model. Data obtained from the 1 mM MgCl2 titration of the Gly285Asp mutant were analyzed using the “sequential binding-six binding sites” function and, for the 20 mM MgCl2 titration, with the “sequential binding-four binding sites” function.

Figure Preparation—The open state model was prepared as described previously (5). Most structural figures were generated using MOLSCRIPT (27) and RASTER3D (28) except that Fig. 5A was rendered in PyMOL (29). The pore contour in Fig. 5C was determined using HOLE (30) and visualized with VMD (31). The sequence alignment in supplemental Fig. 4A was prepared using ClustalW (32) and adjusted manually after comparing a superposition of the TmCorA and AfCorA crystal structures. Structural superpositions, as shown in supplemental Fig. 6, were performed using LSQMAN (33).

RESULTS

Reconstituted TmCorA Mediates Mg2+ Flux—To first establish our function, we developed assays that specifically employed the TmCorA protein. Highly purified TmCorA was reconstituted into phosphatidylcholine-based liposomes loaded with the Mg2+-sensitive fluorescent dye Mag-Fura-2. In the absence of an inwardly directed Mg2+ gradient, TmCorA was capable of mediating Mg2+ influx at ambient temperatures (Fig. 1, B–E). Fig. 1B shows representative experiments where the fluorescence of Mag-Fura-2 increased over time in liposomes containing TmCorA. The addition of valinomycin, a K+-selective ionophore that should allow dissipation of intraliposomal charge accumulation under our experimental conditions, conferred a further increase in Mag-Fura-2 fluorescence and the rate of Mg2+ uptake (Fig. 1B–C, n = 5, p < 0.001). The TmCorA-mediated fluorescence response was dependent on the external MgCl2 concentration (Fig. 1D); there was no calcium-dependent response even in the presence of 10 mM extraliposomal CaCl2 and valinomycin (Fig. 1D, n = 5). Because Mag-Fura-2 exhibits a higher affinity for Ca2+ than for Mg2+, but a similar spectral response (Molecular Probes), these findings suggested that TmCorA is selective for magnesium ions over calcium ions. In fact, the magnesium uptake rate is over 100 times greater than that of calcium. In addition, Co(III)hexammine, a known inhibitor of other CorA superfamily members (15, 16, 34), also blocked Mg2+ influx through TmCorA (Fig. 1E). These results provide the first direct evidence that TmCorA mediates the selective flux of magnesium ions; they also imply that accessory proteins are not required for this function.

TmCorA Complements Mg2+-dependent Cellular Growth—TmCorA was reconstituted into a Salmonella strain devoid of all genomic Mg2+ transport systems, a strain otherwise capable of growth only when supplemented with sufficiently high Mg2+ concentrations (9). Compared with the relevant controls, the full-length WT TmCorA protein was clearly capable of reconstituting Mg2+ transport activity in vivo (Fig. 1F), but the MgCl2 concentrations required for complementation (~1.0–2.5 mM) were 2 orders of magnitude higher than anticipated from studies of other CorA proteins (35, 36). Because TmCorA derives from a bacterium with an optimal growth temperature of ~80 °C, these discrepancies may reflect the high thermostability of TmCorA. However, CorA from Methanococcus jannaschii, an archaean organism with optimal growth temperature at ~85 °C, has transport characteristics almost identical to its
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![Image of the TmCorA gating model and functional complementation assays.](Image)

