Sodium-dependent Extracellular Accessibility of Lys-84 in the Sodium/Dicarboxylate Cotransporter*

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The Na+/dicarboxylate cotransporter transports Na+ with citric acid cycle intermediates such as succinate and citrate. The present study focuses on transmembrane helix 3, which is highly conserved among the members of the SLC13 family. Fifteen amino acids in the extracellular half of transmembrane helix (amino acids 98–112) as well as Lys-84, previously shown to affect substrate affinity, were mutated individually to cysteine and expressed in the human retinal pigment epithelial cell line. Transport specificity ratio analysis shows that determinants for distinguishing succinate and citrate are found at amino acids Lys-84, Glu-101, Trp-103, His-106, and Leu-111. All of the mutants were tested for sensitivity to the membrane-impermeant cysteine-specific reagent (2-sulfonatoethyl) methanethiosulfonate (MTSES), but only K84C was sensitive to MTSES inhibition. The sensitivity of K84C to MTSES was greatest in the presence of sodium, and the inhibition could be prevented by addition of substrate or replacement of sodium, indicating that the accessibility of Lys-84 changes with conformational state. The substrate protection of MTSES inhibition of K84C appears to occur early in the transport cycle, before the large-scale conformational change associated with translocation of substrate. The results point to a new location for Lys-84 within the substrate access pore of the Na+/dicarboxylate cotransporter, either in a transmembrane helix or a reentrant loop facing a water-filled pore.

The Na+/dicarboxylate cotransporter NaDC1 belongs to the SLC13 family, which also includes sodium-coupled transporters for citrate (NaCT) and inorganic sulfate (NaS1) (1, 2). NaDC1 plays a key role in the absorption of tricarboxylic acid cycle intermediates such as succinate and citrate across the apical membrane of the kidney proximal tubule and the small intestine (2). NaDC1 participates in the renal secretion of organic anions, including drugs and xenobiotics, by contributing dicarboxylates to the organic anion/dicarboxylate exchanger (3). The activity of NaDC1 helps to regulate concentrations of citric acid cycle intermediates, which may affect such processes as the development of kidney stones (4) or blood pressure regulation via G-protein-coupled receptors for succinate and α-ketoglutarate in the proximal tubule (5). Mutations in NaDC1 homologs from Drosophila and Caenorhabditis elegans lead to lifespan extension (6, 7), suggesting a potential role for NaDC1 in regulation of metabolism and aging.

The current secondary structure model of NaDC1 contains 11 transmembrane helices (TM) with an extracellular carboxyl terminus containing a site for N-linked glycosylation and the amino terminus facing the cytoplasmic side of the membrane (8). Many of the functionally important residues for substrate and cation recognition are located in the carboxyl-terminal half of the protein (9, 10). In addition, we have identified amino acids in TM9 and the connecting loop whose accessibility to extracellular reagents changes during the transport cycle (11, 12). The amino-terminal half of the protein, in particular TM3, appears to be important in determining substrate specificity and affinity. For example, glutarate transport is determined primarily by residues found in TM3-TM4 (13). The sequence of TM3 and the connecting loops is highly conserved in the SLC13 family (Fig. 1). Lys-84, predicted to be in an intracellular loop at the base of TM3, was found to be important for succinate transport because replacement of Lys-84 with Ala produced a large decrease in succinate affinity (14). His-106, at the extracellular part of TM3, is required for proper targeting of NaDC1 to the membrane (15).

In the present study we investigated the amino acids in the extracellular half of TM3 from Ile-98 to Arg-112, as well as Lys-84, using the substituted cysteine accessibility method used to study structure-function relationships in ion channels and transporters (16, 17). A total of sixteen residues in NaDC1 were mutated one at a time to cysteine, and the sensitivity of the substituted cysteines to the membrane-impermeant cysteine-specific reagents (2-sulfonatoethyl) methanethiosulfonate (MTSES) and [2-(trimethylammonium)ethyl]-methane-thiosulfonate (MTSET) was then determined. TM3 in NaDC1 contains several residues involved in substrate recognition, Lys-84, Glu-101, Trp-103, His-106, and Leu-111, because their mutation to cysteine resulted in a change in transport specificity ratio (TSR). Of all the mutants, only K84C was sensitive to inhibition by MTSES, with differences in accessibility that parallel the exposure of the substrate binding site in NaDC1. The substrate protection of MTSES inhibition of K84C appears to occur early in the transport cycle, before the large-scale conformational change associated with translocation of substrate. The results indicate a new location for Lys-84, either in the
transmembrane helix facing a water-filled pore or in a reentrant loop accessible to the outside.

