Acidic Residues Involved in Cation and Substrate Interactions in the Na^+/Dicarboxylate Cotransporter, NaDC-1[†]

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ABSTRACT: The role of acidic amino acid residues in cation recognition and selectivity by the Na^+/dicarboxylate cotransporter, NaDC-1, was investigated by site-directed mutagenesis and expression in *Xenopus* oocytes. Four of the residues tested, Asp-52, Glu-74, Glu-101, and Glu-332, were found to be unimportant for transport activity. However, substitutions of Asp-373 and Glu-475, conserved residues found in transmembrane domains M8 and M9, respectively, altered transport kinetics. Replacements of Asp-373 with Ala, Glu, Asn, and Gln resulted in changes in sodium affinity and cation selectivity in NaDC-1, indicating that the carbonyl oxygen at this position may play a role in the topological organization of the cation-binding site. In contrast, substitutions of Glu-475 led to dramatic reductions in transport activity and changes in transport kinetics. Substitution with Gln led to a transporter with increased substrate and sodium affinity, while the E475D mutant was inactive. The E475A mutant appeared to have poor sodium binding. Substrate-induced currents in the E475A mutant exhibited a strong voltage dependence, and a reversal of the current was seen at −30 mV. The results suggest that Glu-475 may play a role in cation binding and possibly also in mediating anion channel activity. Remarkably, mutations of both Asp-373 and Glu-475 affected the K_m for succinate in NaDC-1, suggesting dual roles for these residues in determining the affinity for substrate and cations. We propose that at least one of the cation-binding sites and the substrate-binding site are close together in the carboxy-terminal portion of NaDC-1, and thus transmembrane domains M8 and M9 are candidate structures for the formation of the translocation pathway.

The absorption of Krebs cycle intermediates, such as α-ketoglutarate, succinate, and citrate, by the kidney and intestine is mediated by an electrogenic Na^+-dependent transport system present in the apical membrane (*1*, *2*). Three Na^+/dicarboxylate cotransporter cDNAs from rabbit, human, and *Xenopus* (NaDC-1,[1] hNaDC-1, and NaDC-2, respectively) have been isolated in our laboratory (*3*−*5*). These transporters exhibit between 62% and 78% sequence identity and are members of a larger family that includes the rat ortholog, rNaDC-1 (*6*), and the Na^+/sulfate cotransporter (*7*). Hydropathy analysis of the primary amino acid sequence of NaDC-1 suggests that the protein consists of at least 11 α-helical transmembrane domains (*8*). Asn-576 is the sole site of N-glycosylation in NaDC-1, which places the carboxy-terminal domain on the outside of the cell (*8*).

The transport mechanism of NaDC-1 is thought to be an ordered process in which the binding of three Na^+ ions increases the affinity of the transporter for a divalent anion substrate: an essential activator model (*1*). The fully loaded transporter then undergoes a conformational change that permits the release of the substrates into the cell. Lithium is a potent specific inhibitor of Na^+/dicarboxylate transport, most likely due to the transporter assuming a less than optimal conformation for substrate binding upon lithium occupation of one of the Na^+-binding sites (*9*, *10*). However, despite detailed mechanistic knowledge, little is known about the structural determinants of the translocation pathway in NaDC-1.

An important step in elucidating the molecular details of transport mechanisms is the identification of specific amino acid residues involved in cation and substrate binding. Recent site-directed mutagenesis experiments on NaDC-1 have ruled out the involvement of histidine residues in cation or substrate recognition (*11*). Prominent candidates for monovalent cation liganding amino acid residues in ion transporters are conserved acidic residues within transmembrane regions (*12*−*15*). For instance, studies of the melibiose permease of *Escherichia coli* have identified several aspartic acid residues from different transmembrane domains that are thought to form a cation-binding pocket (*12*). Similarly, cation binding to the *c* subunit of the *Propionigenium modestrum* F_1F_0 ATP synthase requires liganding groups provided by a glutamic

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[1] Abbreviations: NaDC-1, rabbit renal Na^+/dicarboxylate cotransporter; hNaDC-1, human renal Na^+/dicarboxylate cotransporter; NaDC-2, *Xenopus* intestine Na^+/dicarboxylate cotransporter; NaSi-1, rat renal Na^+/sulfate cotransporter; M8, M9, transmembrane domains 8 and 9; EAAT family, excitatory amino acid transporter family.

