Arginine-349 and Aspartate-373 of the Na^+/Dicarboxylate Cotransporter Are Conformationally Sensitive Residues†

Xiaozhou Yao and Ana M. Pajor*

Department of Physiology and Biophysics, University of Texas Medical Branch, Galveston, Texas 77555-0641

Received August 13, 2001; Revised Manuscript Received November 19, 2001

ABSTRACT: The conserved residues, Arg-349 and Asp-373, of the renal Na^+/dicarboxylate cotransporter (NaDC-1) have been shown in our previous studies to affect substrate affinity and cation binding. In this study, amino acids surrounding Arg-349 and Asp-373 were individually mutated to cysteines and their sensitivity to methanethiosulfonate reagents (MTS) was tested. Only three of the 21 mutants were sensitive to MTS reagents: R349C, S372C, and D373C. The R349C mutant had reduced activity which was restored by chemical modification with MTSEA. The effect of MTSEA was only observed in the presence of sodium, indicating that Arg-349 is conformationally accessible. The succinate transport activity of the S372C mutant was stimulated by both MTSEA and MTSET. The D373C mutant was very sensitive to inhibition by MTSET ($K_i = 0.5\ \mu M$) in sodium buffer. The inhibition of D373C by MTSET was prevented by substrate, suggesting that the substrate-induced conformational change occludes the residue. We conclude that the accessibility of Arg-349 and Asp-373 is likely to change with the conformational states of the transport cycle.

The Na^+/dicarboxylate cotransporters of the plasma membrane, called NaDC, belong to the SLC13 gene family, and are not related to any other known families of transport proteins (*1*). The major function of the NaDCs is the active transport of Krebs cycle intermediates such as succinate, α-ketoglutarate, and citrate via cotransport with sodium ions (*1*). The first Na^+/dicarboxylate cotransporter to be cloned (NaDC-1)[1] was isolated from a rabbit renal cortex cDNA library using the technique of functional expression in *Xenopus* oocytes (*2*). NaDC-1 is found on the apical membrane in renal proximal tubule and small intestine. In the kidney, one important function of NaDC-1 is to regulate the concentration of urinary citrate, which acts as a chelator of calcium. Hypocitraturia is a risk factor for kidney stone formation (*3*). The intestinal NaDC-1 absorbs Krebs cycle intermediates from the diet. Recent studies suggest that NaDC-1 activity may also be involved in determining life span (*4*).

The secondary structure model of NaDC-1 contains 11 transmembrane domains and an extracellular carboxy terminus (Figure 1) (*5*). The functionally important areas of the protein, including the substrate recognition domain, are located in the carboxy-terminal half of the protein (*6*). The conserved arginine residue at position 349, located at the extracellular end of transmembrane domain (TMD) 7, is likely to play an important role in substrate binding (*7*). The conserved aspartate at position 373 in TMD 8 may be involved in the topological organization of the cation binding site and in the determination of substrate binding affinity (*8*). Therefore, in the study presented here, we examined the amino acids surrounding Arg-349 and Asp-373. A total of 11 residues (Leu-344−Phe-354) around Arg-349 in TMD 7 and 10 residues (Gly-367 and Val-369−Ser-377) around Asp-373 in TMD 8 were mutated one at a time to cysteine using site-directed mutagenesis. The sensitivity of the substituted cysteines via chemical modification by methanethiosulfonate derivative (MTS) reagents was then determined.

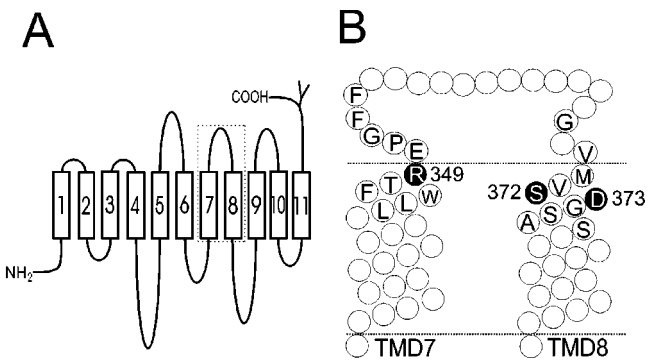

FIGURE 1: (A) Secondary structure model of NaDC-1. The empty rectangles represent 11 transmembrane α-helices joined by hydrophilic loops. The Y represents the N-glycosylation site at Asn-578. The outside of the cell is at the top of the figure. (B) Locations of the 21 amino acids mutated in this study. The circles represent the amino acids in transmembrane domains (TMD) 7 and 8 and the connecting loop. The mutated amino acids are denoted by single-letter abbreviations. Eleven mutants (L344−F354) are located around the outer portion of TMD 7, and 10 mutants (G367 and V369−S377) are located around the outer portion of TMD 8. The C476S mutant was the parental transporter for all the mutants.

† This study was supported by U.S. Public Health Service Grants DK46269 and DK02429.

* To whom correspondence should be addressed: Department of Physiology and Biophysics, University of Texas Medical Branch, Galveston, TX 77555-0641. Telephone: (409) 772-3434. Fax: (409) 772-3381. E-mail: ampajor@utmb.edu.

[1] Abbreviations: NaDC-1, rabbit renal Na^+/dicarboxylate cotransporter; TMD, transmembrane domain; MTS, methanethiosulfonate; MTSEA, (2-aminoethyl)methanethiosulfonate hydrobromide; MTSES, sodium (2-sulfonatoethyl)methanethiosulfonate; MTSET, [2-(trimethylammonium)ethyl]methanethiosulfonate bromide; DTT, dithiothreitol; pCMBS, *p*-chloromercuribenzenesulfonate; SLC13, solute carrier family 13.

