The CorA Mg\(^{2+}\) Transport Protein of *Salmonella typhimurium*

**MUTAGENESIS OF CONSERVED RESIDUES IN THE THIRD MEMBRANE DOMAIN IDENTIFIES A Mg\(^{2+}\) PORE***

(Received for publication, June 19, 1998, and in revised form, August 10, 1998)

Ronald L. Smith‡, Mary Ann Szegedy§, Lisa M. Kucharski, Carin Walker¶, Richard M. Wieti, Allison Redpath, Michelle T. Kaczmarek, and Michael E. Maguire**

*From the Department of Pharmacology, School of Medicine, Case Western Reserve University, Cleveland, Ohio 44106-4965*

The CorA transport system is the major Mg\(^{2+}\) influx pathway for bacteria and the Archaea. CorA contains three C-terminal transmembrane segments. No conserved charged residues are apparent within the membrane, suggesting that Mg\(^{2+}\) influx does not involve electrostatic interactions. We have mutated conserved residues within the third transmembrane segment to identify those involved in transport. Mutation of conserved aromatic residues at either end of the membrane segment to alternative aromatic amino acids did not affect total cation uptake or cation affinity. Mutation to alanine greatly diminished uptake with little change in cation affinity implying that the conserved aromatic residues play a structural role in stabilizing this membrane segment of CorA at the interface between the bilayer and the aqueous environment. In contrast, mutation of Tyr\(^{292}\), Met\(^{299}\), and Tyr\(^{307}\) greatly altered the transport properties of CorA. Y292F, Y292S, Y292C, or Y292I mutations essentially abolished transport, without effect on expression or membrane insertion. M299C and M299A mutants exhibited a decrease in cation affinity for Mg\(^{2+}\), Co\(^{2+}\), or Ni\(^{2+}\) of 10–50-fold without a significant change in uptake capacity. Mutations at Tyr\(^{307}\) had no significant effect on cation uptake capacity; however, the affinity of Y307F and Y307A mutations for Mg\(^{2+}\) and Co\(^{2+}\) was decreased 3–10-fold, while affinity for Ni\(^{2+}\) was unchanged compared with the wild type CorA. In contrast, the affinity of the Y307S mutant for all three cations was decreased 2–5-fold. Projection of the third transmembrane segment as an \(\alpha\)-helix suggests that Tyr\(^{292}\), Met\(^{299}\), and Tyr\(^{307}\) all reside on the same face of the \(\alpha\)-helix. We interpret the transport data to suggest that a hydroxyl group is important at Tyr\(^{307}\), and that these three residues interact with Mg\(^{2+}\) during transport, forming part of the cation pore or channel within CorA.

CorA is a high capacity, constitutively expressed Mg\(^{2+}\) transport system of *Salmonella typhimurium* (1, 2). The CorA locus encodes a single polypeptide of 316 amino acids with a predicted molecular mass of 37 kDa which is sufficient by itself to mediate the uptake of Mg\(^{2+}\). CorA mediates the influx of Mg\(^{2+}\) with an affinity of about 20 \(\mu\)M and can also mediate the influx of Ni\(^{2+}\) and Co\(^{2+}\), albeit only at extracellular concentrations that are toxic to the cell. The amino acid sequence of CorA lacks homology to other known families of proteins (3). Studies of its phylogenetic distribution (4) and the recent plethora of microbial genome sequences have demonstrated that CorA is virtually ubiquitous in bacteria and the Archaea and likely forms the major Mg\(^{2+}\) influx system for these kingdoms (5).

The membrane topology of CorA has been previously studied using C-terminal protein fusions to \(\beta\)-lactamase and \(\beta\)-galactosidase (3). Like its amino acid sequence, its membrane topology is also unlike that of other known transport proteins. The N-terminal 235 residues reside in the periplasm while the remaining 80 amino acids form three transmembrane segments, including a short 6 residue C-terminal sequence in the cytosol. Three transmembrane segments are unlikely to be sufficient to form a transport channel or pore; thus, CorA probably functions as a homoligomer.

