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

MUTAGENESIS OF CONSERVED RESIDUES IN THE SECOND MEMBRANE DOMAIN*

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**The abbreviations used are: TM, transmembrane segment; N-Min, N-minimal media.**

*Salmonella typhimurium* CorA is the archetypal member of the largest family of Mg\(^{2+}\) transporters of the Bacteria and Archaea. It contains three transmembrane segments. There are no conserved charged residues within these segments indicating electrostatic interactions are not used in Mg\(^{2+}\) transport through CorA. Previous mutagenesis studies of CorA revealed a single face of the third transmembrane segment that is important for Mg\(^{2+}\) transport. In this study, we mutated hydroxyl-bearing and other conserved residues in the second transmembrane segment to identify residues involved in transport. Residues Ser\(^{260}\), Thr\(^{270}\), and Ser\(^{274}\) appear to be important for transport and are oriented such that they would also line a face of an $\alpha$-helix. In addition, the sequence YGMNF\(^{280}\), found in virtually all CorA homologues, is critical for CorA function because even conservative mutations are not tolerated at these residues. Finally, mutations of residues in the second transmembrane segment, unlike those in the third transmembrane segment, revealed cooperative behavior for the influx of Mg\(^{2+}\). We conclude that the second transmembrane segment forms a major part of the Mg\(^{2+}\) pore with the third transmembrane segment of CorA.

Mg\(^{2+}\) is the most abundant divalent cation within prokaryotes and eukaryotes, and accordingly it has many functions in the cell. It contributes to membrane stability and is a cofactor with molecules such as ATP (1, 2), tRNA (3) and many other enzymes such as ribonuclease H (4, 5). It is also a signal-cofactor with molecules such as ATP (1, 2), tRNA (3) and many selective ion transport. Therefore, CorA-mediated transport of Mg\(^{2+}\) appears quite different from known mechanisms of selective ion transport.

Previous mutagenesis studies targeting TM3 indicated that a single face of the presumed $\alpha$-helix contained three residues important for transport and therefore appeared to form part of a "Mg\(^{2+}\) pore". This report describes the mutagenesis of TM2. In contrast to studies on TM3, several residues within TM2 are important for transport, and their kinetic properties have led to insights of the transport process.

**MATERIALS AND METHODS**

All media were obtained from Difco. All other reagents were from Sigma unless otherwise specified. Oligonucleotides were purchased from Genosys (The Woodlands, TX). Supplemented N-minimal medium (N-Min) (30) contains 0.1% casamino acids and 0.4% (w/v) glucose. MgSO\(_4\) was used when medium was supplemented with Mg\(^{2+}\). The concentrations of antibiotics used were 50 µg/ml ampicillin, 20 µg/ml chloramphenicol, and 50 µg/ml kanamycin.

**Mutant Construction and Expression**—Mutations were created using one of two similar vectors. Mutations of corA in plasmid pRS170 (29) were made using the Altered Sites$^\text{TM}$ II kit (Promega, Madison, WI). The QuikChange$^\text{TM}$ Mutagenesis System (Stratagene, La Jolla, CA) was used to create mutations of corA in plasmid pMAS29, which is pRS170 with the ampicillin gene repaired. Mutations were verified by sequencing the 3'-portion of the gene, which included the entire membrane domain. The plasmids were propagated as described (29). Transport...
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...and growth by CorA mutants were analyzed in MM281, a Mg\(^{2+}\) transport-deficient strain (18), with Western blot analysis performed as described previously (29). Briefly, overnight cultures of the mutants were lysed by French Press, and the membranes were pelleted by ultracentrifugation at 100,000 \(\times g\) for 1 h. Protein quantitation was done by the Pierce BCA Assay. Membrane samples were analyzed by loading 10 \(\mu\)g of protein on 10% SDS-polyacrylamide gel electrophoresis gels and transferring to nitrocellulose. CorA expression was visualized using an antibody directed to the 16 residues at the amino terminus (29). For those mutants exhibiting altered migration on Western blots, the entire coding region of the gene was sequenced to verify the absence of secondary mutations.