10.1021/bi0156761 CCC: $22.00
Published on Web 12/27/2001

The charged MTS reagents are used to identify substituted cysteine residues that are accessible from the outside of the cell or are located in a water-filled pore. MTSEA and MTSET add a positive charge to the protein, whereas MTSES adds a negative charge. MTSEA is somewhat membrane permeant, but the other two compounds are impermeant (*9*−*11*). In the study presented here, we found that three cysteine-substituted mutants (R349C, S372C, and D373C) were sensitive to either MTSEA or MTSET. In the R349C mutant, treatment with MTSEA restored the activity back to that of the arginine-containing wild-type transporter likely because the MTSEA-modified cysteine is very similar in size and charge to arginine (*12*). The accessibility of the substituted cysteine at position 349 was dependent on the presence of sodium. In the D373C mutant, MTSET treatment inhibited transport in both sodium and choline, but this cysteine was much less accessible in the presence of substrate. Because the residues appear to be alternately accessible and inaccessible to the MTS reagents, we conclude that the reaction cycle of NaDC-1 is likely to involve movements of transmembrane domains 7 and 8.

EXPERIMENTAL PROCEDURES

Site-Directed Mutagenesis. Mutants were prepared with the oligonucleotide-directed technique of Kunkel (*13*), using reagents from the Muta-Gene plasmid kit (Bio-Rad), according to the manufacturer's directions. Mutants were verified by sequencing. The mutants are designated using the single-letter amino acid code followed by the number indicating the position of the residue in NaDC-1. The second letter following the sequence number indicates the amino acid replacement at that position. For example, R349C represents a mutant of NaDC-1 in which the arginine at position 349 is replaced with cysteine. The parental transporter for the mutagenesis reactions was the C476S mutant, to avoid any possible influence of this endogenous cysteine, which mediates inhibition by *p*-chloromercuribenzenesulfonate (pCMBS), a cysteine-specific reagent (*14*). However, neither wild-type NaDC-1 nor the C476S mutant is sensitive to inhibition by the MTS reagents, MTSEA, MTSES, or MTSET (ref *15* and results not shown).

A typical cysteine-scan study is based on a cysteine-less mutant to reduce the possibility that the introduced cysteines alter the conformation of the protein and expose endogenous cysteines. In NaDC-1, there is a direct relationship between the number of cysteines and the expression of the protein on the plasma membrane, and a minimum of three cysteines are required for measurable activity (*14*). Our previous scan of TMD 9 showed no difference in MTS sensitivity in mutants prepared in the C476S background (containing 10 of the 11 endogenous cysteines) compared with a mutant containing only three cysteines (called 3C) (*15*). Since the 3C mutant of NaDC-1 has very low transport activity, this study was carried out using the C476S parental transporter.

In Vitro cRNA Transcription and Oocyte Injections. The cDNAs in the pSPORT1 plasmid were linearized, and in vitro cRNA transcription was carried out using the T7 mMessage mMachine Kit (Ambion) as described previously (*2*). Stage V and VI oocytes were obtained from female *Xenopus laevis* frogs (*Xenopus* I) and treated with collagenase as described previously (*2*). The oocytes were injected with 46 nL of cRNA (1 μg/μL) 1 day following isolation and cultured at 18 °C. Culture dishes and medium were changed daily.

Transport Assays and Data Analysis. The assessment of transport of 100 μM [^{3}H]succinate (DuPont-NEN) was performed 5 days after oocyte injections, as previously described (*2*). The composition of sodium buffer was 100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 10 mM HEPES (pH 7.5, adjusted with Tris base). Choline buffer was prepared by replacing NaCl with choline Cl. Succinate uptake was assessed in sodium buffer for 15 min, unless noted otherwise in the figure legends. Transport was stopped by four washes with 4 mL of ice-cold choline buffer. Counts in control, uninjected oocytes were subtracted from the counts in oocytes injected with cRNA. Kinetic constants were derived by nonlinear regression of the data to the Michaelis−Menten and Hill equations using SigmaPlot 2000 software (Jandel Scientific). The error in the data for kinetic constants represents the error of the fit. Other experimental results are expressed as means ± the standard error of the mean (SEM). Statistical analysis was carried out by a Student's *t* test.

MTS Experiments. In experiments using the MTS reagents, the oocytes were preincubated with the reagent before the succinate transport assay. The oocytes were rinsed once with choline buffer, and then preincubated in 400 μL of sodium buffer containing MTSEA, MTSES, MTSET, or sodium buffer alone (control condition) for up to 10 min at room temperature. In experiments with high concentrations of MTS reagents, the reagents were preweighed and then dissolved in sodium buffer just before use. For experiments using MTSET concentrations of less than 10 μM, a 1 mM stock solution was prepared in water on the day of the experiment, kept in the dark at 4 °C, and then diluted to the final concentration in sodium or choline buffer just before use. After the incubation period, the MTS-containing solution was removed with four washes of choline buffer at room temperature. The transport assays were carried out as described above. Data are expressed as a percentage of the uptake measured in oocytes treated without MTS reagent.

Western Blots of Cell-Surface-Biotinylated Proteins. The cell-surface expression of mutants was tested by biotinylating the proteins on the surface of the oocytes using a membrane-impermeant reagent, sulfo-NHS-LC-biotin (Pierce), as described previously (*16*). For each biotinylation experiment, groups of five oocytes were rinsed three times with 4 mL of ice-cold PBS (pH 8). The oocytes were incubated for 10 min at room temperature in 0.5 mL of PBS containing sulfo-NHS-LC-biotin (0.5 mg/mL) followed by four washes with 4 mL of ice-cold PBS. The oocytes were then dissolved in lysis buffer [150 mM NaCl, 20 mM Tris-HCl (pH 7.6), 1% Triton X-100, and protease inhibitors] for 30 min on ice. The solubilized oocytes were centrifuged for 15 min at 14000*g*, and the supernatants were transferred to new tubes. The biotinylated proteins were incubated with 50 μL of ImmunoPure immobilized streptavidin beads (Pierce) for 1 h at 4 °C. The biotin−streptavidin−agarose bead complexes were washed four times by suspension in lysis buffer followed by centrifugation. The final pellets were resuspended in sample buffer and boiled for 5 min before being loaded onto a Tricine gel containing 8% acrylamide for SDS−PAGE.