The mechanism of ion transport through CorA may also be unique. Unlike many ion transporters and channels, CorA contains only two charged residues, both within the first membrane domain; neither is conserved in other homologs (5). This suggests that the most charge dense of the common biological cations passes through the membrane without involvement of electrostatic bonds. Mg\(^{2+}\) coordinates virtually exclusively with oxygen rather than nitrogen or sulfur (6) which suggests backbone carbonyl groups and hydroxyl bearing residues within the membrane environment would be important. Sequence alignment of the CorA homologs currently available suggest a high degree of conservation of such groups in the second and third transmembrane segments. In this study, conserved residues in the third transmembrane segment (TM3)\(^{3}\) of CorA were mutated. All but one mutation resulted in stable expression of protein and protein insertion into the membrane. Conserved residues Phe\(^{290}\), Tyr\(^{309}\), and Phe\(^{310}\) at the termini of TM3 could be substituted by other aromatic residues without significant change in transport properties suggesting a structural rather than a transport role. In contrast, mutations at Tyr\(^{292}\), Met\(^{299}\), and Tyr\(^{307}\) showed large decreases in transport capacity and/or changes in cation affinity. We suggest that these three residues form part of the Mg\(^{2+}\) transport pathway within CorA.

---

* This work was supported in part by United States Public Health Service Grant GM39447 (to M. E. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by Metabolism Training Grant DK07319. Current address: Dept. of Biology, University of Texas at Arlington, Arlington, TX 76019.

§ Supported by Cell and Molecular Biology Training Grant GM08056 during this work.

¶ Supported by a Summer Undergraduate Research Fellowship from the American Society of Pharmacology and Experimental Therapeutics.

‖ Supported by a Summer Undergraduate Research Fellowship from the American Society of Pharmacology and Experimental Therapeutics.

** To whom correspondence should be addressed: Dept. of Pharmacology, School of Medicine, Case Western Reserve University, 10900 Euclid Ave., Cleveland, OH 44106-4965. Tel.: 216-368-6186; Fax: 216-368-3395; E-mail: mem6@po.cwru.edu.

1 The abbreviation used is: TM3, third transmembrane segment of CorA.
MATERIALS AND METHODS

All media were obtained from Difco (Detroit, MD). All other reagents were from Sigma unless otherwise specified. Oligonucleotides were purchased from Oligos, Etc. (Wilsonville, OR) or Genosys (The Woodlands, TX). N-Minimal medium (7) was modified to include 0.4% (w/v) glucose, 0.1% casamino acids and is referred to as supplemented N-minimal medium. When present, Mg^{2+} was added as MgSO_{4}. In all media, or by automated sequencing at the Cleveland Cloning Laboratory. Mutant verified by sequencing the plasmids with Sequenase (Amersham, Arlington Heights, IL) and propagated in Escherichia coli DH5a. Mutations were verified by sequencing the plasmids with Sequenase (Amersham, Arlington Heights, IL) and propagated in Escherichia coli DH5a. Veriﬁed mutants were transferred by transformation to S. typhimurium JR501 for restriction modiﬁcation before transformation into S. typhimurium MM281, a Mg^{2+} transport-deﬁcient strain (2) for transport assays. For determination of the ability of the various mutant strains to grow, each was streaked on Luria-Bertani (LB) plates containing the appropriate antibiotic overnight. Single colonies were then streaked out on N-minimal plates containing glucose and 0.1 mM leucine and incubated at 37 °C for 48 h.

Western Blot Analysis—Overnight 25-ml cultures grown in LB broth with antibiotic were pelleted and resuspended in 1 ml of 10 mM Tris, 150 mM NaCl, pH 7.5, and passed through a French press at 12,000 psi. Cell debris and unlysed cells were pelleted by centrifugation at 16,000 × g for 20 min. The supernatant was centrifuged at 100,000 × g for 1 h at 4 °C. The membrane fraction was collected by centrifugation of the supernatant at 100,000 × g for 1 h at 4 °C. Membrane samples were resuspended in the same buffer were loaded on 10% SDS-polyacrylamide electrophoresis gels at 10 μg of protein/lane. Protein concentration was determined using the BCA assay (Pierce, Rockford, IL). A polyclonal antibody directed against a peptide of the N-terminal 16 residues of CorA was made in rabbits (Quality Controlled Biochem., Hopkinton, MA) and used for Western blot analysis. Protein was visualized by enhanced chemiluminescence (ECL, Amersham).

Transport—Uptake of 65Ni^{2+} was performed as described previously (8, 9). Briefly, cultures of the appropriate mutant in MM281 were grown overnight in LB broth containing 100 mM Mg^{2+} and the appropriate antibiotic. Cells were washed twice with ice-cold N-minimal medium without added Mg^{2+}, resuspended in supplemented N-minimal medium, and adjusted to an OD_{600} between 1.0 and 2.0 in the same medium. Cells could be kept on ice up to 1 h before initiation of the transport assay without loss of uptake capacity. Transport was initiated by adding 0.1-mL cells to 0.9 ml of supplemented N-minimal medium at 37 °C containing 200 μM Ni^{2+} (or as indicated), 0.1–1.5 μCi of 65Ni^{2+} per tube and the indicated Mg^{2+}, Ni^{2+}, or Co^{2+} concentration. Uptake was terminated after 5 min by addition of 5 ml of ice-cold transport wash buffer (N-minimal medium without glucose and casamino acids, 5 mM Mg^{2+} and 1 mM EDTA). Samples resuspended in the same buffer were filtered through BA85 (0.45 μm) nitrocellulose filters (Schleicher and Schuell, Keene, NH), and washed once with 5 ml of ice-cold wash buffer.