**Growth in Minimal Medium—**Complementation of the Mg\(^{2+}\) transport-deficient strain MM281 was determined by streaking a single colony from an LB plate onto an N-Min plate containing 0.25% (w/v) glucose and 0.5 mM leucine and incubating at 37 °C for 48 h. The growth assay can detect mutants able to take up sufficient Mg\(^{2+}\) to grow but whose transport capacity is too low to be measured by the Ni\(^{2+}\) transport assay. The growth assay also can detect mutations that hinder cell growth yet retain measurable amounts of cation uptake.

Growth in supplemented N-Min medium with varying concentrations of Mg\(^{2+}\) was determined by adding 5 \(\times 10^{7}\) cells/well in a 96-well microtiter plate, assuming 2 \(\times 10^{7}\) cells = 1 A\(_{600}\) unit. The total volume in each well was 100 \(\mu\)l. The inside plate cover was coated with Neva Fog (Atlanta, GA) to prevent condensation during the incubation. MgSO\(_4\) concentrations were 0, 0.01, 0.05, 0.1, 0.25, 0.5, 1, 2.5, 5, 10, 50, and 100 mM. OD\(_{600}\) was measured every 30 min for 16 h using a Molecular Devices THERMOMax™ plate reader held at 37 °C. The Mg\(^{2+}\) transport-deficient strain, MM281, required at least 2.5 mM Mg\(^{2+}\) to grow. MM281 with wild type CorA on plasmid pMAS29 required 10 \(\mu\)M Mg\(^{2+}\) to grow in this assay.

**Transport—**The uptake of \(\text{^{63}Ni}^{2+}\) (NEC Life Science Products) was assayed instead of Mg\(^{2+}\) uptake, as \(\text{^{28}Mg}^{2+}\) is prohibitively expensive and not readily available (19). Methods for transport were as described previously (29, 31, 32). Briefly, cells were grown overnight in LB with 100 mM MgSO\(_4\) and appropriate antibiotics. The MgSO\(_4\) concentration was 100 mM because the background strain MM281 requires high concentrations of Mg\(^{2+}\) for growth. Cells were washed twice in supplemented N-Min (without Mg\(^{2+}\)) and resuspended in the same medium to 1–2 A\(_{600}\) units. Cells were added to tubes containing varying concentrations of inhibitor cation plus 200 \(\mu\)M NiCl\(_2\) and 1–1.2 \(\mu\)Ci of \(\text{^{63}Ni}^{2+}\) in a final volume of 1 ml. The reactions were incubated for 5 min at 37 °C, stopped by the addition of 5 ml of ice-cold N-Min containing 10 mM MgSO\(_4\) and 0.5 mM EDTA, filtered immediately on nitrocellulose filters (Schleicher & Schuell), and washed once with 5 ml of the same solution.

The filters were placed in a 3-ml Biosafe II scintillation mixture (Research Products International Corp., Mount Prospect, IL) and counted using a Beckman LS 6500 scintillation counter with 80% efficiency.

Transport was assayed at 200 mM NiCl\(_2\), the approximate \(K_m\) for wild type CorA (19). If the affinity of a mutant for Ni\(^{2+}\) has decreased, the substrate concentration is no longer near the \(K_m\), and therefore the velocity will be a smaller fraction of the \(V_{\text{max}}\) of the wild type. Unfortunately, the uptake values could not be corrected for affinity changes. As the Ni\(^{2+}\) dose-response curves were irregular for many mutants as described below, the Ni\(^{2+}\) affinity could not be determined accurately. Therefore, reported maximal uptake of each mutant (Table I) as a percentage of wild type CorA represents a minimum value.