**FIGURE 1.** TmCorA gating model and functional complementation assays. A, the presumed closed state of TmCorA (Protein Data Bank code 2HN2) is shown on the left, viewed perpendicular to the plane of the membrane. One protomer is colored as follows: yellow, a1-a4/β1-β7; green, a5; blue, α6; red, α7/TM1; purple, α8/TM2; black spheres, cations bound at the DCS. Regions under investigation in this work: 1, the DCS; 2, the a5 and α6 helices; 3, the α5-α6 loop; 4, the Pro-303 TM1-kink; 5, the Leu-294 hydrophobic pore constriction; 6, the periplasmic loop and GMN motif. A partial model of the open state based on the isolated AICorA FD structure (Protein Data Bank code 2HNI) is presented on the right. The a1-a4/β1-β7 region appears to move as a rigid body, whereas the a5 and α6 helices undergo a rearrangement with respect to one another and relative to α7. Arrow i indicates torque generated along α7 by the rotation of α6; this torque propagates onto the intracellular hydrophobic gate (Leu-294) and possibly activates the periplasmic gate (Pro-303). A proposed electrostatic interaction using 0.1% SDS led to a marked increase in the fluorescence signal (data not shown). Data are shown as mean ± S.E.M., n = 5. D, experiments were performed as described in B. Relative to empty liposomes, TmCorA proteoliposomes mediated significantly increased Mg²⁺ uptake rates with or without valinomycin (n = 5, *p < 0.001, and n = 5, **p < 0.001, respectively). As also indicated, valinomycin-treated TmCorA proteoliposomes mediated higher Mg²⁺ uptake rates compared with proteoliposomes without valinomycin (n = 5, ***p < 0.001). Data are shown as mean ± S.E.M., n = 5. E, a representative experiment (n = 5) showing that Co(III)hexamine inhibits Mg²⁺ flux through TmCorA in the presence of 10 µM valinomycin when 1 mM Co(NH₃)₆Cl₃ is preincubated with the Mag-Fura-2 loaded TmCorA proteoliposomes (intraliposomal: 0 mM MgCl₂, 150 mM KCl; extraliposomal: 5 mM MgCl₂, 150 mM NMDG-Cl). F, S. typhimurium MM281, a strain devoid of all genomic MgCl₂ transport systems (38). Although a detailed substrate profile for TmCorA will require further investigation, these results do not diminish the established roles for the CorA-Alr1-Mrs2 proteins in cellular Mg²⁺ homeostasis (2, 5, 9-11, 15, 16, 39-44). They do,
cytoplasmic fraction, and comparable levels of membrane association were demonstrated for most proteins under study (supplemental Fig. 1B). To diminish the possibility that changes in protein levels may account for some of the observed growth phenotypes, we pointed out the agreement between this complementation assay and other experimental approaches in which in vitro studies use purified proteins that have been normalized for protein concentration.

**Function and Stability of GMN Motif Mutants**—The first mutations we made targeted the universally conserved GMN motif of TmCorA. Extending reports that have indicated an essential role for this motif in the CorA superfamily (12, 35, 43), a LOF was observed for all alanine substitutions examined in the cellular complementation assay (Fig. 1F). For subsequent in vitro reconstitution experiments of the three GMN motif mutants (Fig. 2D), only the G312A protein could be isolated as a stable pentameric species using our standard purification protocol (26). We define a “stable pentameric species” as any TmCorA protein with an analytical gel filtration elution profile superimposable with that of the WT protein (supplemental Fig. 2, A–C; see “Experimental Procedures”). Therefore, irrespective of their confirmed expression and membrane association (supplemental Fig. 1B), we cannot rule out possible misfolding or topological defects in the M313A and N314A mutants. Nevertheless, these mutants did display some population of an apparent pentameric species when tested at higher protein concentrations (supplemental Fig. 2D; data not shown). Considering the high thermostability of all TmCorA proteins analyzed here (−80 °C, supplemental Fig. 2D), our results have implications for the folding and oligomerization of this class of membrane proteins (41, 45). Moreover, these findings warrant caution when interpreting LOF data available within the literature (16, 35, 41–43).

The DCS Is Involved in TmCorA Function—Having established two functional assays that employ TmCorA, we began to investigate components of our proposed CorA gating model. The M1 and M2 sites of the divalent cation sensor have been shown to bind a range of divalent cations, including Mg$^{2+}$, however, oppose the suggestion that TmCorA may function as a divalent cation efflux system (4, 19).

Relative to the WT protein, we have defined a gain-of-function (GOF) phenotype as the ability of TmCorA mutants to complement the growth of this *Salmonella* strain at lower Mg$^{2+}$ concentrations and/or cell densities and a loss-of-function (LOF) phenotype as the requirement for higher Mg$^{2+}$ concentrations and/or cell densities. All growth phenotypes were highly reproducible, and TmCorA proteins appeared to be expressed at similar, albeit extremely low, levels (supplemental Fig. 1). No TmCorA protein could be detected in the soluble fraction, and comparable levels of membrane association were demonstrated for most proteins under study (supplemental Fig. 1B). To diminish the possibility that changes in protein levels may account for some of the observed growth phenotypes, we pointed out the agreement between this complementation assay and other experimental approaches in which in vitro studies use purified proteins that have been normalized for protein concentration.