The transporters precipitated by the biotinylation−streptavidin procedures were identified by Western blotting, also

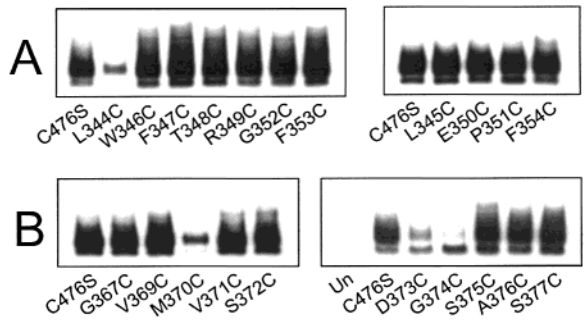

FIGURE 2: Western blots of cell-surface-biotinylated proteins in oocytes expressing cysteine mutants. *Xenopus* oocytes were labeled using a membrane-impermeant derivative of biotin, sulfo-NHS-LC-biotin, as described in Experimental Procedures. Each blot contains an internal control of the C476S mutant, and "Un" refers to control, uninjected oocytes. The blots were incubated with the anti-NaDC-1 antibody at a dilution of 1:5000, followed by horseradish peroxidase-linked anti-rabbit Ig at a dilution of 1:5000. Panels A and B represent the cysteine mutants in TMD 7 and 8, respectively. The two protein bands represent differently glycosylated forms of NaDC-1 expressed in oocytes (*16*).

as described previously (*5*). The nitrocellulose membranes were blocked for a minimum of 1 h in PBS-TM (PBS containing 0.05% Tween 20 and 0.5% Carnation instant dried milk). The Western blots were probed with an antibody directed against a fusion protein of NaDC-1, raised in rabbits (*17*). The primary antibody was diluted 1:5000 in PBS-TM, and applied for 1 h. After being washed with PBS-TM, the nitrocellulose membranes were incubated with the secondary antibody, horseradish peroxidase-linked anti-rabbit Ig (Amersham) diluted 1:5000 in PBS-TM for 1 h. All incubations and washes were carried out at room temperature. Antibody binding was visualized by enhanced chemiluminescence using the Supersignal CL-HRP substrate system (Pierce). Molecular masses were estimated by comparison with prestained protein standards (Bio-Rad).

RESULTS

The residues surrounding Arg-349 and Asp-373 of NaDC-1 were investigated using the substituted-cysteine accessibility method. Eleven residues around Arg-349 (Leu-344−Phe-354) in transmembrane domain (TMD) 7 and the adjacent extracellular loop and 10 residues around Asp-373 (Val-369−Ser-377), including the conserved Gly-367 near the top of TMD 8, were replaced with cysteine, one at a time (Figure 1). The sensitivity of each cysteine-substituted mutant to MTS reagents MTSEA, MTSES, and MTSET was determined to identify the functionally important residues in NaDC-1 that are accessible from the outside of the cell.

Protein Expression and Transport Activity of Cysteine Substitution Mutants. All of the mutant transporters were expressed at the plasma membrane, as shown by cell-surface biotinylation experiments (Figure 2). The cell-surface expression of most of the mutants was similar to that of the C476S mutant, whereas four mutants (L344C, M370C, D373C, and G374C) exhibited lower levels of expression than C476S (Figure 2). The C476S parental transporter had a level of expression similar to that of the wild-type NaDC-1 (results not shown).

The transport activity of the mutants expressed in *Xenopus* oocytes is shown in Figure 3. Many of the mutants had little or no change in transport activity compared with the parental transporter, C476S. The transport activity of the R349C, M370C, S372C, D373C, S375C, and A376C mutants was decreased to approximately 30−50% of that of the parental transporter. Two of these mutants (M370C and D373C) also had decreased levels of protein expression, suggesting that the decrease in activity is related to protein abundance. Also, in accordance with their low levels of protein expression, the L344C mutant exhibited less than 10% of the activity of C476S, whereas the G374C mutant was completely inactive.

Identification of MTS Reagent Sensitive Residues. To investigate whether any of the cysteine-substituted mutants are sensitive to MTS reagents, we compared the transport activity in oocytes preincubated with MTS reagents or with sodium buffer alone (control condition). The concentrations of MTSEA, MTSES, and MTSET were 2.5, 10, and 1 mM, respectively, since MTSET is 2.5 times as reactive as MTSEA and 10 times as reactive as MTSES (*18*). The C476S mutant, the parental transporter of the cysteine substitution mutants, was not sensitive to MTSEA, MTSES, or MTSET (Figure 4A). Only three of the 21 mutants were sensitive to the MTS reagents, and none of the mutants was sensitive to MTSES. As shown in Figure 4A, treatment with MTSEA almost doubled the transport activity of the R349C mutant. Two cysteine-substituted mutants (S372C and D373C) in TMD 8 were sensitive to both MTSEA and MTSET. The activity of D373C was almost completely inhibited by preincubation with MTSEA or MTSET (Figure 4B).

S372C Mutant. The results for the S372C mutant are shown separately in Figure 4C. The transport activity of this mutant was stimulated by both MTSEA and MTSET. However, the stimulatory effect of the MTS reagents was variable, as indicated by the large error bars. In most experiments (five of seven experiments for MTSEA and six of nine experiments for MTSET), there was an increase in the transport activity of 3−4-fold (Figure 4C), but in some batches of oocytes, there was no effect of MTSEA or MTSET. The level of stimulation by MTS reagents appeared to be inversely related to the level of transport activity of the S372C mutant expressed in the oocytes. The stimulation by MTSET or MTSEA was seen in oocyte batches with relatively low levels of expression of S372C, but oocyte batches with high levels of expression of S372C were insensitive to the MTS reagents (results not shown).

The cysteine substitution at position 372 increases the apparent affinity of NaDC-1 for succinate and decreases the apparent affinity for sodium. The K_m for succinate in the S372C mutant was 88 ± 18 μM (mean ± SEM, $n = 3$); that of the C476S mutant was 265 ± 47 μM ($n = 4$, results not shown). The half-saturation constant for sodium (K_{Na}) in S372C was 72 ± 21 mM, and the Hill coefficient was 2.05 (mean ± range, $n = 2$, results not shown). The C476S mutant had a K_{Na} of 36 mM and a Hill coefficient of 1.7 (*15*).