**Inhibition of \(\text{^{63}Ni}^{2+}\) transport was determined for Ni\(^{2+}\), Mg\(^{2+}\), and Co\(^{2+}\).** Inhibition curves are plotted as a percent of the maximal trans-
affinity of Ni$^{2+}$ is sufficiently low to allow changes in affinity to demonstrate the induction at micromolar levels of added Ni$^{2+}$. Induction of Ni$^{2+}$ uptake was displayed for all TM2 mutants except S263A and S275A. The F266Y, P269A, S274A, and S274T mutants demonstrated the largest induction, each reaching approximately 140% of maximal uptake defined at 200 μM Ni$^{2+}$. The peak of induction for most mutants was at approximately 300 μM added NiCl$_2$, corresponding to a total Ni$^{2+}$ concentration of 500 μM. Exceptions were the S274A and S274T mutants, which had a peak of induction with 1 mM added Ni$^{2+}$, corresponding to a total of 1.2 mM Ni$^{2+}$. Such induction may be indicative of cooperativity as has been shown with a Na$^+$/K$^+$-ATPase mutant (33). To investigate this possibility, $^{63}$Ni$^{2+}$ uptake at varying concentrations of NiCl$_2$ was determined for wild type CorA and one of the mutants. This experiment is different from the Ni$^{2+}$ inhibition curves because there was a greater range of Ni$^{2+}$ concentration, and the specific activity of the isotope was maintained. The P268A mutant was chosen for this assay because it maintained a significant amount of uptake, had a normal growth phenotype, yet demonstrated the Ni$^{2+}$ induction. The P268A mutant had a 5-fold shift in the Ni$^{2+}$ and a 4-fold shift for Co$^{2+}$ dose-response curves with no shift in the Mg$^{2+}$ dose-response curve. The velocity versus substrate concentration curve was sigmoidal for both wild type CorA and the P268A mutant (Fig. 5), consistent with positive cooperativity. The $V_{\text{max}}$ and Hill coefficient were estimated using a least square fit. The estimate of $V_{\text{max}}$ is 1800 pmol/μmol/min for wild type and 935 pmol/μmol/min for the P268A mutant. The $n_H$ values are 2.5 for the control and 2.0 for the mutant. Therefore, CorA may contain two or more binding sites for Mg$^{2+}$, and the mutants alter the binding properties of these sites. When Ni$^{2+}$ inhibition for several alamine mutants was assayed with 500 μM NiCl$_2$, rather than 200 μM NiCl$_2$, the induction was either diminished or eliminated altogether (data not shown). Consequently, the induction effect is revealed by the decreased affinity of the mutated CorAs for Ni$^{2+}$. Although the kinetics of transport are more complex than simple inhibition, the dose-response curves are nonetheless appropriate for determining relative affinity changes for the mutants, because all other data follow the wild type simple inhibition curve because the affinities of the different binding sites appear to be altered to a similar extent.

Residue Ser$^{260}$: Mutations Affect Cation Selectivity—The S260A and S260T mutants displayed an unusual affinity profile for the cations tested, suggesting that Ser$^{260}$ plays a role in cation selectivity. Both mutants maintained significant

**FIG. 2.** Western blot analysis of CorA mutants. Western analysis was performed using crude membrane preparations of CorA mutants. All mutants were expressed to a similar extent as wild type (w.t.) except S260V. The sample Western blots shown are representative of at least two experiments.

**FIG. 3.** Ni$^{2+}$ inhibition of $^{63}$Ni$^{2+}$ uptake for the P268A and S274A mutants. Transport was performed as described under “Materials and Methods.” Curves are normalized to the defined “maximal” uptake, which is the amount of uptake with the initial 200 μM Ni$^{2+}$ present in the assay. These data are representative curves from at least two separate experiments.

**FIG. 4.** Mg$^{2+}$ inhibition of $^{63}$Ni$^{2+}$ uptake for the P269A, F266A, and F266Y mutants. Transport was performed as described under “Materials and Methods.” Curves are normalized to the maximal uptake of each mutant. These data are the average of a minimum of three separate uptake experiments.

**FIG. 5.** Cooperativity in wild type CorA and the P268A CorA mutant. Uptake curve of $^{63}$Ni$^{2+}$ as described under “Materials and Methods.” The Hill equation fitted to these data gives a Hill coefficient of 2.5, indicative of positive cooperativity.
amounts of $^{65}$Ni$^{2+}$ uptake and displayed normal growth. However, both mutants also displayed a shift to the left of 4–5-fold in the Mg$^{2+}$ dose-response curve as shown in Fig. 6. No other CorA mutants to date have demonstrated such a left shift. Co$^{2+}$ inhibition was similar to wild type, and Ni$^{2+}$ inhibition displayed 10- and 3-fold shifts to the right for the S260A and S260T mutants, respectively (data not shown). This is in contrast to the behavior seen with other CorA mutants, where the dose-response curves for all three cations shifted in the same direction.