**FIGURE 2. Involvement of the divalent cation sensor in TmCorA function.** A, a view of the DCS with secondary structural elements colored as in Fig. 1A, with bound cations shown as black spheres. The Asp-89 and Asp-253 side chains coordinating the M1 site cation are colored green, where the M1 site forms an intimate interaction across this interface. Residues coordinating the M2 site cation are shown in yellow. B, the M1 site D253K mutation is modeled onto the closed state structure of TmCorA. C, the function of DCS mutants in the Salmonella-based cellular complementation assay with the WT protein and G312A mutant shown for comparison. D, the fluorescence-based Mg$^{2+}$ flux assay for the reconstituted D89K and D253K mutants with the WT and G312A mutant proteins shown for comparison (intraliposomal: 0 mM MgCl$_2$, 150 mM KCl; extraliposomal: 5 mM MgCl$_2$, 150 mM NMDG-Cl; 10 μM valinomycin). Each mutant exhibited a significant reduction in the Mg$^{2+}$ uptake rate relative to the WT protein (p < 0.001). Data are shown as mean ± S.E.M.
FIGURE 3. Involvement of the α5 and α6 helices in TmCorA function. A and B, residues discussed in the main text are shown on neighboring protomers in the closed state (A) and open state (B) models, with secondary structural elements colored as in Fig. 1A. WT residues are depicted on the colored subunit, and the V194E and E206R mutations are modeled on the gray subunit. Dashed lines (in A) indicate potential salt bridges. C, the cellular complementation assay for these α5 and α6 mutants, with the WT, G312A, and D253K mutant proteins shown for comparison. D, the fluorescence-based Mg\(^{2+}\) flux assay of the reconstituted V194E/E206R TmCorA double mutant (intraliposomal: 0 mM MgCl\(_2\), 150 mM KCl; extraliposomal: 5 mM MgCl\(_2\), 150 mM NMDG-Cl; 10 μM valinomycin). This mutant exhibited a significant reduction in the Mg\(^{2+}\) uptake rate relative to the WT protein (n = 5, p < 0.0001). Data are shown as mean ± S.E.M.

Co\(^{2+}\), and Ca\(^{2+}\) (3–5), and the intracellular funnel domain has been suggested to undergo a structural transition that may regulate CorA gating (5). Based on distance constraints, sequence conservation, and apparent binding affinity, the M1 site has been implicated as the primary regulatory metal binding site (3–5, 13). In contrast to the WT protein, a D253K mutation made within the M1 site affords TmCorA strong protection from trypsin proteolysis in the absence of divalent cations (5), suggesting that this lysine side chain mimics a bound cation (Fig. 2, A and B).

Although residual activity remains, the D253K mutant displayed a LOF phenotype in the cellular complementation assay (Fig. 2C). In addition, the rate of change of intraliposomal Mg-uptake rate relative to the WT protein (n = 5, p < 0.0001). Similarly, the reciprocal D89K mutation produced a LOF in both the cellular and fluorescence-based assays (Fig. 2, C and D, n = 5, p < 0.001). These data suggest that a salt bridge across the M1 site can partially maintain TmCorA in a closed or inactive conformation. This conclusion is supported by circular dichroism denaturation studies (Fig. 3A, A and B).

The replacement of Asp-253 by a large hydrophobic residue was predicted to disrupt metal binding at the M1 site (Fig. 2A) and perturb domain closure or packing within the FD. A D253W mutation produced only a small GOF, which was surpassed by the D253F mutant (Fig. 2C). Because these results are counterintuitive based on steric considerations alone, they may reflect the hydrogen-bonding potential of tryptophan and/or the relative hydrophobicity of phenylalanine. In contrast to the complementary results observed with the M1 site lysine mutants, an D89F mutation failed to produce any GOF (Fig. 2C), perhaps suggesting a rotation of the Phe-89 side chain into a position that allows for domain closure.

The above results directly implicate an involvement of the DCS in TmCorA function; they also confirm M1 as an important regulatory metal binding site and highlight the side chain of Asp-253 as a key position within the FD. However, they do not necessarily support a large scale structural rearrangement during gating. A conservative interpretation suggests that only small perturbations within the DCS can propagate effects onto TmCorA function.

A Role for α5 and α6 in TmCorA Function—Next, we focused on the α5 and α6 helices. These helices are juxtaposed in the closed state of TmCorA and postulated to undergo a dramatic structural rearrangement during gating (Figs. 1A and 3, A and B). Keeping sequence perturbations to a minimum, we anticipated that the V194E mutation may form a salt bridge with Arg-210 across the α5-α6 interface, whereas the E206R mutation might generate salt bridges with Glu-198 and Glu-204 (Fig. 3A). If these mutations reinforce the relative orientation of α5
and α6 with respect to the closed state (Fig. 3, A and B), then we would predict a LOF phenotype.