65Ni^{2+} activity was determined by scintillation counting with an efficiency of >80%. Wild type uptake was about 1–1.5 nmol of Ni^{2+} min^{-1} OD_{600}^{-1}. This corresponded to a range of 5 × 10^3 to 1.3 × 10^4 cmp uptake in each sample aliquot depending on the amount of 65Ni^{2+} used. The nonspecific background level of 65Ni^{2+} binding to the filter and cells was 500–3000 cpm over the range of 65Ni^{2+} used.

Transport was generally measured by determining the ability of Mg^{2+}, Ni^{2+}, or Co^{2+} to inhibit the uptake of 65Ni^{2+}. The wild type activity of CorA for Ni^{2+}, Mg^{2+}, and Co^{2+} was about 20, 300, and 30 μmol, respectively. In these assays, unless otherwise indicated, the Ni^{2+} concentration was set at 200 μM. This is approximately equal to the Km of Ni^{2+} uptake by the wild type CorA transporter and is roughly comparable to the Km for an enzyme. The maximal transport capacity of the system will be referred to as V_{max} and is roughly comparable to this same parameter for an enzyme. If K_{i} for Ni^{2+} is not changed by a mutation, then the maximal uptake measured is directly proportional to the V_{max} of the transport system when 65Ni^{2+} is used as the test radiolabeled cation. If K_{i} for Ni^{2+} is changed by a mutation, then the V_{max} or maximal uptake capacity cannot be directly compared with that of the wild type transporter. Conversely, however, regardless of whether a mutation changes V_{max}, the apparent inhibition constant measured in this manner directly reflects the apparent affinity of the cation for the system as a whole as long as the dose-response curves are normalized to the maximal uptake of the individual mutant within each individual experiment. Thus if the dose-response curves are plotted as a percent of the maximal uptake of that particular mutant protein within a given experiment, then the apparent K_{i} values may be directly compared with those determined for the wild type protein. In practice, transport was performed on several strains carrying mutant CorA alleles at the same time. Control dose-response curves measuring cation inhibition of uptake by the wild type CorA allele were performed within each experiment; unless otherwise stated, comparisons of cation inhibition between strains are always within rather than between experiments. Apparent cation affinities between experiments did not vary more than 2-fold for the same mutant allele.

RESULTS

Recent genomic sequence data has provided almost 30 examples of presumed CorA homologs in both bacteria and Archaea (5). Since Mg^{2+} is the most charge dense of biological cations, one might expect it to interact with negatively charged residues during passage through the membrane domains of the transport. However, among the three transmembrane segments of all the CorA homologs, only the first transmembrane segment carries charge. In the S. typhimurium CorA, Glu^{251} is the only negative charge within the membrane, but a negative charge at this or nearby positions is not conserved in the other CorA homologs (5). Moreover, mutation of Glu^{251} to Ala had minimal effect on transport capacity (Table I) or apparent Mg^{2+} affinity (data not shown), suggesting that electrostatic interactions within the membrane domains of CorA are not required for Mg^{2+} uptake.

We therefore turned our attention to more conserved domains within the CorA family of which the most highly conserved is the third transmembrane segment (TM3). An alignment through the TM3 regions of a selection of currently available CorA homologs is shown in Fig. 1. Assuming that the TM3 domain is bounded by the charged residues at each end, it is about 21 residues in length. Within this segment, there is a high degree of conservation for an aromatic residue at position 290, the sequence 290GYPP293, and a methionine at position 299, and more moderate conservation for a tyrosine at position 307 and aromatic residues at positions 309 and 310. We therefore mutated each of these positions and measured the resulting effect on formation of the CorA protein and its activity. Most positions were changed to alanine since this is a relatively benign structural substitution and would not be expected to disrupt the presumed transmembrane α-helix. Additional substitutions were chosen to conserve size or functionality or both as far as was possible. The mutants made and a summary of their properties is listed in Table I.