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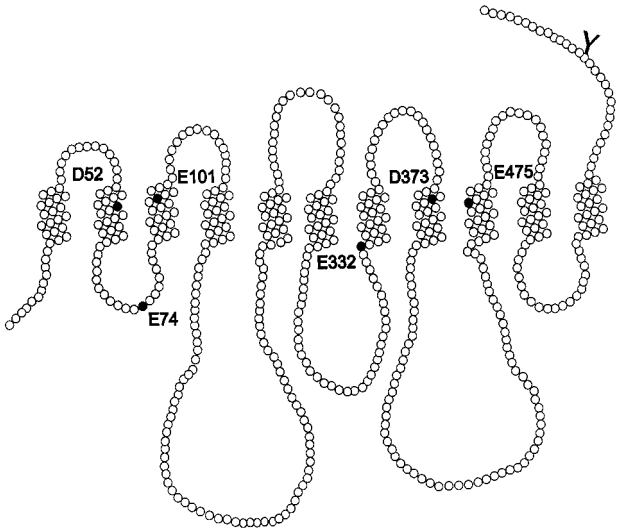

FIGURE 1: Secondary structure model of NaDC-1. The locations of the six mutated acidic amino acid residues are indicated by filled circles. The Y represents the N-glycosylation site at Asn-576. The outside of the cell is at the top of the figure.

Table 1: Oligonucleotides Used for Mutagenesis[a]

mutation	name	sequence (5′-3′) of antisense oligonucleotide
Asp-52 → Ala	D52A	GGG CAG AGC ***AGC*** AGT GCA CCA
Glu-74 → Ala	E74A	CTC AGA AGC ***TGC*** CAT GAT ACC
Glu-101 → Ala	E101A	GTT CCA GTG ***TGC*** CAC GGC GAT
Glu-332 → Ala	E332A	CAC AGC CTT ***TGC*** GGC AAA GGA
Asp-373 → Ala	D373A	CGC TGA TCC ***GGC*** GGA CAC CAT
Asp-373 → Glu	D373E	CGC TGA TCC ***CTC*** GGA CAC CAT
Asp-373 → Asn	D373N	CGC TGA TCC ***GTT*** GGA CAC CAT
Asp-373 → Gln	D373Q	CGC TGA TCC ***CTG*** GGA CAC CAT
Glu-475 → Ala	E475A	GCT AGT GCA ***CGC*** AGT GAA GGT
Glu-475 → Asp	E475D	GCT AGT GCA ***GTC*** AGT GAA GGT
Glu-475 → Gln	E475Q	GCT AGT GCA ***CTG*** AGT GAA GGT

[a] The substituted bases are indicated by boldface letters, and the modified codons are denoted by italic letters.

acid residue (*16*). Aspartic acid and lysine residues participate in substrate binding in the lactose permease of *E. coli* (*17*).

In the present study, we have performed a mutational analysis of the four conserved acidic amino acid residues predicted to be in putative transmembrane domains in NaDC-1: Glu-101, Glu-332, Asp-373, and Glu-475 (Figure 1). Two additional amino acids were also mutated, Asp-52 and Glu-74, which were chosen because an acidic amino acid is found at these positions in the other members of this gene family (Figure 1). We demonstrate that Asp-52, Glu-74, Glu-101, and Glu-332 are not essential for transport activity. In contrast, we find that Asp-373 and Glu-475 play roles not only in cation binding and selectivity but surprisingly, also in determining NaDC-1's apparent affinity for dicarboxylates.

EXPERIMENTAL PROCEDURES

Oligonucleotide-Directed Mutagenesis. Site-directed mutagenesis was performed according to the method of Kunkel (*18*). Uracil-containing single-stranded phagemid DNA derived from the rabbit renal Na^+/dicarboxylate cotransporter cDNA, NaDC-1 (*3*) (Genbank U12186), was used as a template. Point mutations were generated with the mutagenic antisense primers listed in Table 1 using an in vitro mutagenesis kit (Muta-gene; BioRad) according to manufacturer's directions. Mutations were verified by sequencing.