**EXPERIMENTAL PROCEDURES**

*Construction of TM3 Cysteine Mutants—* The cysteine mutants were made using the PCR-based QuickChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions. The C476S mutant of rabbit (rb) NaDC1 in pcDNA3.1 vector was used as a template for mutagenesis. The rbNaDC1 contains an endogenous cysteine at position 476 that is sensitive to labeling by some cysteine-specific reagents such as p-chloromercuribenzenesulfonate (18), although it is insensitive to hydrophilic methanethiosulfonate reagents such as MTSET (11). The cell surface expression of NaDC1 is related to the number of cysteines (18), and the C476S mutant (containing ten of the eleven endogenous cysteines) was used to allow increased protein expression of the cysteine-substituted mutants, because the mutations can sometimes result in decreased activity. Our previous study showed that cysteine mutants made in the C476S background had the same results as constructs containing only three endogenous cysteines, but the protein expression and transport activity were higher in the C476S background (11, 12). All mutants were verified by sequencing at the Protein Chemistry Laboratory of the University of Texas Medical Branch.

*Expression of TM3 Mutants in HRPE Cells—* Human retinal pigment epithelial (HRPE) cells transformed with SV40 (AG 06096; Coriell Institute) were cultured in modified Eagle’s medium containing Glutamax and 25 mM HEPES (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were incubated at 37 °C in 5% CO₂. HRPE cells were plated in 24-well plates for transport assays at a density of 1.2 × 10⁵ cells/well or 6-well plates for biotinylation reactions at a density of 1.5 × 10⁵ cells/well. Twenty-four hours after seeding, cells were transiently transfected with 1.8 μl of FuGENE 6 (Roche Applied Science) and 0.6 μg of plasmid DNA (9:3 ratio) for 24-well plates or with 3 μl of FuGENE 6 and 1 μg of plasmid (3:1 ratio) for 6-well plates (12).

*Transport Assays—* Succinate and citrate transport assays were carried out 48 h after transfections as described previously (12). For the standard assay, each well was washed twice with 1 ml of sodium buffer containing 120 mM NaCl, 5 mM KCl, 1.2 mM MgSO₄, 1.2 mM CaCl₂, 5 mM d-glucose, 25 mM HEPES, pH adjusted to 7.4 with 1 M Tris. Transport was measured by incubating the cells with 250 μl of sodium buffer containing 100 μM succinate (combination of [¹³C]sucinate and non-radioactive succinate). For citrate kinetic measurements, a combination of [¹⁴C]citrate (Amersham Biosciences GE Healthcare) and non-radioactive citrate was used. The surface radioactivity was removed with five washes of 1 ml of sodium buffer, lysed in 250 μl of 1% SDS for 60 min, and then transferred to scintillation vials for liquid scintillation counting. The uptake rates were corrected for background counts in control cells transfected with pcDNA3.1 vector alone. Kinetic constants were calculated by nonlinear regression to the Michaelis-Menten equation using SigmaPlot 2000 software (Jandel Scientific).

**Dual-label Competitive Transport Experiments—** For dual-label transport assays, sodium buffer containing both 10 μM [³H]sucinate and 20 μM [¹⁴C]citrate was added to the cells in 24-well plates and competitive transport of these substrates was measured as described previously (19). The transport specificity ratio was calculated using 

\[
\text{TSR} = \frac{v_{\text{sucinate}}}{v_{\text{citrate}}} \times \left(\frac{[\text{citrate}]}{[\text{sucinate}]}ight)
\]

where \(v_{\text{sucinate}}\) and \(v_{\text{citrate}}\) are the rates of transport of [³H]sucinate and [¹⁴C]citrate, [citrate] and [sucinate] are the concentrations of citrate and succinate (20).

*Chemical Labeling with MTSES—* The NaDC1 mutants in HRPE cells were preincubated with 250 μl of 1 mM MTSES (Toronto Research Chemicals) in sodium buffer. For experiments with 1 mM MTSES, the reagent was weighed out fresh for each experiment, kept on ice, and diluted in buffer just before using. For experiments with 10 μM MTSES, a stock solution of 5 mM MTSES in water was kept dark and on ice and diluted in buffer just before using. Control groups were preincubated in sodium buffer without MTSES. After 20 min of incubation at room temperature (or ice, for temperature-dependence experiment), the cell monolayers were washed three times with sodium buffer and then assayed for succinate transport activity as described above. For cation replacement experiments, choline buffer was substituted for sodium buffer; for substrate protection experiments, the incubation with MTSES was done in the presence or absence of 10 mM succinate.

*Cell Surface Biotinylation and Total Protein Expression—* Cell surface protein expression of NaDC1 was determined using a membrane-impermeant biotin reagent, Sulfo-NHS-LC-biotin (Pierce), and the biotinylated proteins were precipitated using ImmunoPure-immobilized streptavidin beads (Pierce) as described previously (15). The supernatants after centrifugation to separate the biotin-streptavidin-agarose bead complexes were transferred to new tubes for determination of intracellular protein abundance by immunoblotting in parallel with cell surface protein samples. The proteins separated by SDS-PAGE were blotted onto nitrocellulose membranes. Blots were probed with an anti-NaDC1 antibody applied at a dilution of 1:1000 at 4 °C overnight followed by incubation with horseradish peroxidase-linked anti-rabbit Ig (Amersham Biosciences GE Healthcare) at a dilution of 1:5000 for 1 h. Antibody binding to NaDC1 was detected with the Supersignal West Pico chemiluminescent substrate kit (Pierce) as described previously (15). Images were acquired with an Image Station 440CF imager (Eastman Kodak Co.), and the intensity of protein bands was analyzed using Image 1D analysis software.