Restoration of R349C Transport Activity by MTSEA. The cysteine replacement at position 349 resulted in a 54% decrease in transport activity (Figure 3A), whereas MTSEA treatment produced an approximately 2-fold increase in transport activity (Figure 4A), suggesting that exposure to MTSEA restores the activity of R349C. The effect of

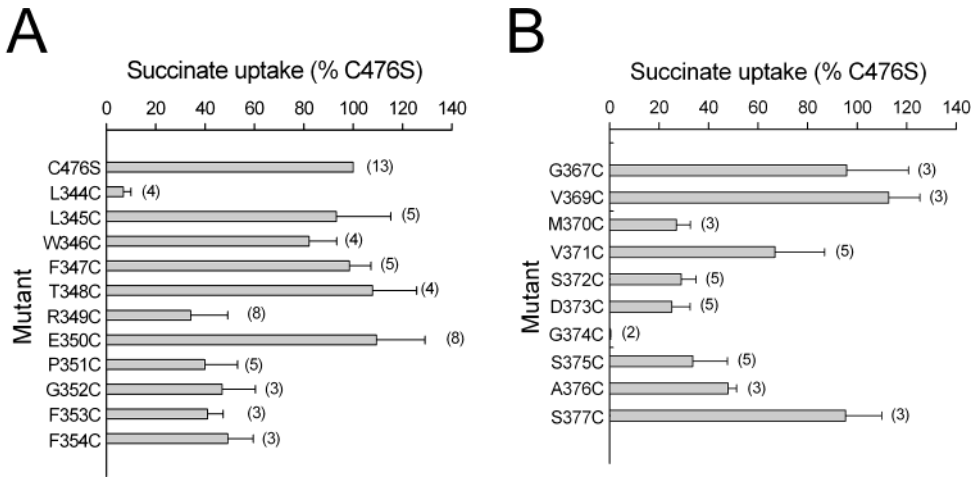

FIGURE 3: Succinate transport activity of cysteine mutants expressed in *Xenopus* oocytes. Uptake of 100 μM succinate was assessed in the mutants in TMD 7 (A) and TMD 8 (B) for a 15 min time period. The data are expressed as a percent of uptakes in the C476S mutant from the same donor frog. The data are means ± SEM, and the numbers in parentheses are the numbers of experiments using different donor frogs.

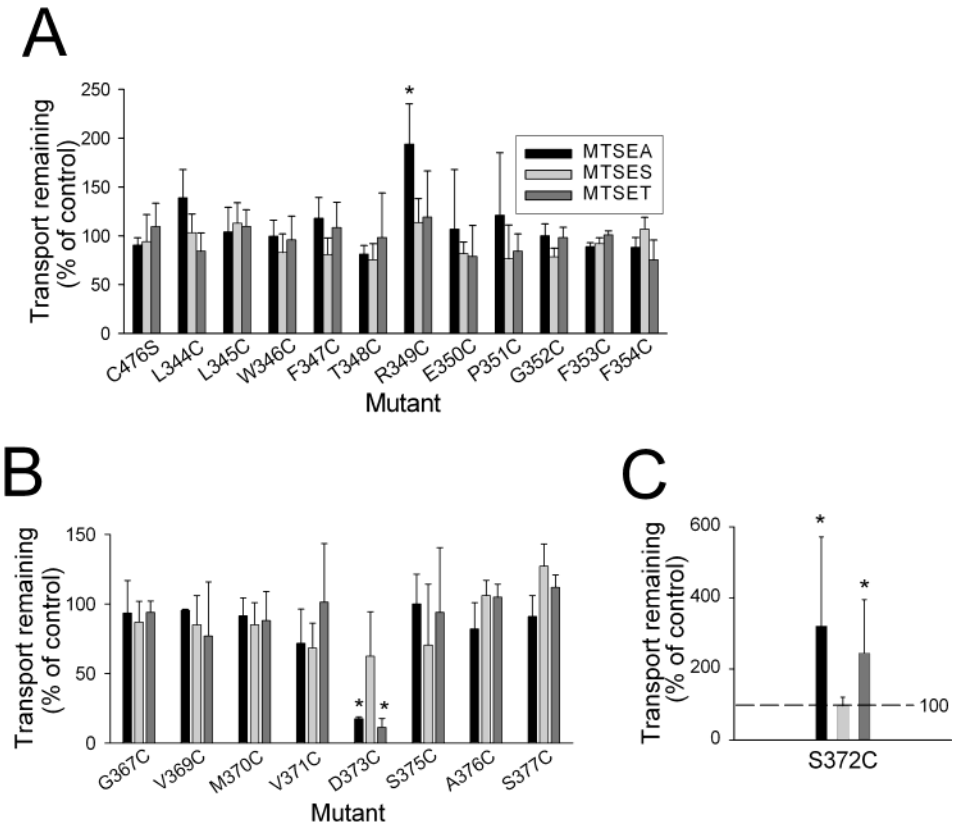

FIGURE 4: Effect of MTS reagents on succinate transport in cysteine mutants. Oocytes expressing mutants in TMD 7 (A) and TMD 8 (B) were preincubated with 2.5 mM MTSEA, 10 mM MTSES, 1 mM MTSET, or sodium buffer alone (control condition) for 10 min. The solutions containing MTS reagents were washed away with choline buffer, and uptake of 100 μM succinate was assessed for a 15 min time period. The parental mutant, C476S, is presented in panel A. The S372C mutant is shown separately in panel C because of the large stimulation with MTS reagents (note the scale). Uptake activity after preincubation with MTS reagents is shown for each mutant as a percentage of the control value measured in sodium buffer alone (the asterisk indicates $P < 0.05$, compared to the control condition measured in sodium buffer alone). The data are means ± SEM ($n = 2-13$ frogs).

MTSEA on succinate kinetics in R349C is shown in Figure 5. In this experiment, the V_{max} for succinate in R349C preincubated with sodium buffer alone was 1594 pmol oocyte^{-1} h^{-1}, and 2924 pmol oocyte^{-1} h^{-1} after pretreatment with MTSEA (Figure 5). In oocytes from the same frog, the V_{max} for succinate in C476S was 3206 ± 429 pmol oocyte^{-1} h^{-1} (±SE of regression, data not shown). MTSEA did not significantly affect the K_m for succinate in R349C (Figure 5). In two experiments, the K_m for succinate was 182 ± 18 μM (control) and 185 ± 29 μM (MTSEA) (mean ± range).