Expression in Xenopus Oocytes. Capped mRNAs transcribed from the cDNAs encoding wild-type or mutant transporters were microinjected into stage V−VI *Xenopus* oocytes (50 ng/oocyte) as described previously (*19*). Succinate uptakes were measured 5 days later. Oocytes were maintained at 18 °C in Barth's medium containing 5% heat-inactivated horse serum, 2.5 mM sodium pyruvate, and 50 mg/L gentamicin sulfate. Medium was changed daily.

Succinate Transport Assays. Succinate uptake was measured at 22−24 °C as described previously (*3*). Briefly, groups of five oocytes were rinsed once in choline buffer and transport was initiated by the addition of 0.4 mL of sodium, lithium, or choline buffer (composed of 100 mM NaCl, LiCl or cholineCl, 2 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM HEPES-Tris, pH 7.5) containing [^{14}C]-succinate (DuPont-NEN) and nonradioactive succinate to the desired concentration. After the appropriate time interval, transport was terminated by washing the oocytes four times with 4 mL aliquots of ice-cold choline buffer. Individual oocytes were then lysed by the addition of 0.25 mL of 10% SDS, and the radioactivity incorporated was determined by liquid scintillation counting. In Na^+ activation experiments, oocytes expressing wild-type (WT) or mutant NaDC-1 transporters were incubated for 5 min with 20 or 100 μM [^{14}C]succinate and seven to eight extracellular concentrations of Na^+ between 0 and 150 mM with iso-osmolarity maintained with cholineCl. Similarly, in succinate kinetic experiments, oocytes were incubated for 5 min with seven concentrations of [^{14}C]succinate in the range 0.05−4 mM in the presence of 100 mM NaCl. The mutants D373E, E475Q, and E475A had low transport activities, and longer time intervals were employed (15 min for D373E and E475Q, and 1 h for E475A). Uptake values were corrected for uptakes in control uninjected oocytes. All experiments were performed on oocytes from 2 to 5 different donor frogs.

The kinetic data were analyzed using SigmaPlot software (Jandel Scientific). Kinetic constants were derived by fitting data to the Michaelis−Menten equation:

$$v = V_{max}[S]/(K_m + [S])$$

where [S] is the substrate concentration, V_{max} is the transport rate at saturating [S], and K_m is the substrate concentration at 0.5 V_{max}, or the Hill equation:

$$v = V_{max}[Na^+]^n/(K_{Na}{}^n + [Na^+]^n)$$

where [Na^+] is the sodium concentration, V_{max} is the transport rate at saturating [Na^+], K_{Na} is the sodium concentration at 0.5 V_{max}, and n is the Hill coefficent. In these least-squares fitting procedures, data points were weighted according to the inverse of their relative experimental errors. Statistical analysis by t-test was performed using Sigma Stat (Jandel Scientific).

Immunodetection of Cell Surface-Expressed Wild-Type and Mutant NaDC-1 Transporters. The cell surface expression of wild-type and mutant NaDC-1 transporters was determined as described previously (*11*) using a membrane impermeant biotin reagent (Sulfo-NHS-LC−Biotin, Pierce) and ImmunoPure Immobilized Streptavidin beads (Pierce). The transporters were detected in the pool of biotinylated oocyte surface proteins by Western blotting (*11*). An anti-NaDC-1

antibody (*8*) was applied at a 1:5000 dilution for 2 h followed by a horseradish peroxidase-linked anti-rabbit Ig (Amersham) at a 1:5000 dilution for 1 h. Antibody binding was visualized by enhanced chemiluminescence using the Supersignal CL-HRP substrate system (Pierce). Film exposure to Western blots was varied to ensure that the intensity of the observed bands was a linear function of exposure time. The relative expression of each mutant transporter was compared with a wild-type control from the same experiment.

Electrophysiology. Two-electrode voltage clamp experiments with oocytes were conducted as described previously (*10*). For chloride replacement experiments, chloride was replaced by gluconate and current offsets were prevented through the use of a 3 M KCl agar bridge. Current−voltage relationships were obtained using a pulse protocol consisting of test voltages applied for 100 ms between +50 and −150 mV in 20-mV decrements, with a holding potential of −50 mV. The results of three runs were averaged for each trial. The voltage pulses were controlled with the pClamp6 program suite (Axon Instruments). The experiments were repeated with similar results using oocytes from at least three donor frogs.