*MTSEA-Biotinylation—* HRPE cells expressing NaDC1 mutants were pretreated with sodium buffer with or without 1 mM MTSES for 20 min at room temperature. The MTSES was removed with three washes of 3 ml of PBS/CM, pH 7.5 (phosphate-buffered saline, pH 7.5, with 1 mM Ca²⁺ and Mg²⁺). For each experiment, a 200-mM stock solution of N-biotinyl aminooethyl methanethiosulfonate (MTSEA-biotin; Toronto Research Chemicals) was prepared in Me₂SO and kept cold and dark. The MTSEA-biotin was diluted to 2 mM with PBS/CM, pH 7.5, just before use and added to the cells for 30 min at room temperature with gentle rocking. The cells were rinsed with cold PBS/CM, pH 7.5. The remaining procedures were identi-
cal to those in the cell surface biotinylation protocol as described above.

RESULTS

Cysteine-scanning Mutagenesis of Conserved Residues in TM3—The sequence alignment of amino acids in NaDC1 with other members of the SLC13 family shows the highly conserved region predicted to form TM3 and the connecting loops (Fig. 1). Fifteen amino acid residues from Ile-98 to Arg-112 as well as Lys-84, previously reported to affect substrate binding (14), were selected for cysteine-scanning mutagenesis in which the amino acids were replaced with cysteine one at a time.

Protein Expression and Transport Activity of Cysteine-substituted Mutants—The cell surface expression of the mutants was monitored by biotinylation with the membrane-impermeant reagent Sulfo-NHS-LC-biotin, followed by Western blotting. Total intracellular expression of the mutant proteins was also determined. Fig. 2 shows single representative blots of cell surface and total intracellular protein expression for each cysteine mutant compared with the parental transporter, the C476S mutant of rbNaDC1. The two bands on the Western blots represent differently glycosylated forms of the transporter (15).

The succinate transport activity and protein expression of the mutants are summarized in Fig. 3. Most of the cysteine substitutions were tolerated, as reflected in some measurable succinate transport activity in most of the cysteine mutants, 30–75% activity compared with the parental C476S. The majority of the mutants were expressed at the plasma membrane. Four of the mutants (V100C, L105C, R108C, and A110C) were either entirely inactive or had activity that was <5% of the parental transporter activity. L105C was absent from the plasma membrane and there was little intracellular protein. R108C was found to be absent from the plasma membrane although total intracellular expression was high, suggesting impairments in protein trafficking or stability. The other two

FIGURE 1. Multiple sequence alignment of TM3 and connecting loops in members of the SLC13 family. The amino acid numbering (76–112) is based on the rbNaDC1 sequence. The sequence alignment was performed using the ClustalW program (default parameters, Gonnet matrix). Other members of the SLC13 family include the high affinity Na+/dicarboxylate cotransporters (NaDC3), Na+/citrate cotransporter (NaCT), Na+/sulfate cotransporters (NaS), and the Drosophila dicarboxylate exchanger (Indy). The GenBank accession numbers for nucleotide sequences are next to the names. Positions of conserved amino acid residues in the proteins are highlighted in gray. Amino acids in rbNaDC1 mutated in this study are indicated by * above the sequence. The amino acids before the predicted TM3 sequence are part of the intracellular loop connecting TM2 and 3.

FIGURE 2. Western blots of cell surface (A) and total (B) protein expression. HRPE cells expressing cysteine mutants were treated with Sulfo-NHS-LC-biotin as described under “Experimental Procedures.” The upper panel shows the cell surface protein expression, and the lower panel shows the intracellular protein expression. Western blots were probed with anti-NaDC1 antibodies (1:1000 dilution). Each blot includes an internal control of the parental transporter C476S. The positions of the molecular mass standard (MagicMark XP) are indicated on the left. Two immunoreactive bands at ~70 and 55 kDa represent differently glycosylated forms of rbNaDC1 (15).