The V194E and E206R mutations produced a slight LOF in the cell-based assay, as did the V194E/E206R double mutant (Fig. 3C). Confirming our design, the V194E/E206R double mutant demonstrated a marked impairment of function in the fluorescence-based assay (Fig. 3D, n = 5, p < 0.0001). Circular dichroism denaturation studies were also consistent with a stabilization of the presumed closed state for both single and double mutants (supplemental Fig. 3C). Therefore, it appears that these mutations indeed reinforced the relative orientation of α5 and α6 with respect to the closed state. These LOF mutations were made far from the DCS (Fig. 1A), indicating that a second strategy could be used to engineer LOF phenotypes within the FD. Taken together, the above results may implicate a large scale structural rearrangement during gating.

**Decoupled Gating Mutants**—We have suggested previously that CorA gating occurs in a concerted manner, where gating events are initiated in one region of the structure and subsequently propagated across the molecule (5). As such, we reasoned that it should be possible to “decouple” these gating movements. Pursuing an insertional mutagenesis regime, four consecutive glycine residues were inserted at different positions within the TmCorA structure (Fig. 4A). These Gly4 insertions were placed between TM2 and the conserved C-terminal basic ring sequence (Gly4KKKKWL); within the periplasmic MPEL motif loop connecting TM1 to TM2 (Gly4MPEL); in the loop connecting α6 to α7 (Gly4α6α7); and within the α5-α6 loop (Gly4α5α6). A more detailed description of these insertion mutants is provided in supplemental Fig. 4.

LOF was observed for three of the Gly4 insertion mutants (Fig. 4B). Because these proteins did not form stable pentameric species as per our above definition (supplemental Fig. 2D, data not shown), we cannot conclude the precise nature of these defects, i.e., “decoupling” of gating or protein misfolding/destabilization. However, because the Gly4KKKKWL mutant maintained a moderate capacity to complement cell growth (Fig. 4B), it is possible that this insertion decoupled TmCorA gating to a small extent. By contrast, the Gly4 insertion within the α5-α6 loop resulted in a dramatic GOF (Fig. 4B). This result may support our claim that the dynamics of this loop region (the so-called CorA elbow) are critically involved in transmitting a structural transition within the FD that allosterically modulates TmCorA function (5).

**Evidence for a Structural Transition within the FD**—We next examined the effect of placing shorter glycine insertions into the α5-α6 loop. A biologically relevant threshold appears to be surpassed upon inserting two or more glycines into this loop, because the Gly1 insertion performed similar to the WT protein in the cell complementation assay (Fig. 4B). Implying that the threshold is biophysical in nature, and in accordance with predictions of our gating model (Fig. 1A), analytical gel filtration chromatography demonstrated an increase in Stokes radius for all mutants within this series except for the Gly1 insertion protein (Fig. 4C). Because these experiments were performed in the absence of added divalent cations, the elution profiles shown in Fig. 4C should represent a time-averaged conformation. Assuming that these glycine insertions perturb the packing

**FIGURE 4. Glycine insertion gating mutants.** A, initially, four consecutive glycine residues (Gly4) were inserted at the indicated locations (red asterisks) around the TmCorA structure. B, function of the glycine insertion mutants was investigated using the *Salmonella*-based cellular complementation assay. The WT protein and G312A mutant are shown for comparison. C, analytical gel filtration chromatography of the α5-α6 loop glycine insertion mutants compared with the WT protein. Equal volumes and concentrations of all proteins were run as described under “Experimental Procedures.” D, the established protease digestion assay of TmCorA demonstrates Mg2+-dependent protection from trypsin proteolysis (5). The α5-α6 loop Gly insertion mutants retain the ability to bind Mg2+ and become protected from proteolysis in a Mg2+ concentration-dependent manner but with an apparent reduced affinity for Mg2+ relative to the WT protein. E, fluorescence-based Mg2+ flux assay of the reconstituted α5-α6 loop glycine insertion mutants (intraliposomal: 0 mM MgCl2, 150 mM KCl; extraliposomal: 1 mM MgCl2, 150 mM NMDG-Cl, 10 μM valinomycin). Rates of Mg2+ uptake are shown relative to the WT protein. Except for the Gly4α5α6 protein (ND, no difference), each mutant exhibited an enhanced uptake rate (n = 5, p < 0.001). Data are shown as mean ± S.E.M.
and/or structural dynamics within this loop region, we may have anticipated a more titratable type of result. This expectation was essentially born out using the established protease protection assay (Fig. 4D) (5). It was also recapitulated in the fluorescence-based assay (Fig. 4E). In addition, isothermal titration calorimetry showed a clear alteration in the Mg$^{2+}$ binding properties of the Gly$_4$ insertion mutant relative to the WT protein (supplemental Fig. 5). We therefore propose that glycine insertions into the α5-α6 loop shift the TmCorA equilibrium toward the open state and that a significant structural transition does occur within the FD during gating.