RESULTS

Site-directed mutagenesis of several conserved acidic amino acid residues in NaDC-1 was conducted to evaluate their role in cation and substrate binding. Five of these acidic amino acid residues (Asp-52, Glu-101, Glu-332, Asp-373, and Glu-475) are predicted to be in putative transmembrane domains, and one residue (Glu-74) is in a cytoplasmic loop between transmembrane domains M2 and M3 of NaDC-1 (Figure 1). The mutant transporters were expressed in *Xenopus* oocytes, and the effect of these substitutions on transport activity was determined. All mutant transporters catalyzed Na^+-dependent succinate transport, which suggests that the mutated acidic amino acid residues are not absolutely essential for cation-coupled transport by NaDC-1. Nevertheless, the data presented below support a role for Asp-373 and Glu-475 in the transport mechanism of NaDC-1.

Immunoblots. Cell surface biotinylation of *Xenopus* oocytes expressing wild-type and mutant NaDC-1 proteins was done using a membrane impermeant derivative of biotin, sulfo-NHS-biotin. The biotinylated proteins were blotted and treated with anti-NaDC-1 antibodies (*11*). The two immunoreactive proteins seen in oocytes expressing NaDC-1 correspond to two differentially glycosylated forms of NaDC-1 (*11*). As seen in previous studies, there was no immune reaction with control, uninjected oocytes (Figure 2) (*11*). All of the mutants in this study were expressed at the plasma membrane (Figure 2), indicating that the mutations used in this study do not affect protein stability or trafficking.

Mutant Transporters with Na^+ Activation and Succinate Kinetics Comparable to Wild-Type NaDC-1. In the initial mutagenic screen, the six acidic amino acid residues were individually replaced with alanine. Several of the mutant transporters (D52A, E74A, E101A, and E332A) showed sodium interaction properties and succinate kinetics comparable to those of the wild-type NaDC-1 transporter (Table 2). Therefore, Asp-52, Glu-74, Glu-101, and Glu-332 are not required for transport in NaDC-1.

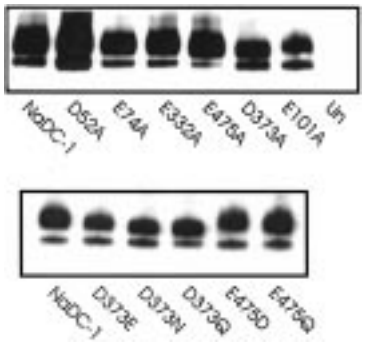

FIGURE 2: Immunodetection of wild-type and mutant NaDC-1 transporters in the plasma membrane of *Xenopus* oocytes. Western blots of cell-surface biotinylated membranes from *Xenopus* oocytes expressing wild-type or mutant transporters were made as described in Experimental Procedures. Samples from control, uninjected oocytes are labeled "Un". The blots, shown in the upper and lower panels, contain proteins from two different batches of oocytes. The blots were incubated with anti-NaDC-1 antibody at a 1:5000 dilution, followed by horseradish peroxidase-linked anti-rabbit Ig at a 1:5000 dilution. The two protein bands in each sample represent differently glycosylated forms of NaDC-1 expressed in oocytes (*8*).

Table 2: Mutant Transporters Exhibiting Na^+ Activation and Succinate Kinetics Comparable to Wild-type (WT) NaDC-1[a]

	Na^+ activation		succinate kinetics	
mutation	K_{Na} (mM)	n	K_m (mM)	V_{max} (nmol oocyte^{-1} h^{-1})
WT	21 ± 2	2.6 ± 0.3	0.34 ± 0.04	10.8 ± 0.4
D52A	20 ± 2	2.4 ± 0.2	0.23 ± 0.07	8.1 ± 0.7
E74A	25 ± 4	2.1 ± 0.1	0.19 ± 0.02	6.9 ± 0.2
E101A	31 ± 5	2.6 ± 0.3	0.28 ± 0.03	5.7 ± 0.3
E332A	20 ± 1	2.3 ± 0.6	0.38 ± 0.01	6.0 ± 0.1

[a] Sodium activation and succinate kinetic experiments were performed as described under Experimental Procedures. Values shown represent the results (mean ±sem, $n = 5$ oocytes) from one experiment representative of at least 2 experiments in which the mean values were not significantly different.