FIGURE 3. Transport activity and protein expression of cysteine-substituted mutants. The data are shown as a percentage of the C476S control from the same blot or uptake experiment. Transport of 100 μM [3H]succinate was measured with 30 min of incubation in sodium-containing buffer. Transport results shown are mean ± S.E. (n = 3–11). Protein abundance was determined by quantitating the intensities of NaDC1 protein bands from Western blots, such as those shown in Fig. 2. The bars represent mean ± range or S.E. (n = 2–4 blots, separate transfections).
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FIGURE 4. Dual-label competitive transport of cysteine-substituted NaDC1 mutants. A, competitive uptake of [3H]succinate (10 μM) and [14C]citrate (20 μM) by cysteine-substituted mutants. The transport assay was performed for 20 min. B, transport specificity ratios (TSR succinate: citrate) of NaDC1 mutants, calculated from data shown in panel A. Bars are mean ± S.E., N is sample size. The * denotes significant difference compared with control group.

TABLE 1

Succinate and citrate kinetics in cysteine-substituted mutants

The kinetics of succinate and citrate transport were determined in HRPE cells expressing the parental transporter C476S or the cysteine mutants exhibiting changes in TSR. Six-minute uptakes were measured. The kinetic values shown are the mean ± S.E., N is sample size. The * denotes significant difference compared with the parental C476S (p < 0.05). Because of differences in transporter expression between the succinate and citrate kinetics experiments, the Vmax values are shown relative to internal controls. The C476S Vmax values are 7.9 (succinate) and 49.1 (citrate) pmol/well-min.

| NaDC1 mutant | Succinate kinetics | Citrate kinetics |
|--------------|-------------------|------------------|
|              | K<sub>m</sub> | V<sub>max</sub> | N | μμ | K<sub>m</sub> | V<sub>max</sub> | N |
| C476S        | 25 ± 2           | 100 ± 40        | 5 | % of C476S | 203 ± 6      | 100 ± 12        | 3 |
| K84C         | 36 ± 8           | 63 ± 33         | 3 | % of C476S | 617 ± 151*   | 78 ± 5          | 3 |
| E101C        | 50 ± 4*          | 83 ± 31         | 4 | % of C476S | 672 ± 126*   | 16 ± 1*         | 3 |
| W103C        | 20 ± 3           | 102 ± 44        | 3 | % of C476S | 372 ± 52*    | 111 ± 34        | 3 |
| H106C        | 47 ± 8           | 42 ± 16         | 3 | % of C476S | 1075 ± 91*   | 36 ± 2*         | 3 |
| L111C        | 24 ± 0.3         | 50 ± 6          | 3 | % of C476S | 487 ± 40*    | 74 ± 8          | 3 |

FIGURE 5. Effect of MTSES on succinate transport by cysteine-substituted NaDC1 mutants. HRPE cells expressing mutants were preincubated with 1 mM MTSES in sodium buffer or with sodium buffer alone (control) for 20 min. The [3H]succinate uptake activity remaining after the preincubation was then measured. Uptake activities in cells pretreated with MTSES are expressed as a percentage of the uptakes in cells preincubated with sodium buffer alone. Data shown are means ± S.E., N = 3 experiments. *, p < 0.05, significantly different from control group for that mutant.

mutants, V100C and A1110C, were completely inactive despite a high level of cell surface expression, implying that these two residues have functionally important roles in succinate transport in NaDC1.

TSR Analysis of Cysteine Mutants—TSR analysis was used to detect substrate-selective perturbations in catalytic specificity in the cysteine mutants. TSR is a method to compare the effects of site-directed mutagenesis on function by monitoring relative changes in catalytic efficiency (k<sub>cat</sub>/K<sub>m</sub>) of two substrates (20). One great advantage to TSR analysis is that it is independent of protein expression, so that mutants with low expression can be analyzed even when the expression is too low for kinetic analysis. TSR is also valid over a wide range of substrate concentrations (20). To investigate whether substrate specificity determinants exist within TM3, cells expressing cysteine mutants with measurable transport activity were assessed by measuring competitive uptakes of [3H]succinate and [14C]citrate (Fig. 4A). The succinate: citrate TSR of most cysteine mutants and the C476S parental transporter were ~3 (Fig. 4B), similar to wild-type rbNaDC1 (19). Five mutants showed significant increases in TSR: K84C, E101C, W103C, H106C, and L111C (Fig. 4B). Consequently, these five residues might play an important role in distinguishing succinate from citrate in NaDC1.

Functional Characteristics of Cysteine Mutants—The kinetics of succinate and citrate transport were measured in the cysteine mutants showing differences in TSR (K84C, E101C, W103C, H106C, and L111C). The time courses of uptake, measured between 1–15 min, were linear up to 6 min (data not shown); therefore, 6-min uptakes were chosen for subsequent kinetic study. As shown in Table 1, the mean apparent K<sub>m</sub> for succinate in K84C, W103C, H106C, and L111C was similar to that of the parental C476S transporter. The K<sub>m</sub> in E101C was significantly greater than the parental, by ~2-fold. There were no significant differences between succinate V<sub>max</sub> values in any of the mutants compared with the parental C476S transporter. However, the decreased cell-surface protein abundance in E101C, W103C, H106C, and L111C (Fig. 3) indicates that the succinate k<sub>cat</sub> values of these mutants are likely to be increased relative to C476S.