We point out the likelihood that no gross conformational distortions have occurred within the α5-α6 loop glycine insertion mutants. These proteins all form pentamers (Fig. 4C), and their thermostability is comparable with the WT protein (supplemental Figs. 2D and 3D). With an anticipated reduction in binding affinity, these mutants also bind Mg$^{2+}$ (Fig. 4D, supplemental Fig. 5). Moreover, the Gly$_4$ insertion protein can be crystallized under the high divalent cation condition established for their thermostability is comparable with the WT protein (supplemental Fig. 1). As expected, the E316K and E320K mutants both showed a GOF in the cellular complementation assay (Fig. 5E), which was significant for the L294V and L294A substitutions ($n = 5, p < 0.001$, and $n = 5, p < 0.001$, respectively). In addition, the protease protection assay suggested that the Leu-294 mutations might be influencing the “open probability” of TmCorA (Fig. 5F). These observations match predictions made by our gating model, specifically, that loss of metal binding at the DCS should communicate through a conformational change to intracellular gating residues (5); only here, the reverse scenario also appears to be true. Isothermal titration calorimetry confirmed that the L294V mutant has altered Mg$^{2+}$ binding properties compared with the WT (supplemental Fig. 5).

**Activated TmCorA Proteins Can Rescue LOF Phenotypes**—We were interested to see whether the activated α5-α6 loop glycine insertion proteins described in this work might be useful tools to probe further structure-function relationships in TmCorA. The role of the short periplasmic MPEL motif-containing loop connecting TM1 to TM2, particularly the role of the highly conserved acidic residues, remains controversial. We have suggested that these acidic residues aid in protein folding and concentrate cations near the entrance of the CorA pore; we also noted that a structural transition within the FD might be transmitted to the mouth of the pore through TM2 and this intervening loop (5). Other authors have suggested that these highly conserved acidic residues might function as part of the “selectivity filter” in CorA (3, 13) or have misinterpreted our previous statements (37). To address these issues, we mutated Glu-320 of the MPEL motif, and because it appears to reside closer to the pore entrance (Fig. 6A), we mutated Glu-316 as well.

As expected, the E316K and E320K mutants both showed a LOF (Fig. 6B); however, the E316K mutant retained a slight capacity to complement cellular growth, and the E320A mutant showed an even greater level of complementation (Fig. 6B). The E316K protein could be isolated as a stable pentameric species and, although to a lesser extent, so too could the E320A mutant (supplemental Fig. 2C). Although the membrane association of these mutant proteins does not appear to have been compromised (supplemental Fig. 1B), the E320K protein could not be readily isolated as a stable pentameric species (supplemental Figs. 2, C and D). Nevertheless, our data indicate that these highly conserved acidic residues are not essential for TmCorA ion conduction pathway, where the narrowest one is formed by the highly conserved residues Leu-294 and Met-291 (Fig. 5C, supplemental Fig. 4A) (3–5). Although values vary slightly among the available crystal structures, the pore diameter measures ~2 Å at this location, raising the possibility that a “hydrophobic gate” may exist in TmCorA. To test this hypothesis we mutated Leu-294 to smaller hydrophobic residues.