Since MTSEA is somewhat membrane permeable, high concentrations of extracellularly applied reagent can diffuse into the cell to modify intracellular cysteines (*10*). Therefore, we examined the effects of microinjecting 50 μL of 20 mM MTSEA into oocytes expressing R349C (results not shown). The transport of succinate was unchanged after the MTSEA

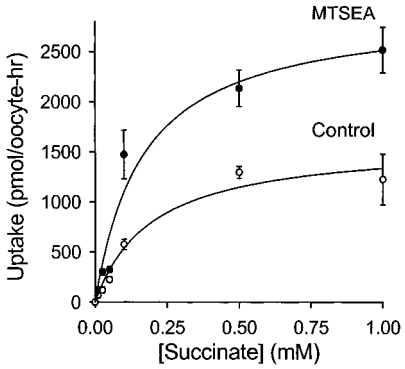

FIGURE 5: Effect of MTSEA on the kinetics of succinate transport in the R349C mutant. Oocytes expressing R349C were preincubated with sodium buffer (Control) or 2.5 mM MTSEA for 10 min. The solutions containing MTSEA were washed away, and succinate uptake was assessed for 5 min in sodium-containing buffer. The succinate concentrations were between 10 μM and 1 mM. In controls incubated with sodium buffer, the K_m is 189 ± 47 μM and V_{max} is 1594 ± 155 pmol oocyte^{-1} h^{-1} (±SE of regression). In oocytes pretreated with MTSEA, the K_m and V_{max} are 164 ± 58 μM and 2924 ± 330 pmol oocyte^{-1} h^{-1} (±SE of regression), respectively. Each data point represents the mean ± SEM (n = 4).

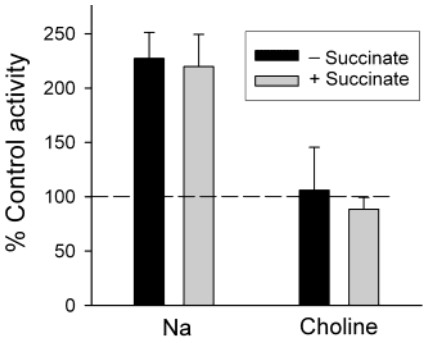

FIGURE 6: Cation and substrate dependence of MTSEA stimulation of succinate transport in R349C. Oocytes expressing R349C were preincubated for 10 min in 2.5 mM MTSEA dissolved in sodium or choline buffer, with or without 5 mM succinate. Control groups were preincubated in the same buffers without MTSEA. The preincubation solution was washed away, and 15 min uptakes of 100 μM succinate were assessed in sodium-containing buffer. The data are expressed as a percentage of the control. The data are means ± SEM (n = 3 frogs).

injection, verifying that the MTSEA stimulates transport by modifying an extracellularly accessible cysteine.

The preincubation of oocytes expressing R349C with MTSEA was also tested under different conditions to determine whether cations or substrate has any influence on the accessibility of this residue. As shown in Figure 6, the stimulatory effect of MTSEA was only observed in the presence of sodium, but not in choline. Furthermore, there was no effect of substrate on the stimulation of activity by MTSEA (Figure 6).

Functional Characterization of the D373C Mutant. The transport of 1 mM succinate in oocytes expressing the D373C mutant was linear up to 20 min; therefore, 15 min uptakes were measured (data not shown). The cysteine substitution at Asp-373 resulted in a decrease in the apparent K_m for succinate. In the experiment whose results are shown in Figure 7A, the K_m for succinate was 58 μM and the V_{max} was 440 pmol oocyte^{-1} h^{-1}. In four experiments, the mean K_m for succinate was 47 ± 12 μM and the V_{max} was 397 ± 30 pmol oocyte^{-1} h^{-1} (mean ± SEM). The apparent sodium

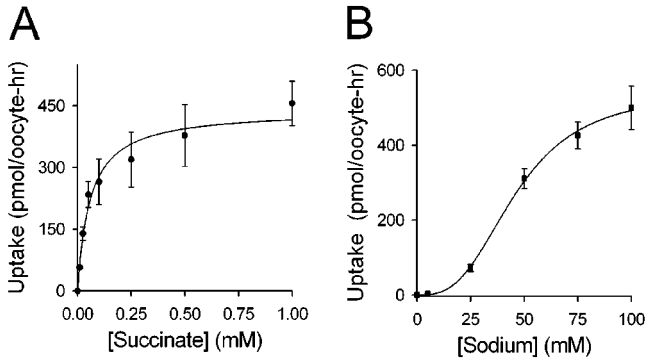

FIGURE 7: Kinetics of the D373C mutant. (A) Kinetics of succinate transport in oocytes expressing D373C. Succinate concentrations were between 10 μM and 1 mM. Uptakes were assessed in sodium-containing medium for 15 min. The K_m for succinate is 58 ± 11 μM, and V_{max} is 439 ± 23 pmol oocyte^{-1} h^{-1} (±SE of regression). Each data point represents the mean ± SEM (n = 5). (B) Sodium activation of succinate transport in oocytes expressing the D373C mutant. Uptake of 100 μM succinate was assessed for a 15 min time period in increasing concentrations of sodium (from 0 to 100 mM). Sodium was replaced with choline. The K_{Na} is 46 ± 2 mM, and the Hill coefficient (n_H) is 2.9 ± 0.3 (±SE of regression). Each data point represents the mean ± SEM (n = 5).