There were differences in citrate kinetics between the substituted cysteine mutants and the C476S parental transporter (Table 1). All of the mutants had increased citrate K<sub>m</sub> values compared with C476S. Two of the mutants, E101C and H106C, also had much lower V<sub>max</sub> values, which were comparable with the abundance of these transporter proteins on the plasma membrane (Fig. 3). The increased TSR in the K84C, E101C, W103C, H106C, and L111C mutants indicates an increase in relative catalytic efficiency (k<sub>cat</sub>/K<sub>m</sub>) of succinate compared with citrate. Therefore, for many of the mutants, the TSR value
reflects a combination of an increased catalytic efficiency for succinate and a decreased catalytic efficiency for citrate.

It should be noted that the succinate $K_m$ value in C476S in the present study is ~10-fold lower than we have previously reported. The succinate $K_m$ in wild-type rbNaDC1 is similar to that of C476S when expressed in HRPE cells (results not shown), and the succinate: citrate TSR values are also similar. Under two-electrode voltage clamp conditions, the $K_m$ for wild-type rbNaDC1 expressed in Xenopus oocytes is ~180 $\mu$M at ~50 mV (21). In radiotracer uptake assays with Xenopus oocytes, not under voltage clamp, the $K_m$ for wild-type rbNaDC1 or the C476S mutant is between 0.2 and 0.5 mM (11, 22). We have previously measured a $K_m$ of 0.5 mM for wild-type NaDC1 expressed in HRPE cells (19). At present we do not know why the $K_m$ values in this study are lower than in our previous studies. The cDNA has been resequenced to make sure there are no mutations, both authors of this report have done kinetic measurements of C476S and obtained similar results, and we have tested the effects of transport conditions (time points, preincubations) and cell passage number. The effect may be specific to the rbNaDC1 ortholog since the succinate $K_m$, for hNaDC1 expressed in HRPE cells is similar to that in oocytes, ~0.7 mM (results not shown). All of the experiments reported in this study have compared mutant values with C476S in the same transfection experiment.

**MTSES Sensitivity of Cysteine Mutants**—The twelve cysteine mutants with measurable transport activity were screened for their sensitivity to the membrane-impermeant thiol-reactive reagent MTSES. The mutant T482C, which is highly sensitive to MTSET, MTSEA, and MTSES (11), was used as a positive control in each experiment. Of all the TM3 mutants, only K84C showed a decrease in transport activity after chemical labeling with MTSES (Fig. 5). None of the cysteine mutants between positions 98 and 112 was sensitive to inhibition by MTSES (1 mM (Fig. 5) or 10 mM (data not shown)). We also tested the cysteine mutants with MTSET (1 mM), which adds a positive charge, but it had no effect on any of the cysteine mutants, including K84C (data not shown). However, the MTSET does react with K84C, since pretreatment of cells expressing K84C with 1 mM MTSET prevented subsequent inhibition by MTSES (data not shown).

**Concentration Dependence of MTSES Inhibition**—The concentration dependence of MTSES inhibition of K84C transport activity was next examined. As shown in Fig. 6, K84C was very sensitive to inhibition by MTSES, with an IC$_{50}$ value of ~6 $\mu$M. Therefore, a concentration of 10 $\mu$M MTSES was used in subsequent experiments.

**Effect of Substrate and Cations on the Accessibility of K84C Mutant**—The transport process of NaDC1 follows an ordered binding mechanism in which three sodium ions bind first, followed by substrate (23, 24). To determine whether K84C is accessible to MTSES in different conformational states, the preincubation with MTSES was conducted in either sodium or choline buffer with or without succinate. The inhibition by MTSES in K84C was only seen when the preincubation was done in sodium buffer (Fig. 7A). When the sodium was replaced by
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choline, or when substrate was present, the effect of MTSES was prevented.

Effect of Temperature on MTSES Inhibition—Substrate protection of MTSES labeling could be due to substrate binding preventing the access of MTSES to the cysteine or there could be a conformational change after substrate binding that occludes the substituted cysteine. At cold temperatures, large scale conformational changes of transporters are slowed down but substrate binding is not affected (25, 26). If the major effect of substrate protection is a conformational change due to translocation, then we would predict a reduction in substrate protection of MTSES inhibition at cold temperature. However, there was no change in substrate protection at cold temperatures in the K84C mutant (Fig. 7B). The $K_{0.5}$ of substrate protection in K84C was $\sim 73 \mu M$ at room temperature compared with $79 \mu M$ at 4°C. The $K_{0.5}$ value agrees well with the $K_{0.5}$ value for succinate in this mutant (Table 1). At both temperatures, there was almost complete protection of transport activity with high concentrations of succinate. This result suggests that the effect of substrate protection in K84C is likely to be steric hindrance of chemical labeling by MTSES.