The L294I, L294V, and L294A mutations all produced a GOF in the cellular complementation assay that increased with decreasing side-chain volume (Fig. 5D). Considering the more intracellular pore constrictions found at Leu-280 and Met-291 (Fig. 5C), it is noteworthy that the L280A and M291A mutants both failed to produce any GOF (Fig. 5D). The effect seen at Leu-294 therefore indicates that a hydrophobic gate may be operational within TmCorA at this location. The smaller hydrophobic residues at the 294 position also produced a GOF in the fluorescence-based assay (Fig. 5E), which was significant for the L294V and L294A substitutions ($n = 5, p < 0.001$, and $n = 5, p < 0.001$, respectively). In addition, the protease protection assay suggested that the Leu-294 mutations might be influencing the “open probability” of TmCorA (Fig. 5F). These observations match predictions made by our gating model, specifically, that loss of metal binding at the DCS should communicate through a conformational change to intracellular gating residues (5); only here, the reverse scenario also appears to be true. Isothermal titration calorimetry confirmed that the L294V mutant has altered Mg$^{2+}$ binding properties compared with the WT (supplemental Fig. 5).
function, which is consistent with recent work on the eukaryotic Alr1 and Mrs2 homologues (41, 42).

We combined the Gly_4 insertion with the MPEL motif loop mutants. In this context, both the E316K and E320A proteins became activated, and the Gly_4/E316K/E320A triple mutant could also complement cellular growth (Fig. 6B). However, the Gly_4 insertion could not rescue any function for the E320K mutants (Fig. 6B). We therefore suggest that the E320K mutation affects the proper folding and/or stability of TmCorA (supplemental Fig. 2, C and D) and, by extension, that an analogous mutation may impart similar defects in Mrs2 (16). Nevertheless, the activating Gly_4 insertion can clearly rescue LOF mutations in a dominant manner. Critically, the apparent selectivity for Mg^{2+} over Ca^{2+} has not been drastically compromised in the Gly_4/E316K/E320A triple mutant relative to the WT protein (n = 5) or the Gly_4/E316K mutant (Fig. 6C, n = 5). In fact, the triple mutant still fails to support Ca^{2+} influx even at 10 mM extraliposomal CaCl_2 concentration.
FIGURE 6. Non-essential role of acidic residues in the periplasmic loop. A, based on the electron density observed for the periplasmic loop connecting TM1 to TM2 (5), the approximate locations of Glu-316 and Glu-320 are shown. The α5-α6 loop Gly5 insertion is also indicated for reference. B, basic or neutral substitutions were examined at the Glu-316 and Glu-320 positions in the cell complementation assay, with the WT protein and G312A mutant shown for comparison. Single and double mutants were then combined with the activating α5-α6 loop Gly5 insertion mutation and also studied. C, in vitro Mg2+ and Ca2+ fluorescence-based flux assays (see “Experimental Procedures”). First, the rate of fluorescence change/uptake in response to Ca2+ (intraliposomal: 0 mM CaCl2, 150 mM KCl; extraliposomal: 1 mM CaCl2, 150 mM NMDG-Cl; 10 mM Val) for the Gly5 mutant was increased compared with the WT protein (n = 5, *, p < 0.05). However, this likely reflects an increase in open time for this mutant, in addition to the high affinity of Mag-Fura-2 for Ca2+ (Molecular Probes). Considering the extent of Mg2+ flux activation afforded by this insertion (n = 5, *, p < 0.001), the change in Ca2+ flux is simply proportional. Second, there is a significant effect (n = 5, *, p < 0.001) on the Mg2+ uptake rate for the Gly5/E316K/E320A triple mutant (intraliposomal: 0 mM MgCl2, 150 mM KCl; extraliposomal: 1 mM MgCl2, 150 mM NMDG-Cl; 10 µM valinomycin) but no significant difference in the Ca2+-dependent fluorescence compared with the WT protein (n = 5) or the Gly5 insertion mutant (n = 5). These results suggest that the E316K and E320A substitutions do not drastically affect the selectivity of TmCorA for Mg2+. Data are shown as mean ± S.E.M. ND, no difference.
centrations (n = 5, data not shown). Thus, our present data do not support the suggestion that these acidic loop residues might function as part of the selectivity filter in TmCorA (3, 13). However, the effect of the Gly_{α5α6}/E316K/E320A triple mutant on the Mg^{2+} uptake rate (Fig. 6C, n = 5, p < 0.001) is consistent with the notion that Glu-316 and Glu-320 play a role in concentrating cations near the periplasmic pore entrance (5, 16). Thus, CorA appears to utilize at least one type of electrostatic sink, a property that it shares with numerous other ion channel and transporter proteins (46).