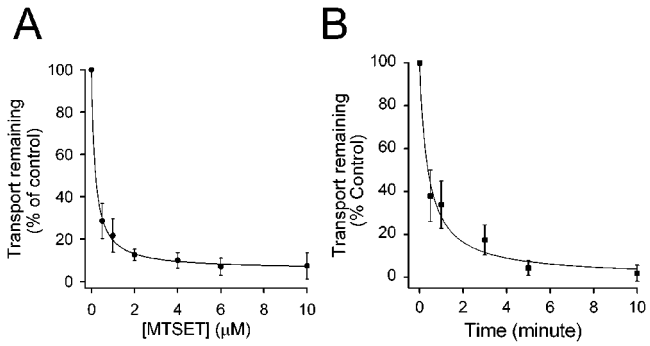

FIGURE 8: (A) Concentration dependence of MTSET inhibition of the D373C mutant. Oocytes expressing the D373C mutant were preincubated for 10 min with different concentrations of MTSET up to 10 μM or sodium buffer alone (control condition). The solutions containing MTSET were washed away, and the uptake of 100 μM succinate was assessed for a 15 min time period. Data are expressed as a percentage of the control. The K_i was 0.3 ± 0.05 μM, and maximal inhibition was 99.6 ± 5.7% (±SE of regression). (B) Time course of MTSET inhibition in the D373C mutant. Oocytes expressing the D373C mutant were preincubated with 10 μM MTSET or sodium buffer alone (control condition) for time periods from 30 s to 10 min, and the data are expressed as a percentage of the control. The $t_{1/2}$ was 0.3 ± 0.1 min (±SE of regression). The data points represent means ± SEM (n = 5 oocytes).

affinity remained relatively unchanged by the cysteine substitution at position 373. In the experiment whose results are shown in Figure 7B, the K_{Na} was 56 mM, and in a second experiment (not shown), the K_{Na} was 39 ± 6 mM (±SE of regression). The mean Hill coefficient (n_H) was 2.3 ± 0.2 (mean ± range, n = 2).

MTSET Concentration and Time Dependence in D373C. Although the D373C mutant is sensitive to inhibition by both MTSET and MTSEA, subsequent experiments were carried out using MTSET since it is less likely to cross the membrane than MTSEA. The D373C mutant is very sensitive to inhibition by low concentrations of MTSET. As shown in Figure 8A, the K_i for MTSET was 0.3 μM with a 10 min preincubation time and the maximal inhibition was 99%. In three experiments, the mean K_i was 0.5 ± 0.1 μM and the

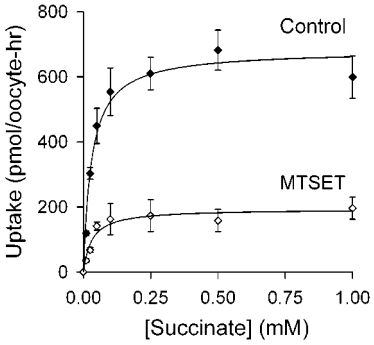

FIGURE 9: Effect of MTSET on the kinetics of succinate transport in the D373C mutant. Oocytes expressing D373C were preincubated with sodium buffer (Control) or 1 μM MTSET for 10 min. Uptakes of [^{3}H]succinate between 10 μM and 1 mM were assessed for a 15 min period in sodium-containing buffer. In control oocytes, the K_m was 32 ± 19 μM and the V_{max} was 707 ± 109 pmol oocyte^{-1} h^{-1} (the errors represent the SE of regression). In oocytes treated with MTSET, K_m and V_{max} were 37 ± 14 μM and 188 ± 16 pmol oocyte^{-1} h^{-1}, respectively (±SE of regression). Each data point represents the mean ± SEM (n = 5 oocytes).

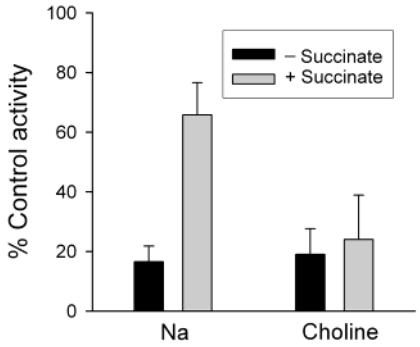

FIGURE 10: Cation and substrate dependence of MTSET inhibition of D373C. Uptakes (15 min) of 100 μM succinate were assessed in sodium-containing buffer after preincubation for 10 min with MTSET (10 μM) in sodium or choline buffer with and without 5 mM succinate. Control experiments were carried out by preincubation in the same buffers without MTSET. The data are expressed as a percentage of the control (mean ± SEM, n = 3 frogs).

maximal inhibition was 99 ± 3% (mean ± SEM). The time course of MTSET inhibition of succinate transport in D373C is shown in Figure 8B. The $t_{1/2}$ was 0.3 min at 10 μM MTSET, and in a second experiment (not shown), the $t_{1/2}$ was 0.4 ± 0.1 min (value ± SE of regression). Coincubation of oocytes with 10 mM dithiothreitol (DTT) during the MTSET exposure completely prevented the inhibition by MTSET (data not shown), verifying that MTSET inhibition occurs through disulfide bond formation.

Effect of MTSET on D373C Kinetics. The kinetics of succinate transport were measured in oocytes expressing the D373C mutant with and without preincubation with 1 μM MTSET (Figure 9). As expected for an irreversible inhibitor, the V_{max} for succinate was reduced to almost 30% of the control value after MTSET treatment, whereas the K_m was unchanged. In three experiments, the mean V_{max} was reduced to 32 ± 7% in the presence of MTSET (mean ± SEM, n = 3). However, there was no significant change in K_m; the mean K_m was 48 ± 15 μM (mean ± SEM, n = 3) in the control group and 38 ± 8 μM (mean ± SEM, n = 3) after preincubation with MTSET.

The influence of cations and substrate on the sensitivity of D373C to MTSET was also tested. As shown in Figure 10, D373C was equally sensitive to inhibition by MTSET in sodium or choline buffer. However, there was much less inhibition in oocytes preincubated with MTSET in 5 mM succinate in sodium buffer. There was no substrate protection in choline buffer.