Mutation of virtually all of these conserved residues had little effect on formation and membrane association of CorA (Fig. 2). Only the F290A mutant could not be detected in significant quantity; extended exposure indicated a small amount of F290A CorA present, roughly estimated at ~3% of the wild type level (not shown). The fact that each mutant was expressed at about the same level as the wild type CorA indicated that a direct comparison of wild type and mutant transport activity was largely reflective of the actual transport capacity of each CorA molecule and did not reflect any significant contribution from varying levels of protein expression. Since each mutant was being expressed from a multicopy plasmid, we also minimal plates containing some of the most mutable cases. In all cases, including the wild type CorA expressed from pRS170, some immunoreactive material was found in inclusion bodies; however, the amount did not appear to vary between the various CorA proteins expressed (not shown). We concluded that comparison of total uptake normalized to OD_{600} between mutants was therefore valid as a measure of relative uptake capacity.

Mutations of Aromatic Residues at the Membrane Interface—Phe^{290}, on the periplasmic side of TM3, was mutated to another
CorA $\text{Mg}^{2+}$ Transport Protein of S. typhimurium

**Table I**

Properties of mutant CorA transport systems

| Residue | Mutation | Protein expression at wild type level | Fold decrease in $\text{Mg}^{2+}$ affinity | Percent of wild type transport |
|---------|----------|-------------------------------|---------------------------------|-------------------------------|
| Glu$^{251}$ | A        | Y                             | 1                               | 87 ± 2                        |
| Phe$^{290}$ | W        | Y                             | 1                               | 99 ± 6                        |
| Gly$^{291}$ | A        | N                             | 3                               | <2                           |
| Tyr$^{292}$ | C        | Y                             | >10                             | 48 ± 14                       |
|          | F        | Y                             | >10                             | <2                           |
|          | I        | Y                             | >10                             | <2                           |
|          | S        | Y                             | >10                             | <2                           |
| Pro$^{293}$ | A        | Y                             | 50                             | 7.2                          |
| Met$^{299}$ | C        | Y                             | 50                             | 3 ± 1                        |
| Tyr$^{307}$ | A        | Y                             | 10                             | 75 ± 10                      |
|          | S        | Y                             | 3                              | 57 ± 4                       |
| Tyr$^{309}$ | A        | Y                             | 1                              | 102 ± 21                     |
|          | F        | Y                             | 1                              | 84 ± 2                       |
| Phe$^{310}$ | A        | Y                             | 10                             | <2                           |
|          | Y        | Y                             | 1                              | 69 ± 24                      |
|          | W        | Y                             | 1                              | 40 ± 7                       |

*See Fig. 2 for representative examples. "Y" means that the mutant allele was expressed at levels approximately that of wild type. "N" means that little or no protein was detected on Western blot (<3% of wild type). Intermediate levels of expression were not seen for any of the mutants discussed.

Cation inhibition curves measured at 200 µM $\text{Ni}^{2+}$ were plotted for each mutant as shown in Figs. 3–7. The number shown is an average from multiple experiments with each mutant allele, estimated by taking the concentration of cation required to give 50% inhibition of the mutant allele and dividing by the concentration of cation required to give 50% inhibition of the wild type allele measured in the same experiment. A value greater than 1 indicates that the mutant allele exhibited an apparent decrease in cation affinity compared to wild type while a value of 1 indicates no change in cation affinity compared to wild type. No mutant tested exhibited an increase in affinity.

The value given is the percent of uptake of the mutant allele compared to the uptake of a wild type allele measured in the same experiment (see "Materials and Methods" and comments on specific mutations in the text).

**Fig. 1.** Conserved residues in the third transmembrane segment of CorA. Currently available genomic sequence data shows >23 genes encoding CorA-like proteins in bacteria and the Archaea (5). An alignment of the third transmembrane domain of CorA is shown for a representative selection. The numbering is that of the CorA sequence. Residues conserved in most CorA family members are in bold-face type. E. coli; HI, Hemophilus influenzae; MT, Mycobacterium tuberculosis; ML, Mycobacterium leprae; PA, Pseudomonas aeruginosa; SY, Synechocystis sp. 6803; and TM, Thermatoga maritima. AF denotes the Archaeal *Archeoglobus fulgidus* CorA sequence. Residues conserved in the most CorA family members are in bold-face type (5). E. coli; P. aeruginosa, and Synechocystis sp. 6803 contain one or more CorA-like proteins more distantly related that are not shown.

**Fig. 2.** Western blot analysis showing expression of CorA mutant alleles. Crude membrane preparations from strain MM281 carrying the various plasmid borne mutant CorA alleles were analyzed by Western blot as described under "Materials and Methods." Gels were scanned, and the contrast was slightly adjusted using Adobe Photoshop 4.0. The Western blots shown are a composite of 2 different gels. Overexposure shows a small amount of F290A CorA protein (see text). The G291A CorA appears to be expressed at a level about 50% greater than that of wild type. In various experiments, all other mutants were expressed at levels comparable to wild type levels.