DISCUSSION

Our main goal was to investigate the structure-function relationships underlying the gating mechanisms operating in TmCorA. We first demonstrated that the DCS is unequivocally involved in TmCorA function (Fig. 2, C and D). The DCS can be engineered to promote an inactive or closed conformation, likely through salt bridge formation across the M1 cation binding site. It was also possible to access an activated or open state by placing a bulky hydrophobic residue at the Asp-253 location, perhaps by perturbing the closed state structure. Beyond the DCS, we have implicated the funnel domain (FD) in TmCorA function in a more general way. Presumably by engineering salt bridges across the closed state α5-α6 interface, we created a second LOF mutant series (Fig. 3, C and D). Furthermore, our biophysical characterization of the α5-α6 loop glycine insertion mutants supports the notion that a significant structural transition within the FD influences TmCorA activity (Figs. 4, B–E, and 6, B and C, and supplemental Fig. 5). Taken together, these data are consistent with the structural features postulated in our gating model, and they confirm that the FD constitutes an intracellular allosteric regulatory module.

Because we previously established that the DCS is capable of sensing Mg^{2+} concentrations within a physiologically meaningful range (5), TmCorA conforms to the modular architecture of an intracellular regulatory domain linked to a membrane-embedded catalytic pore. This organization has emerged as a common structural theme in ion channel and transport proteins, including the MgtE Mg^{2+} transport system (6), and it is reminiscent of the acetylcholine receptor (47, 48) and K+ channel structures (49, 50). Critically, the positioning of ligand binding sites at subunit or domain interfaces may serve to propagate concerted conformational changes across these proteins. In this way, ligand binding (or unbinding) can be fundamentally and thermodynamically coupled to gating events.

We further aimed to identify residues with specific functional roles in TmCorA gating. The Asn-314 side chain of the GMN motif tapers over the periplasmic pore entrance in two of the available crystal structures (supplemental Fig. 6) (3, 5); however, the N314A mutation detrimentally affected both the function and stability of TmCorA (Fig. 1E, supplemental Fig. 2B). Mutations to Pro-303 in TM1 were also destabilizing but provided a dramatic GOF (Fig. 5B). Extending the view that proline residues may be functionally linked to the gating mechanism of a channel or transporter, a proline appears to be universally conserved across the CorA-Alr1-Mrs2 superfamily near this position in TM1 (supplemental Fig. 4A, data not shown). Our results imply that a “straightening” of the TM1 helix is sufficient to gate CorA; and modeling of the P303I mutation further indicates a displacement of TM2 (Fig. 5A). Therefore, in addition to the FD, the region surrounding Pro-303 appears to undergo a conformational transition during gating. This should be compatible with the opening of a “carbonyl funnel” at the periplasmic mouth of the TmCorA pore (4), as suggested by the available crystal structures (supplemental Fig. 6). Thus, Pro-303 and its surrounding region may operate in ways that are analogous to the “gating hinge” and “activation gate” which are well known in K+ channels (22, 50, 51). This notion is consistent with the high conservation of Gly-312 as well as the universal conservation of Gly-312 in the GMN motif. Accordingly, we have shown that Gly-312 is required for TmCorA function (Figs. 1F and 2D).

Our present analysis suggests that at least three distinct regions in TmCorA could have functionally coupled roles in gating. The FD constitutes an intracellular regulatory module that might communicate through different structural elements to an intracellular hydrophobic gate located at Leu-294 and to the Pro-303-based periplasmic gate (Fig. 7). To our knowledge, the concept of a hydrophobic gate was first substantiated by structural studies of the acetylcholine receptor (ACR) (47). Compared with the ACR open state structure, hydrophobic side chains are rotated into the ion conduction pathway in the ACR closed state structure (48). Although these hydrophobic gating residues do not sterically impede ion conduction per se, computational studies describe an energetic barrier to ion conduction (52, 53), possibly through the occlusion of water (53). Biophysical analyses of the ACR have implicated these hydrophobic gating residues in determining the channels open probability (54), suggesting an intimate coordination between gating events and distal ligand binding sites (55).