Labeling of Substituted Cysteines with MTSEA-Biotin—Even though most of the TM3 cysteine mutants were insensitive to MTSES, it is possible that some residues might be accessible to MTSES labeling without any functional consequences. We therefore examined whether some of the substituted cysteines could be labeled directly with MTSEA-biotin. I98C and R112C were chosen because they are at both ends of the cysteine scan and exhibit relatively high protein expression (see Fig. 2). We have found in previous studies that proteins with low expression are difficult to detect after labeling with MTSEA-biotin; the sensitivity appears to be lower than that of sulfo-NHS-biotin labeling (12). Additionally, we verified the accessibility of the cysteine substitution at position 84. The mutant T482C from our previous study was used as a positive control (12). There is some background binding of MTSEA-biotin to the C476S control, but this was not decreased by preincubation with MTSES (Fig. 8). The positive control, T482C, showed a large decrease in the MTSEA-biotin signal after incubation with MTSES. There was specific binding of MTSEA-biotin to the K84C and R112C mutants, verifying that they are accessible from the outside of the cell, but there was no specific binding to I98C (Fig. 8).

DISCUSSION

Predicted TM 3 of the rabbit Na$^+$/dicarboxylate cotransporter NaDC1 is highly conserved with other members of the SLC13 family. Our previous studies have shown that individual amino acids from TM3 and connecting loops in NaDC1 have important functions. For example, Ala replacement of Lys-84 decreases affinity for substrate (14). His-106 at the extracellular part of the helix affects targeting of NaDC1 expressed in Xenopus oocytes (15). Differences in glutarate affinity between the rabbit and mouse NaDC1 are determined by residues in TM3 and 4 (13). In the present study, we found that cysteine substitutions at the extracellular portion of TM3 (amino acids 98–112) do not produce transport inhibition by MTS reagents, although several of the amino acids in that region are involved in determining substrate selectivity. Lys-84, in contrast, was very sensitive to chemical labeling by MTSES and exhibited its greatest accessibility to the reagent in the presence of sodium. Lys-84 is likely to be located within the substrate binding pocket, and it undergoes conformational changes in accessibility to the outside during the transport cycle. The results indicate a new location for Lys-84 either in a membrane helix facing a water-filled pore or in a reentrant loop.

TSR analysis is used to identify mutations in transporters that produce changes in relative catalytic efficiency or specificity ($k_{cat}/K_m$) of one substrate compared with another (20). The mutations at amino acids Glu-101, Trp-103, His-106, and Leu-111 resulted in increases in succinate:citrate TSR values. These amino acids are highly conserved among the members of the SLC13 family (Fig. 1). The increased TSR could be, at least in part, determined by an increased $k_{cat}$ for succinate because there was no change in $V_{max}$ but a decrease in cell surface protein expression. The changes in TSR could also be determined.
by decreased catalytic efficiency for citrate because all of the mutants with altered TSR had decreased citrate affinity. Because the mutations themselves did not alter succinate $K_m$ and treatment with MTSES or MTSET had no effect on the activity of the mutants, it is possible that the residues determine substrate selectivity indirectly by affecting transporter flexibility or by holding other key residues in position. Mutations far from the active site in proteins can perturb catalytic rate by altering conformational mobility of the entire protein (27). Cysteine replacement of two residues in TM3, Val-100 and Ala-110, resulted in inactive proteins although the proteins were present on the plasma membrane, suggesting that these amino acids are critical for transport activity in NaDC1. The positions of these critical amino acids are adjacent to Glu-101 and Leu-111, both of which are involved in determining substrate selectivity, confirming that this region is important functionally.

Cysteine replacement of Lys-84 had no effect on succinate kinetics, indicating that this residue is not likely to be directly involved in succinate binding, but the citrate $K_m$ was increased ~2-fold. The increase in TSR, therefore, reflects changes in citrate transport by this mutant. In our previous study, alanine substitution at position 84 had no effect on sodium affinity but resulted in a large decrease in succinate affinity with a $K_m$ of 2.2 mM (14). It is not clear why the cysteine substitution at position 84 would have no effect on succinate $K_m$ since it also represents a charge neutralization and replacement with a much smaller side chain. There was also no effect of chemical labeling of K84C with MTSET, which should restore the positive charge. However, charge reversal at position 84 by chemical modification with MTSES, which adds a negative charge, produced inhibition of transport.

The substrate binding sites of all of the known structural models of ion-coupled transport proteins, including the H$^+$/Cl$^-$-coupled lactose permease (28), Na$^+$-dependent leucine transporter LeuT$_{Aa}$ (29), and the Na$^+$-dependent aspartate transporter Glt$_{p}$ (30), are located within aqueous cavities formed by multiple helices. In LeuT$_{Aa}$, the cation and substrate binding sites are located very close together and are formed, in part, by unwound regions of transmembrane helices (29). Several of the MTS derivatives, including MTSES and MTSET, are membrane-impermeant reagents that label cysteine residues in an aqueous environment (16, 17). The impermeant MTS reagents can be used to map cysteine residues located in extracellular loops or in water-filled pores, such as the substrate access and binding pores of membrane proteins.