Aromatic residue, tryptophan, with little effect on transport capacity (Table I) or cation affinity (Fig. 3). In contrast, mutation of Phe$^{290}$ to alanine resulted in loss of expression, the only mutant to show such a result. This position therefore appears to need a bulky, presumably aromatic residue. The lack of change in cation affinity further suggests that this position plays a largely structural role in CorA. On the cytosolic side of TM3, mutations at Tyr$^{309}$ and Phe$^{310}$ also had relatively little effect on cation uptake. Cation affinity was unchanged by mutation to Phe or Ala at Tyr$^{309}$ (Fig. 4), and uptake capacity was unaffected (Table I). Likewise, the F310W (Fig. 4) and F310Y (not shown) mutants exhibited no change in cation affinity and maintained 40–70% of wild type uptake capacity. The F310A mutant exhibited properties similar to those seen with the F290A mutation, greater than a 95% decrease in transport capacity and a 10-fold decrease in apparent cation affinity (not shown). These results again suggest a requirement for a bulky, probably aromatic residue at position 310. Position 309 appears more flexible since the Y309A mutation had little effect.

**Mutation of Gly$^{291}$ and Tyr$^{292}$**—The sequence GYP in the periplasmic half of TM3 is highly conserved in CorA, although in some homologs the proline is replaced by a bulky hydrophobic residue. A G291A mutation had no effect on cation affinity but decreased uptake capacity about 50% (Table I). A
glycine may be conserved at this position in most CorA homologs (5) because of its small size, positioned as it is among three amino acids with large side chains. Substitution of the tyrosine at position 292 had dramatic effects on CorA function (Fig. 5 and Table I). The sterically conservative substitution from Tyr to Phe reduced uptake to less than 2% that of wild type CorA. Mutation to Ser, another hydroxyl-bearing residue, was similarly deleterious as were mutations to Cys or Ile (Fig. 5). Because of the extremely low levels of uptake in these mutants, shifts in apparent cation affinity could not be accurately measured but appeared to be at least 10-fold for Mg$^{2+}$ (Fig. 5), Co$^{2+}$, and Ni$^{2+}$ (data not shown). Mutation of the adjacent Pro$^{290}$ residue to Ala decreased uptake to about 30% of wild type and also decreased affinity for Mg$^{2+}$ about 10-fold (Table I and Fig. 3).

Mutations at Tyr$^{307}$—Alteration of Tyr$^{307}$ to Ser, Phe, or Ala; capacities of each of these mutants were all >50% of wild type uptake at 200 $\mu$M Ni$^{2+}$ (Table I). The Y307A and Y307S mutants showed a 5–10-fold decrease in Mg$^{2+}$ affinity while the Y307F mutant showed a 3-fold decrease compared with wild type (Fig. 7). With Co$^{2+}$ (not shown), apparent affinity changes from wild type were apparent for all three mutations but were less than for Mg$^{2+}$. In contrast, neither the Y307F nor the Y307A mutant showed any change in Ni$^{2+}$ affinity (Fig. 7), whereas Ni$^{2+}$ affinity remained slightly decreased for the Y307S mutant. Thus, we conclude that Tyr$^{307}$ also apparently interacts with cation as it moves through the membrane and to at least a small degree determines cation selectivity.

Growth on Minimal Media—Growth on N-minimal medium plates containing 0.4% glucose as carbon source roughly reflected the ability of the various mutants to transport magnesium. The F290A mutant, as would be expected from its lack of production of protein, was unable to grow. Likewise, the Y292F, Y292C, Y292I, and Y292S mutants grew only on media supplemented with a high concentration of Mg$^{2+}$. Other muta-
The CorA family of $\text{Mg}^{2+}$ transporters lacks similarity in sequence and in apparent structure to other known transport systems (3, 5). The most charge dense of the biological cations, $\text{Mg}^{2+}$, might be expected to interact with negatively charged amino acids in passing through the membrane. Despite this expectation, the sequence and determined membrane topology of the $S.\text{typhimurium}$ CorA put only a single non-conserved glutamate within the membrane. Mutation of this residue had no effect on transport. Therefore, as part of a systematic effort to determine membrane residues involved in passage of $\text{Mg}^{2+}$ through the membrane, we have mutated highly conserved amino acids within the third transmembrane segment (Fig. 1).