Mutant Transporters with Altered Function: Na^+ Activation Kinetics. Substitutions of Asp-373 and Glu-475 altered the transport characteristics of NaDC-1. A summary of the transport properties of carriers with mutations at these positions is shown in Table 3. The key observations contained in Table 3 are illustrated in the form of normalized representative Na^+ activation and succinate kinetic curves in Figures 3 and 4.

The initial substitution of alanine for Asp-373 (D373A) led to a moderate reduction in the apparent affinity for Na^+, that is, ~2.5-fold increase in the K_{Na} value (Table 3, Figure 3A). To examine the properties of Asp-373 that are important for the interaction with sodium, we made additional replacements of this residue. The neutral conservative substitution of Asp-373 with asparagine (D373N), which retains both the residue's size and carbonyl oxygen, resulted in an increase in the K_{Na} similar to that seen with the alanine replacement (Table 3, Figure 3A). Replacement of Asp-373 with glutamine (D373Q), an amino acid containing a bulkier side chain than asparagine, resulted in approximately a 6-fold reduction in the transporter's apparent affinity for Na^+ (Table 3, Figure 3A), suggesting that side chain volume at this position may be important. Succinate transport mediated by the D373Q transporter was not saturated at 150 mM Na^+, the highest

Table 3: Mutant Transporters Exhibiting Altered Na^+ Activation and Succinate Kinetics[a]

	Na^+ activation					
	20 μM succinate		100 μM succinate		succinate kinetics	
mutation	K_{Na} (mM)	n	K_{Na} (mM)	n	K_m (mM)	V_{max} (nmol oocyte^{-1} h^{-1})
WT	27 ± 4	2.0 ± 0.3	26 ± 3	2.4 ± 0.2	0.30 ± 0.04	8.8 ± 1.1
D373A	64 ± 5[b]	3.5 ± 0.3[b]			0.11 ± 0.01[b]	3.0 ± 0.7[b]
D373E			19 ± 3[b]	2.4 ± 0.3	0.13 ± 0.06[b]	0.5 ± 0.2[b]
D373N	75 ± 4[b]	2.7 ± 0.3			0.14 ± 0.03[b]	2.9 ± 0.5[b]
D373Q	154 ± 46[b]	3.0 ± 0.9			0.34 ± 0.08	1.7 ± 0.4[b]
E475A			>100	>1	0.38 ± 0.02	0.4 ± 0.1[b]
E475D	nd	nd	nd	nd	nd	nd
E475Q			9 ± 2[b]	1.8 ± 0.3	0.08 ± 0.01[b]	1.1 ± 0.4[b]

[a] Na^+ activation and succinate kinetic experiments were performed as described under Experimental Procedures. Values shown represent the mean ±sem of 3−4 determinations. [b] $P < 0.05$ relative to WT values. nd, not determined because of low activity.