The S260V mutant had no measurable transport. However, it was functional because it required only 50 $\mu$M Mg$^{2+}$ to grow in supplemented N-Min medium, as shown in Table I. The increased Mg$^{2+}$ requirement could be related to the decreased stability of the S260V mutant as noted above. Alternatively, this may be because of a slight decrease in affinity for Mg$^{2+}$ with a severely decreased affinity for Ni$^{2+}$, causing no measurable $^{65}$Ni$^{2+}$ transport. The latter seems more likely because it correlates with the properties of other Ser$^{260}$ mutants. Thus, Ser$^{260}$ is likely involved in cation selectivity, but the hydroxyl moiety is not essential.

**Residue Thr$^{270}$: Size of the Residue Affects Transport**—For the Thr$^{270}$ mutants constructed, as the size of the side chain increased, both transport capacity and affinity for cation also increased. The T270A mutant had no measurable transport activity. The T270C mutant had 4% of wild type uptake, a greater than 5-fold shift in the Mg$^{2+}$ dose-response curve (Fig. 7) and a 10-fold shift to the right in the Ni$^{2+}$ inhibition curve (data not shown), indicating significant decreases in affinity for both Mg$^{2+}$ and Ni$^{2+}$. The T270S mutant, which maintains the hydroxyl moiety, had 17% of wild type uptake and demonstrated a smaller shift to the right in the Mg$^{2+}$ dose-response curve of about 3–4-fold (Fig. 7). The shift for Ni$^{2+}$ inhibition was also moderate at 3-fold (data not shown). The T270V mutant, which maintains the relative size of the residue while eliminating the hydroxyl group, maintained 35% of wild type ion uptake and had a minimal shift to the right in both Mg$^{2+}$ and Ni$^{2+}$ dose-response curves. All three mutants appeared to have the same 5-fold shift to the right for Co$^{2+}$ (data not shown). The growth data indicated that all Thr$^{270}$ mutants, including T270A, were functional because they all complemented MM281. The T270A mutant required 50 $\mu$M Mg$^{2+}$ to grow, whereas the T270C, T270S, and T270V mutants required only 10 $\mu$M Mg$^{2+}$, the same as wild type. Thus, the T270V mutant maintained the greatest amount of CorA function of all the mutants made at this residue. Valine best approximates the size of threonine but is hydrophobic. This suggests that the size of the residue at Thr$^{270}$ is more important for transport than the hydroxyl group.

FIG. 6. Mg$^{2+}$ inhibition of $^{65}$Ni$^{2+}$ uptake for the S260A and S260T mutants. Transport was performed as described under “Materials and Methods.” Curves are normalized to the maximal uptake of each mutant. These data are the average of a minimum of three separate uptake experiments.