Mutation of conserved aromatic residues at each end of TM3 suggests that these residues play largely a structural role, likely stabilizing the interaction of the membrane segment at the interface of the bilayer and the aqueous solution. Alteration of Phe$^{290}$, Tyr$^{309}$, and Phe$^{310}$ to alternative aromatic residues had little effect on either cation affinity or transport capacity. In contrast, mutation to alanine had significant deleterious effects with Phe$^{290}$ and Phe$^{310}$. Substitution of a small, non-aromatic residue at the membrane interface provides little stabilization within this energetically difficult region. Since other aromatic residues can substitute, this suggests that the specific residue is not particularly important as long as an aromatic ring is present. This result is consistent with results from other membrane proteins. In the photosynthetic reaction center, intramembrane aromatic residues are markedly concentrated at the membrane interfaces (11, 12) and appear to be involved in the structural transition from the hydrophobic to the hydrophilic environment. This is presumably because the electron cloud of the aromatic ring can accommodate both types of environment.

In contrast to the results with the aromatic residues at the ends of TM3, mutagenesis of Tyr$^{292}$ and Met$^{299}$ had profound effects on function while mutations at Tyr$^{309}$ had differential effects on cation affinity. Changes at the first two of these positions greatly decreased cation affinity. Results from mutations at Tyr$^{292}$ argue strongly that both the hydroxyl group and the aromatic ring at this position are essential. A conservative change to Phe almost abolished uptake and appeared to decrease cation affinity at least 10-fold, thus implicating the hydroxyl residue. Substitution of a Ser also abolished uptake, suggesting that the phenolic ring is required. The severe phenotype of the Y292C and Y292I mutants is fully consistent with this interpretation. The relative lack of transport by the Tyr$^{292}$ mutants is presumably not due to misfolding of the protein as all of the mutants appear to insert into the membrane properly (data not shown).

The effect of mutations at Gly$^{291}$ and Pro$^{293}$ are relevant here. The sequence at this site in CorA consists of a large aromatic residue, Phe$^{290}$, followed by Gly$^{291}$, lacking any side chain, followed by two bulky residues, Tyr$^{292}$ and Pro$^{293}$. A Gly may be present at position 291 primarily because of the need for a small residue. In agreement with this hypothesis, the G291A mutation is not severely affected but is diminished by at least half in uptake capacity. The decrease in capacity may be even greater since the G291A CorA mutant appears to be somewhat overexpressed relative to wild type (Fig. 2). A proline or a bulky hydrophobic residue is conserved at position 293 in CorA (5). Proline is known to bend or kink an $\alpha$-helix but also has significant side chain bulk. Thus its presence at residue 293 may be to position Tyr$^{292}$ appropriately, and the relatively mild phenotypes of the Gly$^{291}$ and Pro$^{293}$ mutations may be indirect, resulting from improper positioning of Tyr$^{292}$.

Mutations at Met$^{299}$ also have major effects. Cation accumu-
lation in the M299C mutant is decreased by 90–95% at a substrate concentration of 200 μM Ni\(^{2+}\), and cation affinity is decreased 10–30-fold. The decrease in uptake capacity is largely due to the decrease in cation affinity. If transport is measured at increased extracellular Ni\(^{2+}\) concentrations, accumulation is greatly increased, consistent with a decrease in cation affinity. Similar results were seen with the M299A mutation which is somewhat more severely affected. Thus Met\(^{299}\) also likely interacts with Mg\(^{2+}\) during passage through the membrane. The involvement of a methionine residue is surprising. Mg\(^{2+}\) normally coordinates with oxygen via its free electrons. It rarely exhibits coordination with free electrons on nitrogen with the obvious exception of chlorophyll and is not known to coordinate with sulfur in biological systems (6, 13). Since substitution of an Ala at this site had a major effect, the apparent requirement for an alkylated sulfur atom at this location would appear to be of some importance.

The Tyr\(^{307}\) mutant is also of interest because of the slight but significant alterations in cation selectivity with the different mutants. Mutations at this site do not significantly alter uptake capacity, and absolute Mg\(^{2+}\) affinity is decreased 3–10-fold among the various mutants, less than effects of mutation at some other sites. However, affinity changes for Ni\(^{2+}\) and Co\(^{2+}\) are different than with Mg\(^{2+}\). Y307A, Y307F, and Y307S CorA mutants show significant decreases in affinity for Mg\(^{2+}\). In contrast, the Y307A and Y307F mutants show no change in affinity for Ni\(^{2+}\), whereas the Y307S mutant shows a slight decrease in affinity for Ni\(^{2+}\). Changes in Co\(^{2+}\) affinity are intermediate in effect. The molecular basis for this selectivity is unclear and is presumably related to the precise microenvironment within the membrane. Nonetheless, the observation that alterations in cation selectivity occur with mutation at this site suggests strongly that Tyr\(^{307}\) is an additional residue with which Mg\(^{2+}\) interacts during transport.