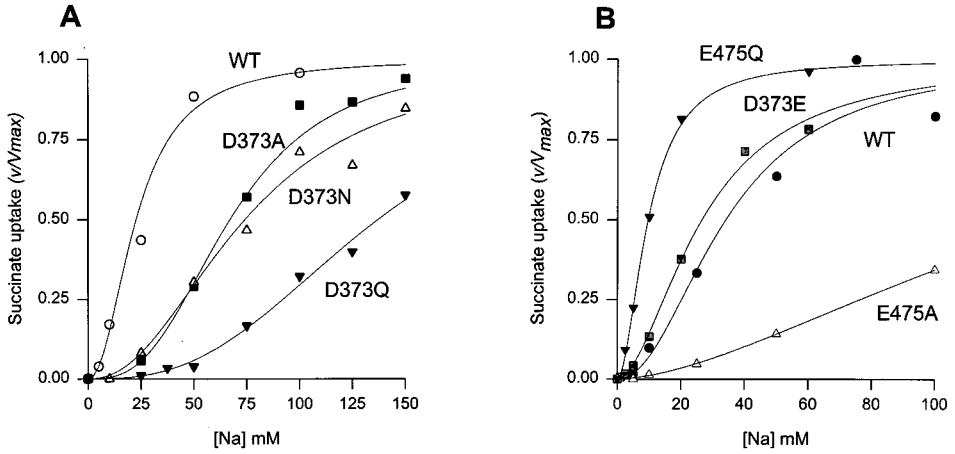

FIGURE 3: Na^+ activation of succinate transport by wild-type (WT) and mutant NaDC-1 transporters. Na^+ activation curves were determined as described under Experimental Procedures at (A) 20 μM and (B) 100 μM succinate. Data represent single experiments in which the rate of transport (v) is normalized to the V_{max} for comparison between mutants. The kinetic constants used to generate the curves (K_{Na} (mM) and n, respectively) were the following: (A) 23 ± 2 and 2.1 ± 0.1 WT, 67 ± 11 and 2.9 ± 0.6 D373A, 74 ± 15 and 2.2 ± 0.4 D373N, and 137 ± 32 and 2.8 ± 0.4 D373Q; and (B) 29 ± 3 and 2.0 ± 0.1 WT, 24 ± 2 and 1.8 ± 0.1 D373E, 147 ± 37 and 1.7 ± 0.1 E475A, and 10 ± 4 and 1.3 ± 0.2 E475Q (± standard error of regression).

concentration of Na^+ used, and consequently the kinetic values reported for this mutant are estimates. The D373E mutant transporter, which retains the carboxylate functional group but as part of a bulkier side chain, showed sodium interaction properties comparable to wild-type NaDC-1 (Figure 3B). Taken together these results suggest that the negatively charged oxygen of the Asp-373 carboxylate group may be involved in the topological organization of the cation-binding sites.

Replacement of Glu-475 with alanine resulted in a transporter with greatly reduced activity, although the protein was expressed at the plasma membrane (Table 3, Figure 3). The E475A mutant transporter appeared to have an increased K_{Na} (Table 3 and Figure 3B), suggesting a role for this residue in Na^+ binding. The kinetic values for the E475A mutant should be considered as estimates based on the low transport activity and lack of transport saturation at the highest Na^+ concentration, 150 mM, employed in these experiments. In one experiment, the apparent K_{Na} was 147 mM (Figure 3B). However, in three other experiments, although the transport was clearly sigmoidal (similar to the data shown in Figure 3B), it was not possible to fit the data to the Hill equation, likely because of the lack of saturation. To explore further the role of Glu-475, we made the additional substitutions of aspartic acid and glutamine. The E475D mutant transporter exhibited a pronounced defect in transport function. Succinate uptake by oocytes expressing the E475D mutant was barely detectable above background, although the E475D transporter was well expressed at the oocyte cell surface (Figure 2). The more conservative neutral substitution of glutamine for Glu-475 resulted in a transporter with greater transport activity than either the E475A or E475D mutants and a significantly decreased K_{Na} value compared with wild-type NaDC-1 (Table 3, Figure 3B).

Mutant Transporters with Altered Function: Succinate Kinetics. Na^+/dicarboxylate cotransport in the apical membrane of the renal proximal tubule exhibits transport properties consistent with an essential activator model (*1*). According to this model, the cotransporter's affinity for succinate depends on the extent of occupancy of its Na^+-binding sites, with the highest apparent succinate affinity being observed when its cation-binding sites are saturated. Therefore, it is striking that a number of the mutant transporters exhibiting reduced affinities for Na^+ showed succinate K_m values that were either similar to (D373Q, E475A) or lower than (D373A, D373N) wild-type NaDC-1 (Figure 4 and Table 3). The essential activator model would predict that the apparent affinity of the mutant transporters for succinate would be increased if succinate transport were to be assayed in the presence of saturating sodium concentra-