DISCUSSION

The substituted-cysteine accessibility method has been used extensively to identify functionally important residues and the topology of various transporter proteins, including NaDC-1 (*15*, *19*−*22*). In the study presented here, we used this approach to examine the regions surrounding Arg-349 and Asp-373 in transmembrane domains (TMD) 7 and 8 of NaDC-1. Arg-349 and Asp-373 were previously found to be important in substrate or cation binding (*7*, *8*). In this study, only three (R349C, S372C, and D373C) of 21 cysteine substitution mutants were sensitive to chemical modification by MTSEA or MTSET. MTSEA restored the transport activity of the R349C mutant. Both MTSEA and MTSET stimulated the transport activity in the S372C mutant, but completely inhibited the transport activity in the D373C mutant. However, the inhibitory effect of MTSET on D373C was prevented by the protein conformational change induced by substrate binding.

The Arg-349 residue at the extracellular end of TMD 7 in NaDC-1 (Figure 1) is conserved in all members of the SLC13 gene family, which includes both dicarboxylate and sulfate transporters (*1*). The results of our previous study suggested that Arg-349 is important in substrate recognition, particularly in the outward-facing configuration. Replacement of Arg-349 with alanine produced a transporter that was sensitive to substrate inhibition at succinate concentrations of >100 μM. Charge reversal was not tolerated at position 349, but lysine replacement produced an active transporter (*7*). In the study presented here, the substitution of Arg-349 with cysteine decreased the transport activity by approximately 50% and only moderately decreased the K_m for succinate. Although Arg-349 appears to be functionally important for substrate binding or translocation, this residue is not absolutely required for activity since mutations at this position produce functional transporters.

The succinate transport activity of the R349C mutant was approximately half of that of the parental transporter, C476S. Treatment of R349C with MTSEA restored its activity almost to the level of the parental transporter, with an increase in the V_{max} of almost 2-fold. The MTSEA modification is likely acting as a direct replacement of the arginine residue. The volume of a cysteine modified by MTSEA is almost identical to that of arginine, 125 Å^3 (*12*). In contrast, chemical modification by MTSET, which adds a bulkier side group to the cysteine, does not restore activity to the R349C mutant of NaDC-1. Therefore, in addition to the charge, the volume of the residue at position 349 appears to play an important role in the transport function of NaDC-1. Similar results of MTSEA have been shown with two other citrate transport proteins. In the mitochondrial citrate transport protein from *Saccharomyces cerevisiae*, mutation of Arg-181 or Arg-189 abolishes transport, but some activity could be restored by incubation with MTSEA (*12*). In the bacterial citrate/lactate exchanger, CitP, the R425C mutation decreases activity through an increase in K_m (*23*), which is restored by chemical modification with MTSEA.

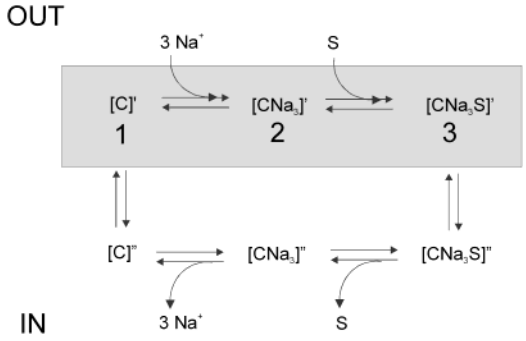

FIGURE 11: Simplified model of Na^+/dicarboxylate cotransport by NaDC-1. The three states, 1−3, with binding sites accessible from the outside are highlighted at the top of the figure. C represents the carrier or transporter, and S represents the substrate. OUT refers to the outside of the cell, and IN refers to the inside. Three sodium ions bind first to the transporter, which increases the affinity for the substrate. The substrate then binds to the transporter, and the fully loaded transporter reorients so that the binding sites are exposed to the inside of the cell. The substrate and sodium are then released on the inside. Finally, the empty carrier undergoes another conformational change that reorients the binding sites so they are accessible from the outside.

The current model of NaDC-1 function involves the ordered binding of three sodium ions followed by a divalent anion substrate, such as succinate (*24*). A simplified version of the model is shown in Figure 11. The binding of sodium triggers a conformational change in the protein that increases its affinity for substrate. Sodium binding is cooperative, and there may be intermediate conformational changes before the optimal substrate binding site is formed. Once the transporter is fully loaded with sodium and substrate, an additional conformational change reorients the binding sites and translocates the sodium and substrate to the inside of the cell. The conformational states in which the substrate or cation binding sites are exposed to the outside of the cell are states 1−3 (Figure 11). Since the effects of MTSEA on the R349C mutant occur only when the MTSEA is applied extracellularly in sodium buffer and are not affected by the presence of substrate, it seems likely that Arg-349 is accessible from the outside of the cell in conformational states 2 and 3 (Figure 11). In the absence of sodium, when the transporter is in conformational state 1, the cysteine substituted at position 349 is not accessible to MTSEA. The lack of substrate protection indicates that Arg-349 is not located in the substrate binding site and is not affected by conformational changes induced by substrate binding.

The conserved aspartate at position 373 in NaDC-1 is located near the extracellular surface of TMD 8 (Figure 1). Our previous study suggested that Asp-373 has a dual role in determining the K_m for substrate and cations (*8*). Substitution with alanine and asparagine produced mutant transporters with increased substrate affinity and decreased cation affinity; substitution with glutamate increased the affinity for succinate without affecting cation affinity, and substitution with glutamine only decreased the sodium binding affinity. In this study, the cysteine substitution of Asp-373 reduced the K_m for succinate considerably to ~50 μM, compared with 265 μM for the C376S mutant, without changing the affinity for sodium.

The aspartate at position 373 is accessible from the outside of the cell membrane either near the cell surface or in a water-filled pore since the D373C mutant was very sensitive to inhibition by the membrane-impermeant reagent, MTSET. It seems likely that the D373C mutant is accessible to MTSET in conformational states 1 and 2 (Figure 11). In conformational state 1, seen in the absence of sodium, the sodium binding sites are accessible from the outside of the cell. The presence of sodium produces conformational state 2, in which the substrate binding site is accessible from the outside of the cell. The results are consistent with a dual role of D373 in both substrate and cation binding, either participation in the formation of the binding sites or transduction of conformational changes between substrate and cation binding sites. Once the substrate is added, however, the inhibition by MTSET is prevented, suggesting that the Cys-373 becomes occluded during the substrate-induced conformational change in the protein. There is evidence from studies with the *lac* permease that substrate binding and translocation produces changes in the tilt and rotation of transmembrane helices in transporters (*25*). An alternate explanation for the results is that Asp-373 is located near the substrate binding site and substrate binding prevents access of MTSET to the substituted cysteine.