A Pathway for Mg\(^{2+}\) through the Membrane—The transport results discussed above suggest that residues Tyr\(^{292}\), Met\(^{299}\), and Tyr\(^{307}\) within TM3 directly interact with Mg\(^{2+}\) during the transport process. A simple model of TM3 as an α-helix is supportive of a role for these three residues in interaction with Mg\(^{2+}\) (Fig. 8). All three residues are on a continual face of the presumed α-helix. In this context, it makes sense that mutations at Tyr\(^{307}\) do not alter transport, as opposed to those at Tyr\(^{309}\), because Tyr\(^{309}\) would reside about halfway around the α-helix. Such a model for a pathway involving TM3 also suggests additional experiments. For example, Ala\(^{295}\) and 302AG\(^{-}\)303 are all small residues roughly one turn around the α-helix from the Tyr\(^{292}\)-Met\(^{299}\)-Tyr\(^{307}\) axis. Substitution of a large hydrophobic residue at one or more of these positions might not provoke gross changes in the overall structure of CorA since the side chain of the substitution might simply protrude into an already formed transport channel; however, such a protrusion would likely afford some degree of steric hindrance to cation passage.

The Remainder of the Mg\(^{2+}\) Pathway—Even in the context of an oligomeric CorA transporter, residues within TM3 are unlikely to form the entire transport pathway. Although some multimeric ion channels appear to form a transport pore using similar or identical transmembrane segments from 5 or more monomers, formation of a Mg\(^{2+}\) pore using only TM3 seems less likely with CorA as it would necessitate, by analogy with ion channels, at least a pentamer of 15 transmembrane segments. Likewise, the three transmembrane segments of the monomer form of CorA seem too few to form a functional pore. Thus, a homologomer of some order is the likely functional form of CorA. Preliminary evidence suggests that CorA is indeed an oligomer. This would provide 6 or more total membrane segments. TM2 also has a large number of hydroxyl-bearing residues, and we have preliminary evidence that Thr\(^{270}\) is important. This suggests that TM2 and TM3 are involved in forming a transport pathway. The involvement of TM1 is more problematic. We have shown previously that the E. coli and S. typhimurium CorA proteins possess three transmembrane segments (3). Nevertheless, phylogenetic and sequence analysis suggests that some members of the family may contain only 2 transmembrane segments, corresponding to TM2 and TM3 in the S. typhimurium CorA (5). In certain CorA family members, the TM1 domain contains multiple charged residues, as many as 9, making it most unlikely that this segment would be resident within the membrane. Thus, while the most parsimonious model for CorA would involve a homodimer with all three transmembrane segments of each monomer participating in forming the transport pathway, additional models involving only TM2 and TM3 must be considered. These will require not only additional mutagenesis studies as described in this report, but also structural studies to resolve the oligomeric structure of CorA and the arrangement of its transmembrane segments.

Comparison with Other Systems—S. typhimurium contains two other Mg\(^{2+}\) transporters, MgtA and MgtB, both of which are P-type ATPases (14–16) and both of which are highly

---

2 P. F. Grulich, M. A. Szegedy, and M. E. Maguire, manuscript in preparation.
3 M. A. Szegedy and M. E. Maguire, unpublished data.
homologous with Ca\(^{2+}\)- and H\(^{+}\)-transporting P-type ATPases of eukaryotic cells (14, 16–19). Mutational studies have suggested that several conserved charged residues within the transmembrane segments of the Ca\(^{2+}\)- and Na\(^{+}\),K\(^{+}\)-ATPases form part of the cation pathway (20–25). These residues are largely conserved in the two Mg\(^{2+}\)-transporting P-type ATPases. Mutagenic studies suggest that some, although not all, of these conserved charged residues are involved in Mg\(^{2+}\) transport.\(^4\) Thus, Mg\(^{2+}\) movement through the membrane via the P-type ATPases is likely to involve different mechanisms than movement via CorA.

The lack of involvement of charged residues within the transmembrane domains in transport proteins mediating the movement of cations is unusual. Most transport systems that mediate ion movement, e.g. the proton/sugar symporter lactose permease (26), have charged residues within transmembrane domains. However, although unusual, the lack of charged residues within the transmembrane domains of a transporter is not unique to CorA. For example, the movement of cation through the K\(^{+}\) channel of *Streptomyces lividans* involves coordination of two cations within the channel with closely spaced backbone carbonyl oxygen atoms rather than negatively charged residues or free electrons on hydroxyls or nitrogen atoms (27). Nonetheless, movement of cation through a membrane without interaction with charged residues within the transmembrane segments is relatively unusual. The Mg\(^{2+}\) ion is unique among the common biological cations with its small ionic radius and large hydrated radius, and we have previously hypothesized that because of these unique properties of the Mg\(^{2+}\) ion that Mg\(^{2+}\) transport systems would be highly unusual or even unique members of the transport protein family. These mutational studies on the CorA Mg\(^{2+}\) transporter strongly support this hypothesis (5, 10, 28, 29).