The cysteine-scanning approach, particularly when compared with protein crystal structures, is beginning to provide detailed information on conformational changes in ion-coupled transport proteins. The mammalian Na$^+$/Cl$^-$-serotonin transporter SERT exhibits changes in reactivity of substituted cysteines that relate well to the crystal structure of its bacterial homolog LeuT$_{Aa}$ (31). For example, cysteine substitutions of residues in TM5 of SERT, predicted to form part of the cytoplasmic substrate permeation pathway, exhibit decreased accessibility with inhibitor binding, which locks the transporter in an outward-facing conformation. The same substituted cysteines in SERT have increased reactivity with MTS reagents in the presence of ions and substrate, reflecting conformational changes associated with transport (32). The mammalian glutamate transporters EAAT1 and GLT-1 also exhibit changes in conformation that can be detected by accessibility of substituted cysteines. For example, the relative positions of the two opposing hairpin loops, thought to form substrate access gates in the crystal structure of the archael homolog Glt$_{p}$ (30), change during the transport cycle in both EAAT1 and GLT-1 (33, 34). The lactate permease also exhibits changes in substituted cysteine accessibility upon substrate binding (35). When superimposed on the crystal structure of the inward-facing conformation of the lactose permease, residues that exhibited increased N-ethylmaleimide labeling in the presence of substrate were found to be located away from the substrate binding site and near the periplasmic surface of the protein. In contrast, decreased N-ethylmaleimide labeling in the presence of substrate (substrate protection) was seen in cysteines located near the inward-facing substrate binding cavity and at the cytoplasmic ends of helices, also an indication of conformational changes that occur with substrate binding.
Transmembrane Helix 3 of NaDC1

The transport cycle of NaDC1 involves ordered binding of three sodium ions followed by a divalent anion substrate (23, 24). The accessibility of K84C to MTSES seems to parallel the exposure of the substrate binding site. The inhibition by MTSES was seen in the presence, but not the absence, of sodium, and the addition of substrate produced substrate protection. The succinate $K_{0.5}$ for substrate protection of MTSES inhibition was $\sim 79 \mu M$, similar to the transport $K_m$ of 25 $\mu M$. In the absence of sodium the transporter is in a conformational state that prevents accessibility of MTSES to the substituted cysteine at position 84 by placing it in a lipid environment or between two helices that do not have an aqueous pore open to the outside of the cell. Sodium binding triggers a conformational change in NaDC1 in which the helices may tilt or rotate to allow exposure of the substrate binding site and subsequent increase in substrate affinity. This is also the conformation in which the cysteine at position 84 is most accessible to extracellularly applied MTS reagents. The fully loaded transporter containing the sodium and substrate undergoes a large conformational change to translocate all substrates to the opposite side of the membrane. Substrate protection could occur either by occlusion of the cysteine at position 84 as a result of the conformational change or by steric hindrance produced by substrate binding. The large scale conformational changes of transporters are inhibited in the cold, although substrate binding is not affected by temperature (25, 26). Because cold temperature did not affect substrate protection in K84C, it is likely that substrate binding produces steric hindrance of MTSES binding and this occurs at an early state in the transport cycle prior to translocation. Therefore, the Lys-84 residue is most likely located in the water-filled cavity that contains the substrate binding site.

Our previous model of TM3 was based on hydropathy analysis and placed Lys-84 in an intracellular loop between TM2 and 3 (8, 36). However, the present study provides experimental evidence that Lys-84 is accessible to the outside of the cell, indicating that it is located either on the extracellular side of the membrane or in an aqueous pore or cavity. We also found that R112C is located extracellularly, since it can be labeled with MTSEA-biotin, and I98C is located either intracellularly or within the membrane in a transmembrane helix, since it was not labeled by MTSEA-biotin. Two alternative models of the TM3 region of NaDC1 are shown in Fig. 9. In one model, Lys-84 is located in the transmembrane helix, with part of the helix forming the substrate binding cavity or access pore. If this model is correct, the size of the substrate access pore would be large enough to accommodate MTSEA-biotin, at least as far as Lys-84. In the second model, Lys-84 is located in a reentrant loop that lines an aqueous pore forming the substrate binding pocket. Further experiments will be needed to distinguish between the two models.

In conclusion, this study has shown that the conserved residue at Lys-84 and amino acids at the extracellular half of TM3 are functionally important in NaDC1. Residues in TM3 include Ala-100 and Val-110, which produced inactive proteins when mutated to cysteine, and Glu-101, Trp-103, His-106, and Leu-111, which contain determinants of substrate selectivity although these residues may participate indirectly in substrate binding and translocation. Lys-84 is probably located within the substrate binding cavity of NaDC1. The sensitivity of the K84C mutant to MTSES inhibition was greatest in the presence of sodium, and inhibition could be prevented by addition of substrate or replacement of sodium, indicating that the accessibility of Lys-84 changes with conformational state. The substrate protection of MTSES inhibition of K84C appears to occur early in the transport cycle, before the large scale conformational change associated with translocation of substrate. The results indicate a new location for Lys-84 in the substrate access pore formed in part by the transmembrane helix or a reentrant loop.