**TABLE I**  Properties of CorA mutants

| Mutant   | Growth on minimal medium$^a$ | Minimal [Mg$^{2+}$] required for growth$^b$ (mM) | % Wild type transport$^c$ | Fold shift in Mg$^{2+}$ dose-response curve$^d$ |
|----------|-----------------------------|-----------------------------------------------|---------------------------|-----------------------------------------------|
| S260A    | ++                          | 0.01                                          | 32 ± 7                    | 4 × Left                                      |
| S260A    | ++                          | 0.01                                          | 13 ± 3                    | 5 × Left                                      |
| S260V    | +                           | 0.05                                          | 1 ± 3                     | —                                             |
| S263A    | ++                          | 0.01                                          | 68 ± 12                   | None                                          |
| F266A    | +                           | 0.25                                          | 47 ± 7                    | None                                          |
| F266Y    | ++                          | 0.05                                          | 25 ± 6                    | 3×                                            |
| F268A    | ++                          | 0.01                                          | 27 ± 6                    | None                                          |
| F299A    | +                           | 0.25                                          | 11 ± 12                   | None                                          |
| T270A    | ++                          | 0.05                                          | 1 ± 1                     | —                                             |
| T270C    | ++                          | 0.01                                          | 1 ± 1                     | 4×                                            |
| T270S    | ++                          | 0.01                                          | 17 ± 6                    | 4×                                            |
| T270V    | ++                          | 0.01                                          | 35 ± 9                    | <2×                                           |
| S274A    | ++                          | 0.01                                          | 5 ± 2                     | 2–10×$^e$                                     |
| S274C    | +                           | 0.01                                          | 1 ± 1                     | —                                             |
| S274T    | ++                          | 0.01                                          | 17 ± 4                    | 10×                                           |
| S274V    | −                           | 2.5                                           | 0 ± 0                     | —                                             |
| S275A    | ++                          | 0.01                                          | 85 ± 11                   | <2×                                           |
| Y276A    | −                           | 1                                             | 1 ± 1                     | —                                             |
| Y276F    | −                           | 0.25                                          | 1 ± 1                     | —                                             |
| Y276W    | ++                          | 0.01                                          | 49 ± 12                   | <2×                                           |
| G277A    | −                           | 2.5                                           | 0 ± 0                     | —                                             |
| M278A    | −                           | 2.5                                           | 0 ± 0                     | —                                             |
| M278C    | −                           | 2.5                                           | 0 ± 0                     | —                                             |
| M278I    | −                           | 2.5                                           | 1 ± 1                     | —                                             |
| M278L    | −                           | 2.5                                           | 2 ± 1                     | —                                             |
| N279A    | −                           | 2.5                                           | 2 ± 0                     | —                                             |
| N279L    | −                           | 2.5                                           | 2 ± 0                     | —                                             |
| N279Q    | −                           | 2.5                                           | 1 ± 1                     | —                                             |
| F280A    | −                           | 2.5                                           | 0 ± 0                     | —                                             |
| F280R    | −                           | 2.5                                           | 1 ± 0                     | —                                             |
| F280W    | −                           | 1.0                                           | 1 ± 0                     | —                                             |
| F280Y    | −                           | 1.0                                           | 0 ± 0                     | —                                             |

$^a$ Growth on plates was the ability to grow on N-Min plates supplemented with leucine and glucose. Mutants that grew as well as wild type are designated by “+” and mutants with slow growth are designated by “−.”

$^b$ The minimum concentration of Mg$^{2+}$ required for growth was tested in N-Min medium supplemented with casamino acids and glucose.

$^c$ Transport was measured as the amount of $^{65}$Ni$^{2+}$ transported into cells compared to wild type uptake using 200 $\mu$M NiCl$_2$. Values are the average of at least two independent experiments. The values reported here are minimum values because the Ni$^{2+}$ affinity decreased in many mutants, which would cause underestimation of transport capacity. See comments in “Materials and Methods.”

$^d$ The shift in the Mg$^{2+}$ dose-response curve is a right shift for except where noted.

$^e$ The S274A mutant demonstrated an uneven shift in the dose-response curve for Mg$^{2+}$, as shown in Fig. 5.

FIG. 7. Mg$^{2+}$ inhibition of $^{65}$Ni$^{2+}$ uptake for the T270C, T270S, and T270V mutants. Transport was performed as described under “Materials and Methods.” Curves are normalized to the maximal uptake of each mutant. These data are the average of a minimum of three separate uptake experiments.

Residue Ser$^{274}$: The Hydroxyl Moiety Is Important—All mutations at Ser$^{274}$ displayed considerably decreased cation affinity and decreased transport capacity. S274A and S274T mu-

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These data suggest that a hydroxyl moiety provides optimal
an even shift throughout the curve and did not reflect the
to grow in minimal medium was Ser.
grew as well as wild type. Therefore, all mutations at Ser274
the transport assay. The S274A mutant and the S274T mutant
the growth curve assay, although uptake was undetectable by
Ni2+ and Co2+ inhibition were similar to the Mg2+
inhibition curves (data not shown). Thus all of the mutations at Ser274 affected the transport properties of CorA, with the S274T mutant retaining the greatest amount of uptake.