The serine residue at position 372, adjacent to Asp-373, also appears to affect substrate affinity. The K_m for succinate in the S372C mutant was decreased to ~90 μM, compared with 265 μM in the parental C476S mutant. Interestingly, the effect of both MTSEA and MTSET was a large stimulation of transport activity in the S372C mutant, although the results were quite variable. At present, we have no explanation for the results. It is possible that the effect of MTS reagents on the S372C mutant is indirect, through the activation of a second protein. At low expression levels, the activation of the second protein is enough to produce a stimulation of S372C transport, but at high expression levels, the amount of the second protein is limiting and does not have much of an effect on S372C. However, this remains to be tested.

The current secondary structure model of NaDC-1 contains 11 transmembrane domains, with an intracellular amino terminus and an extracellular carboxy terminus (see Figure 1). The carboxy terminus contains the N-glycosylation site, which is conserved in all members of the SLC13 gene family (*1*). The intracellular locations of the N-terminus and the large loop between TMD 4 and 5 have been verified using antibodies (*26*). However, the rest of the model remains largely untested. Our previous study showed that the four amino acids at the top of TMD 9 are accessible to charged MTS reagents, verifying the orientation of TMD 9 (*15*). The results of this study are also in accordance with our secondary structure model, which places Arg-349 near the extracellular surface of TMD 7 and Ser-372 and Asp-373 near the top of TMD 8. Since the R349C, S372C, and D373C mutants were accessible to MTSET or MTSEA applied from the outside of the cell, these residues are likely to be either on the outside or in a water-filled pore.

In conclusion, Arg-349 and Asp-373 are conformationally sensitive residues, exposed during different states of the reaction cycle. Arg-349 is important in substrate binding, particularly in the outward-facing configuration. The charge distribution and molecular volume at this site are important for function, and the MTSEA-modified cysteine can substitute for arginine at position 349. Arg-349 is exposed to the extracellular surface in conformational state 2, when the

substrate binding site is exposed, and also in state 3, when the substrate binding site is reorienting to the inside of the cell. The aspartate at position 373 is important in both cation and substrate affinity in NaDC-1. This residue is accessible from the outside of the cell in conformational state 1, when the cation binding site is exposed, as well as in state 2, when the substrate binding site is exposed. Substrate binding, however, decreases the reactivity of the D373C mutant, suggesting that it is occluded in conformational state 3. Because these residues appear to be alternately accessible and inaccessible to the MTS reagents, it is likely that the transport cycle of NaDC-1 involves movements of transmembrane domains 7 and 8.

ACKNOWLEDGMENT

We thank Channon Hudgins for assistance with preparation of oocytes and solutions.

REFERENCES

1. Pajor, A. M. (2000) *J. Membr. Biol. 175*, 1−8.
2. Pajor, A. M. (1995) *J. Biol. Chem. 270*, 5779−5785.
3. Pak, C. Y. C. (1991) *Am. J. Kidney Dis. 18*, 624−637.
4. Rogina, B., Reenan, R. A., Nilsen, S. P., and Helfand, S. L. (2000) *Science 290*, 2137−2140.
5. Pajor, A. M., and Sun, N. (1996) *Am. J. Physiol. 271*, C1808−C1816.
6. Kahn, E. S., and Pajor, A. M. (1999) *Biochemistry 38*, 6151−6156.
7. Pajor, A. M., Kahn, E. S., and Gangula, R. (2000) *Biochem. J. 350*, 677−683.
8. Griffith, D. A., and Pajor, A. M. (1999) *Biochemistry 38*, 7524−7531.
9. Akabas, M. H., Stauffer, D. A., Xu, M., and Karlin, A. (1992) *Science 258*, 307−310.
10. Holmgren, M., Liu, Y., Xu, Y., and Yellen, G. (1996) *Neuropharmacology 35*, 797−804.
11. Stauffer, D. A., and Karlin, A. (1994) *Biochemistry 33*, 6840−6849.
12. Xu, Y., Kakhniashvili, D. A., Gremse, D. V., Wood, D. O., Mayor, J. A., Walters, D. E., and Kaplan, R. S. (2000) *J. Biol. Chem. 275*, 7117−7124.
13. Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 488−492.
14. Pajor, A. M., Krajewski, S. J., Sun, N., and Gangula, R. (1999) *Biochem. J. 344*, 205−209.
15. Pajor, A. M. (2001) *J. Biol. Chem. 276*, 29961−29968.
16. Pajor, A. M., Sun, N., and Valmonte, H. G. (1998) *Biochem. J. 331*, 257−264.
17. Pajor, A. M., Sun, N., Bai, L., Markovich, D., and Sule, P. (1998) *Biochim. Biophys. Acta 1370*, 98−106.
18. Karlin, A., and Akabas, M. H. (1998) *Methods Enzymol. 293*, 123−145.
19. Pascual, J. M., and Karlin, A. (1998) *J. Gen. Physiol. 111*, 717−739.
20. Frillingos, S., Sahin-Toth, M., Wu, J., and Kaback, H. R. (1998) *FASEB J. 12*, 1281−1299.
21. Golovanesky, V., and Kanner, B. I. (1999) *J. Biol. Chem. 274*, 23020−23026.
22. Seal, R. P., Leighton, B. H., and Amara, S. G. (2000) *Neuron 25*, 695−706.
23. Bandell, M., and Lolkema, J. S. (2000) *J. Biol. Chem. 275*, 39130−39136.
24. Yao, X., and Pajor, A. M. (2000) *Am. J. Physiol. 279*, F54−F64.
25. Kaback, H. R., and Wu, J. (1999) *Acc. Chem. Res. 32*, 805−813.
26. Zhang, F. F., and Pajor, A. M. (2001) *Biochim. Biophys. Acta 1511*, 80−89.

BI0156761