\(^4\) D. G. Kehres and M. E. Maguire, unpublished observations.

**REFERENCES**

1. Hmiel, S. P., Snively, M. D., Miller, C. G., and Maguire, M. E. (1986) *J. Bacteriol.* **168**, 1444–1450
2. Hmiel, S. P., Snively, M. D., Florer, J. B., Maguire, M. E., and Miller, C. G. (1989) *J. Bacteriol.* **171**, 4742–4751
3. Smith, R. L., Banks, J. L., Snively, M. D., and Maguire, M. E. (1993) *J. Biol. Chem.* **268**, 14071–14080
4. Smith, R. L., and Maguire, M. E. (1995) *J. Bacteriol.* **177**, 1638–1649
5. Kehres, D., Lawyer, C. H., and Maguire, M. E. (1998) *Comp. Microbiol. Genom.* 3, 151–169
6. Martin, R. B. (1990) *Metal Ions Biol.* **26**, 1–13
7. Nelson, D. L., and Kennedy, E. P. (1971) *J. Biol. Chem.* **246**, 3042–3049
8. Grubbs, R. D., Snively, M. D., Hmiel, S. P., and Maguire, M. E. (1989) Methods Enzymol. **173**, 546–563
9. Snively, M. D., Florer, J. B., Miller, C. G., and Maguire, M. E. (1989) *J. Bacteriol.* **171**, 4761–4766
10. Smith, R. L., and Maguire, M. E. (1998) *Mol. Microbiol.* **28**, 217–226
11. Roth, M., Lewit-Bentley, A., Michel, H., Deisenhofer, J., Huber, R., and Oesterhelt, D. (1989) *Nature* **340**, 659–662
12. Deisenhofer, J., and Michel, H. (1991) *Annu. Rev. Biophys. Biophys. Chem.* **20**, 247–266
13. Diebler, H., Eigen, M., Ilgenfritz, G., Maass, G., and Winkler, R. (1969) *Pure Appl. Chem.* **20**, 93–115
14. Snively, M. D., Miller, C. G., and Maguire, M. E. (1991) *J. Biol. Chem.* **266**, 815–823
15. Tao, T., Gruhlich, P. F., Kucharski, L. M., Smith, R. L., and Maguire, M. E. (1998) *Microbiology* **144**, 655–664
16. Tao, T., Snively, M. D., Farr, S. G., and Maguire, M. E. (1995) *J. Bacteriol.* **177**, 2654–2662
17. Green, N. M., and MacLennan, D. H. (1989) *Biochim. Biophys. Acta* **953**, 28815–28818
18. Saier, M. H., Jr. (1994) *Microbiol. Rev.* **58**, 71–95
19. MacLennan, D. H., Clarke, D. M., Loo, T. W., and Skerjanc, I. S. (1992) *Acta Physiol. Scand.* **146**, Suppl. 607, 141–150
20. Clarke, D. M., Loo, T. W., Inesi, G., and MacLennan, D. H. (1989) *Nature* **339**, 476–478
21. Rice, W. J., and MacLennan, D. H. (1996) *J. Biol. Chem.* **271**, 31412–31419
22. Rice, W. J., and MacLennan, D. H. (1997) *J. Biol. Chem.* **272**, 28815–28818
23. Lingrel, J. B, Arguello, J. M., Van Huysse, J., and Kuntzweiler, T. A. (1997) *Ann. N. Y. Acad. Sci.* **834**, 194–206
24. Kuntzweiler, T. A., Arguello, J. M., and Lingrel, J. B. (1996) *J. Biol. Chem.* **271**, 29682–29687
25. Kaback, H. R., and Wu, J. (1997) *Q. Rev. Biophys.* **30**, 333–364
26. Doyle, D. A., Cabrall, J. M., Pfuetzner, R. A., Kuo, A., Gulbis, J. M., Cohen, S. L., Chait, B. T., and MacKinnon, R. (1998) *Science* **280**, 69–77
27. Grubbs, R. D., and Maguire, M. E. (1987) *Magnesium* **8**, 113–127
28. Maguire, M. E. (1990) *Metal Ions Biol.* **26**, 135–153