REFERENCES

1. Markovich, D., and Murer, H. (2004) Pflugers Arch. Eur. J. Physiol. 447, 594–602
2. Pajor, A. M. (2006) Pflugers Arch. Eur. J. Physiol. 451, 597–605
3. Wright, S. H., and Dantzler, W. H. (2004) Physiol. Rev. 84, 987–1049
4. Pak, C. Y. (1991) Am. J. Kidney Dis. 18, 624–637
5. He, W., Miao, F. J., Lin, D. C., Schwander, R. T., Wang, Z., Gao, J., Chen, J. L., Tian, H., and Ling, L. (2004) Nature 429, 188–193
6. Rogina, B., Reenan, R. A., Nilsen, S. P., and Helfand, S. L. (2000) Science 290, 2137–2140
7. Fei, Y. J., Inoue, K., and Ganapathy, V. (2003) J. Biol. Chem. 278, 6136–6144
8. Zhang, F. F., and Pajor, A. M. (2001) Biochim. Biophys. Acta 1511, 80–89
9. Kahn, E. S., and Pajor, A. M. (1999) Biochimie 81, 6151–6156
10. Pajor, A. M., Sun, N., Bai, L., Markovich, D., and Sule, P. (1998) Biochim. Biophys. Acta 1370, 98–106
11. Pajor, A. M. (2001) J. Biol. Chem. 276, 29961–29968
12. Pajor, A. M., and Randolph, K. M. (2005) J. Biol. Chem. 280, 18728–18735
13. Oshiro, N., King, S. C., and Pajor, A. M. (2006) Biochemistry 45, 2302–2310
14. Pajor, A. M., Kahn, E. S., and Gangula, R. (2000) Biochem. J. 350, 677–683
15. Pajor, A. M., Sun, N., and Valmonte, H. G. (1998) Biochem. J. 331, 257–264
16. Javitch, J. A. (1998) Methods Enzymol. 296, 331–346
17. Karlin, A., and Akabas, M. H. (1998) Methods Enzymol. 293, 123–145
18. Pajor, A. M., Krajewski, S. J., Sun, N., and Gangula, R. (1999) Biochem. J. 344, 205–209
19. Joshi, A. D., and Pajor, A. M. (2006) Biochemistry 45, 4231–4239
20. King, S. C. (2004) BMC Biochem. 5, 16
21. Pajor, A. M., Hirayama, B. A., and Loo, D. D. (1998) J. Biol. Chem. 273, 18923–18929
22. Griffith, D. A., and Pajor, A. M. (1999) Biochemistry 38, 7524–7531
23. Wright, S. H., Hirayama, B., Kaunitz, J. D., Kippen, I., and Wright, E. M. (1993) J. Biol. Chem. 258, 5456–5462
24. Yao, X., and Pajor, A. M. (2000) Am. J. Physiol. 279, F54–F64
25. Parent, L., Supplisson, S., Loo, D. D., and Wright, E. M. (1992) J. Membr. Biol. 125, 63–79
26. Wadiche, J. I., and Kavanaugh, M. P. (1998) J. Neurosci. 18, 7650–7661
27. Wong, K. F., Selzer, T., Benkovic, S. J., and Hammes-Schiffer, S. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 6807–6812
28. Abramson, J., Smirnova, L., Kasho, V., Verner, G., Kacskov, H. R., and Iwata, S. (2003) Science 301, 610–615
29. Yamashita, A., Singh, S. K., Kawate, T., Jin, Y., and Gouaux, E. (2005) Nature 437, 215–223
30. Yernool, D., Boudker, O., Jin, Y., and Gouaux, E. (2004) Nature 431, 811–818
31. Rudnick, G. (2006) J. Membr. Biol. 213, 101–110
32. Zhang, Y. W., and Rudnick, G. (2006) J. Biol. Chem. 281, 36213–36220
33. Leighton, B. H., Seal, R. P., Watts, S. D., Skyba, M. O., and Amara, S. G. (2006) J. Biol. Chem. 281, 29788–29796
34. Shlaifer, I., and Kanner, B. I. (2007) Mol. Pharmacol. 71, 1341–1348
35. Kaback, H. R., Dunten, R., Frillingos, S., Venkatesan, P., Kwaw, J., Zhang, W., and Ermolova, N. (2007) Proc. Natl. Acad. Sci. U. S. A. 104, 491–494
36. Pajor, A. M. (1995) J. Biol. Chem. 270, 5779–5785