Growth on minimal medium reflected the ion uptake experiment for the S274V mutant as it did not complement MM281 and required 2.5 mM Mg2+ to grow. The S274C mutant allowed slow growth on minimal plates and grew as well as wild type in the growth curve assay, although uptake was undetectable by the transport assay. The S274A mutant and the S274T mutant grew as well as wild type. Therefore, all mutations at Ser274 greatly affected transport properties. The transport data reflected an order of Ser > Thr > Ala >> Cys = Val in terms of maintaining 65Ni2+ transport, whereas the order for the ability to grow in minimal medium was Ser > Thr = Ala > Cys >> Val. These data suggest that a hydroxy moiety provides optimal transport and that size is not the dominant factor.

Residues 276-280 of CorA: Critical Structural Motif for Mg2+ Transport—This five amino acid sequence has the highest conservation among the CorA homologues, effectively acting as a CorA signature sequence (21). In keeping with this high degree of conservation, this motif is critical for CorA function as even the most conservative mutations at these residues abolished or markedly diminished transport.

A Y276A CorA mutant had no measurable 65Ni2+ uptake, nor did the conservative Y276F mutant. This would imply input from the hydroxyl group of the tyrosine; however, the Y276W mutant maintained 50% ion transport capacity. The Y276W mutant also had a small shift to the right in the Mg2+ dose-response curve, a 2-fold shift for Ni2+, and a 5-fold shift for Co2+ (data not shown). The amount of Mg2+ required for growth also varied with mutations at Y276. The Y276A mutant required 1 mM, the Y276F mutant required 250 µM, and the Y276W mutant required only the wild type level of 10 µM Mg2+ to grow. This roughly correlates with transport, as the Y276W mutant, the only mutant with measurable transport, required the least Mg2+ for growth.

Gly277 was only mutated to alanine, as this was the only conservative mutation possible; even so, the G277A mutant demonstrated no transport of 63Ni2+ and required 2.5 mM Mg2+ for growth, the amount required for the Mg2+ transport-deficient strain. The G277A CorA mutant is therefore a nonfunctional protein, suggesting that any side chain at this position likely sterically interferes with a critical structure in CorA.

M278A and the conservative M278C and M278I mutants were not functional. None had measurable ion uptake, and all required 2.5 mM Mg2+ for growth. Similarly, the N279A, the sterically conservative N279L, and the functionally conservative N279Q mutations were all nonfunctional in either assay. Mutations at Phe280 also lacked measurable transport. The F280W and F280Y mutants, both conservative mutations maintaining an aromatic ring, required 1.0 mM Mg2+ for growth suggesting minimal functionality. The F280A and F280R mutants required 2.5 mM Mg2+ and therefore appeared completely nonfunctional.

The results from mutations in the 279-281 sequence clearly show that these residues are essential for CorA function. It is striking that in practically all cases even the most conservative mutations could not be tolerated. Because this sequence would comprise more than a full turn of an α-helix, all of these residues could not come in direct contact with a cation as it traversed the pore. Therefore it is likely that these residues form a critical structural motif within CorA.

Residues Phe266 and Pro279: Unusual Growth Phenotype—For the mutants discussed above, the Mg2+ requirement for growth reflected the ability of the mutants to transport cation. However, the F266A and P269A mutants exhibited significant transport but had a decreased the ability to grow in minimal medium. The P269A mutant maintained 40% of wild type ion uptake with no shift in the Mg2+ (Fig. 4) and Co2+ dose-response curves and only a small shift in the Ni2+ dose-response curve (data not shown). However, growth in liquid medium required 0.25 mM Mg2+. The F266A mutant also required 0.25 mM Mg2+ for growth. It had approximately 47% of wild type ion uptake with no shift in the Mg2+ inhibition curve (Fig. 4) and 2- and 5-fold changes in the affinity for Co2+ (data not shown). To further investigate the phenotype of mutations at Phe266, an F266Y mutant was made. This mutation resulted in approximately a 3-fold shift in the Mg2+ dose-response curve (Fig. 4) with a 10-fold shift for Co2+ and a 5-fold shift for Ni2+ inhibition curves (data not shown). It retained 25% of wild type cation uptake. The growth phenotype, however, was similar to wild type. Therefore, the aromatic group may be important at this position. The disparity in transport and the growth phenotype seen with the F266A and P269A mutants could be because of an alteration in efflux of Mg2+, although this cannot be tested without 28Mg2+ (see “Discussion”).