We have provided evidence that a hydrophobic gate exists within TmCorA. Our functional assays imply that reducing the side chain volume at Leu-294 may decrease the energetic and/or kinetic barrier to ion permeation (Fig. 5, D and E). In addition to local hydrophobicity, these data implicate steric occlusion as another possible level of control. Considering results from the protease protection assay and isothermal titration calorimetry (Fig. 5E, supplemental Fig. 5), and by analogy with the ACR (48, 54, 55), we suggest that the Leu-294 hydrophobic gate may also influence the open probability of TmCorA (see below). Further studies will be needed to confirm or refute these possibilities. Nevertheless, it is noteworthy that the ACR is primarily a monovalent cation channel, whereas TmCorA is a divalent cation transport system. This raises the possibility that a common gating mechanism may be operating in both monovalent and divalent cation systems. In fact, a hydrophobic gate has been indicated in K+ (50), Na+ (56), ammonia (57), and mechanosensitive channels (25), as well as the protein-conducting translocon (58), ABC transporters (24, 59), and voltage sensors (60). Therefore, these studies begin to highlight the “hydrophobic gating” hypothesis as a general principle underlying the structure and function of diverse channel and transporter proteins.

It remains unproven whether TmCorA functions as an ion channel or transporter. We submit that the biophysical properties and activation afforded by the α5-α6 loop glycine inser-
tion mutants (Figs. 4, B–E, and 6, B and C, and supplemental Fig. 5) would be inconsistent with the “alternating access” models that are commonly indicated for many transporters (23, 24, 61). We have provided the first direct evidence that purified, reconstituted TmCorA mediates the transport of magnesium ions and that TmCorA can complement Mg\(^{2+}\) influx \textit{in vivo}. As anticipated for an ion channel, and like its bacterial and eukaryotic counterparts (14–16, 44), Mg\(^{2+}\) influx by TmCorA appears to be electrogenic and driven by the membrane potential. Our results seem difficult to reconcile with the suggestion that TmCorA would function as a metal ion efflux system or that its substrate selection occurs before the hydrophobic gate, and assuming that substrate selection occurs before the hydrophobic gate, we propose that the selectivity filter of CorA resides in the neighborhood of the periplasmic Pro-303 gate. Beyond the GMN motif and Pro-303, we find it curious that there are no universally conserved residues found along the pore but note that the distribution of hydrophobic and hydroxyl-bearing pore-lining residues appears to be largely maintained (supplemental Fig. 4A). This raises the possibility that selectivity for Mg\(^{2+}\) perhaps arises from some “bulk” property of the ion conduction pathway.

In summary, we have identified three distinct regions in TmCorA that may have tightly coupled roles in gating and have highlighted general principles that underlie the structure and function of diverse channel and transport proteins. The present work extends our previously proposed gating model for the CorA-Alr1-Mrs2 superfamily (Fig. 7) and reveals features that are characteristic of an ion channel. Future studies must focus on fundamental mechanistic questions that remain unresolved within the CorA-Alr1-Mrs2 superfamily, hopefully in parallel to the elucidation of a high-resolution open state structure.

Acknowledgments—We thank Michael Maguire (Case Western Reserve University) for providing the Salmonella strain used in this work and Nilu Chakrabarti (University of Toronto) for help in preparing Fig. 5C. We also acknowledge the kind support of Emily Cowan.

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FIGURE 7. \textbf{Schematic TmCorA gating model.} Structural elements are \textit{colored} as in Fig. 1A (the \(\alpha1-\alpha4/\beta1-\beta7\) region has been omitted), and only two subunits are shown for clarity. \textit{Black spheres} represent the locations of cations bound in the available crystal structures (3–5). The closed state (\textit{A}) reflects high or adequate intracellular Mg\(^{2+}\) concentrations, with cations bound at the DCS. When intracellular Mg\(^{2+}\) concentrations decline, loss of cation binding at the DCS produces a charge repulsion that destabilizes interactions between the intracellular electrostatic sink residues (ESR) and neutralizing residues (NR), initiating a structural transition to the open state (\textit{B}). This reorganizes the acidic ring (AR) found on the FD exterior, which may interact with the basic ring (BR) that sits atop TM2; this possibly communicates gating events to the periplasmic mouth of the pore through the MPEL motif loop. Rotation of \(\alpha6\) around \(\alpha7\) generates torque along \(\alpha7\), which produces a gating force on the hydrophobic gate (HG) and possibly the periplasmic Pro-303 gate. The Asn-314 side chains are ultimately displaced from the pore entrance as the periplasmic gate region undergoes a structural transition, which also exposes a carbonyl funnel (CF). The carbonyl funnel should provide coordination sites for cations (probably at least partially hydrated) entering the CorA pore. Although Glu-316 and Glu-320 in the periplasmic loop are not absolutely essential for function or selectivity, these acidic residues likely concentrate cations (and repel anions) near the periplasmic pore entrance.