Residues Ser263 and Ser274: Hydroxyl-bearing Residues Not Required for Transport—Ser263 and Ser274 were mutated to alanine with few changes in transport properties. There were no shifts in the dose-response curves for all cations tested, unlike results seen at other hydroxyl-bearing residues in TM2. The S263A and S274A mutants retained 68 and 85% wild type transport, respectively. Accordingly, the S263A and S274A mutants both had growth phenotypes similar to wild type CorA.
DISCUSSION

Our working hypothesis is that CorA functions as a homolog with residues along the α-helical TM2 and TM3 segments, including many of the hydroxyl-bearing residues, coordinating Mg\(^{2+}\) as it passes through a pore or channel. Preliminary data indicate that CorA is a pentamer, and we have previously shown that three residues along a single face of the presumed TM3 α-helix appear to be required for Mg\(^{2+}\) transport. Herein, we continue the mutagenesis of the membrane domain targeting highly conserved and hydroxyl-bearing residues in TM2 basing our selection upon alignments of homologues such as those shown in Fig. 1. We first used alanine-scanning mutagenesis and then, for those residues that showed a significant effect, changed the residues to more conserved amino acids in size or functional moieties. Western analysis revealed that all mutants were expressed to a similar extent as CorA except S260V, which may not have been stable during membrane isolation. Functionality was determined by \(^{63}\)Ni\(^{2+}\) uptake and its inhibition by Mg\(^{2+}\), Co\(^{2+}\), and Ni\(^{2+}\). Phenotype was assayed by complementation of the Mg\(^{2+}\) transport-deficient strain MM281 and by growth assays. We have determined that in TM2, conserved residues 276YGMNF280 and the hydroxyl-bearing residues Ser\(^{260}\), Thr\(^{270}\), and Ser\(^{274}\) are important for transport through the \(S.\) typhimurium CorA. These latter residues may line part of a pore of an oligomeric CorA in conjunction with TM3. Furthermore, several mutations in TM2 alter the affinity for Ni\(^{2+}\) and allow an induction to be seen in the dose-response curves revealing cooperative behavior.

Cooperativity—The Ni\(^{2+}\) induction seen with several residues in TM2 is an unexpected finding of this study because no similar transport phenomenon was seen with any mutation in TM3 (29). Such substrate stimulation has been seen previously in which transport occurs in a cooperative manner, with two or more binding sites in CorA. Possibly, two or more binding sites in CorA act in a cooperative manner. An alternative interpretation is that CorA is a channel rather than the hydroxyl group is required for CorA function.

Growth Phenotype—The growth phenotype generally reflected the ability to transport Ni\(^{2+}\). However, for certain mutants, cell growth did not correspond with transport. The S260V, T270A, S274C, Y276A, Y276F, F280W, and F280Y CorA mutants had no measurable transport but required less Mg\(^{2+}\) to grow than MM281, the transport-deficient strain. These mutants apparently permit sufficient Mg\(^{2+}\) entry to sustain cell growth but do not have sufficient transport to allow measurement under the conditions assayed, the mutations decreasing Ni\(^{2+}\) uptake to a level below the sensitivity of the assay. The most likely explanation for this difference is a markedly decreased maximal transport capacity, with possible contribution from decreased affinity. Conversely, less likely, this phenotype could be because of a highly increased affinity for Ni\(^{2+}\) in which the cation binds too tightly for transport to occur.

The opposite growth phenotype occurred with the F266A, F266Y, P269A, and Y276W mutants. These mutants maintained at least 25% total uptake compared with wild type CorA and had minimal changes in affinity for Mg\(^{2+}\). Yet these mutants required more Mg\(^{2+}\) for growth than other mutants with significantly less ion transport capacity. We interpret this phenotype as most likely because of inappropriate Mg\(^{2+}\) efflux. CorA mediates efflux of Mg\(^{2+}\) (25), but efflux is “gated” by Mg\(^{2+}\) and does not occur with wild type CorA except at high extracellular Mg\(^{2+}\) concentrations, greater than or equal to 1 mM (26). If a mutation altered this gating mechanism to constitutively activate efflux or simply allowed leakage of Mg\(^{2+}\), Mg\(^{2+}\) efflux would occur at low extracellular concentrations. If this loss of Mg\(^{2+}\) were of sufficient magnitude, growth could be compromised. However, the unavailable isotope \(^{28}\)Mg\(^{2+}\) is required for efflux studies, as CorA does not mediate Ni\(^{2+}\) or Co\(^{2+}\) efflux (26), possibly because of high affinity binding of these metals within the cell. Therefore this supposition cannot currently be tested directly.

Hydroxyl-bearing Residues—Many of the hydroxyl-bearing residues in TM2 are not essential for Mg\(^{2+}\) transport. The S263A and S275A mutants had significant uptake capacity and no apparent change in cation affinity. In addition, whereas Thr\(^{270}\) is important for transport, the size of the side chain rather than the hydroxyl group is required for CorA function. Tyr\(^{276}\) also plays a role in cation transport. The Y276F mutant was not functional, which might suggest that the hydroxyl moiety is important. However, the Y276W mutant maintained a significant amount of activity with only a slight shift in affinity, suggesting that a hydroxyl moiety is not critical. The requirement here may be sufficient delocalization of charge within the aromatic ring. We conclude that the hydroxyl moiety itself at Ser\(^{260}\), Ser\(^{274}\), Thr\(^{270}\), and Tyr\(^{276}\) does not play a role in transport, even though the latter two residues have important roles in transport.

In contrast, the hydroxyl moieties of Ser\(^{260}\) and Ser\(^{274}\) appear to play significant roles in Mg\(^{2+}\) transport. Alanine mutations at either position had drastic phenotypes. Mutations even to relatively conserved residues had major effects. Alanine and threonine mutations at Ser\(^{260}\) caused the affinity for Co\(^{2+}\) and Ni\(^{2+}\) to decrease significantly compared with the Mg\(^{2+}\) affinity, whereas the valine mutant did not exhibit transport. This difference in cation affinities suggests that Ser\(^{260}\) plays a role in cation selectivity with the hydroxyl moiety conferring optimal activity. Although the alanine mutant did have significant uptake, as did the threonine mutant, the alanine might...
CorA contains multiple cation binding sites, such as the Na\(^{+}\), K\(^{+}\)-ATPase, SR Ca\(^{2+}\)-ATPase (34), and the KcsA ion channel (35). The alanine mutant may have altered the Mg\(^{2+}\) affinity as a gate or filter for Mg\(^{2+}\) uptake in the transport assay. The valine may be too large for transport to occur. In addition, the asymmetry of the shift in the S274A mutant Mg\(^{2+}\) inhibition curve may indicate that CorA contains multiple cation binding sites, such as the Na\(^{+}\), K\(^{+}\)-ATPase, SR Ca\(^{2+}\)-ATPase (34), and the KcsA ion channel (35). The alanine mutant may have altered the Mg\(^{2+}\) affinity of only one of the binding sites. The presence of multiple binding sites is compatible with the cooperative behavior derived from the kinetic analysis above and suggests cooperative binding may occur, although other mechanisms cannot be ruled out.

The CorA Signature Sequence—The 276YGMNF280 sequence is the most highly conserved sequence in all CorA homologues. As might be expected, all of the residues in this sequence are required for transport; even conservative mutations completely eliminated cation transport. The only conservative mutation of the 276YGMNF280 sequence that retained activity in the ion uptake assay was the Y276W mutant, although the even more conservative Y276F mutant was completely nonfunctional. Similarly, aromatic substitutions at Phe280 are functional, but only minimally. Thus, at Tyr276 and Phe280, a bulky aromatic moiety. The alanine mutant may be too small to eliminate transport, whereas the valine mutant is too large to allow transport to occur. In addition, the asymmetry of the shift in the S274A mutant Mg\(^{2+}\) inhibition curve may indicate that CorA contains multiple cation binding sites, such as the Na\(^{+}\), K\(^{+}\)-ATPase, SR Ca\(^{2+}\)-ATPase (34), and the KcsA ion channel (35). The alanine mutant may have altered the Mg\(^{2+}\) affinity of only one of the binding sites. The presence of multiple binding sites is compatible with the cooperative behavior derived from the kinetic analysis above and suggests cooperative binding may occur, although other mechanisms cannot be ruled out.

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