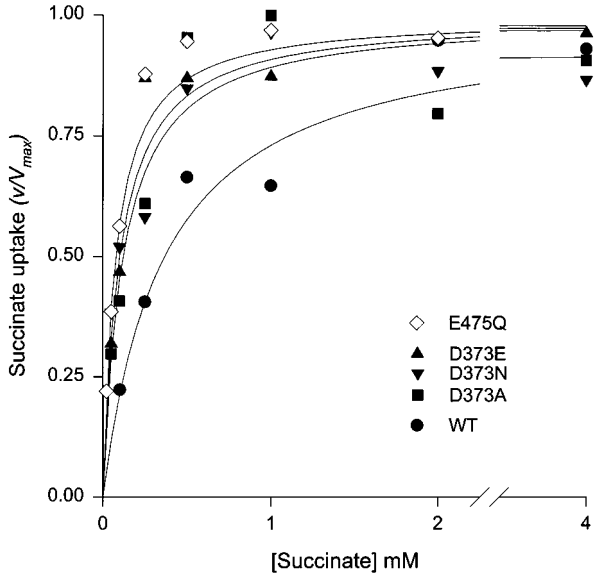

FIGURE 4: Kinetics of succinate transport in wild-type (WT) and mutant NaDC-1 transporters. The concentration dependence of succinate transport by WT and mutant NaDC-1 transporters was determined as described under Experimental Procedures. Data from a single experiment normalized to each V_{max} are compared. The K_m values (in mM) used to generate the curves were 0.33 ± 0.03 WT, 0.12 ± 0.04 D373A, 0.10 ± 0.02 D373E, 0.10 ± 0.03 D373N, and 0.076 ± 0.019 E475Q (± standard error of regression).

tions. Furthermore, it is likely that even the D373Q and E475A mutants when assayed under such conditions would have apparent affinities for succinate higher than wild-type NaDC-1. Despite having similar Na^+ affinities to that of wild-type NaDC-1, the mutant transporters D373E and E475Q exhibited decreases in their K_m values for succinate (Table 3). Therefore, these results suggest roles for Asp-373 and Glu-475 in determining the apparent K_m of NaDC-1 for succinate.

Lithium Effects on Wild-Type and Mutant NaDC-1 Transporters. Succinate transport by NaDC-1 is sensitive to inhibition by lithium ($K_i \sim 2$ mM) (*19*), which competes with sodium at one of the cation-binding sites (*10*). However, in the absence of sodium, lithium can drive the transport of succinate in NaDC-1 although at a much lower rate (*9*, *10*). Figure 5 shows the effect of lithium on the transport of succinate by Asp-373 and Glu-475 mutant transporters compared with wild-type NaDC-1. As seen in our previous studies, succinate transport by wild-type NaDC-1 in the presence of 100 mM lithium was between 3% and 8% of the uptake measured in sodium (Figure 5). A similar result was seen in the E475A and D373E mutants, whereas the D373N, D373Q, and E475Q mutants had slightly higher uptakes of approximately 10% of the transport in sodium (Figure 5). In contrast, the D373A mutant had a significantly higher proportional transport in lithium: approximately 24% of the uptake seen in sodium, indicating an alteration in cation selectivity (Figure 5A). In the presence of sodium, wild-type NaDC-1 and the D373E mutant were inhibited approximately 60% by 25 mM Li^+ (Figure 5). The other mutants at positions 373 and 475 were insensitive to inhibition by lithium (Figure 5). Although mutant D373Q appeared have increased transport when lithium and sodium were both present, the increase was not statistically significant when compared with the 100% sodium group (Figure 5B).

Two-Electrode Voltage Clamp Measurements. The voltage dependence of succinate-induced currents in NaDC-1, D373A, and E475A was tested using a two-electrode voltage clamp. Figure 6 shows the steady-state, substrate-induced currents in the presence of sodium, lithium, or choline of the wild-type and mutant transporters. As shown previously, NaDC-1 exhibits large inward currents in the presence of sodium and succinate (Figure 6A, ref *10*). Substrate-induced inward currents were also observed in lithium in NaDC-1 (Figure 6A, ref *10*). By comparison, the D373A mutant appeared to have altered cation selectivity in substrate-induced currents, similar to the transport activity (Figure 6). The substrate-induced currents in lithium compared with sodium in D373A were larger than in the wild-type NaDC-1 (Figure 6B). In contrast, the steady-state substrate-induced currents in the

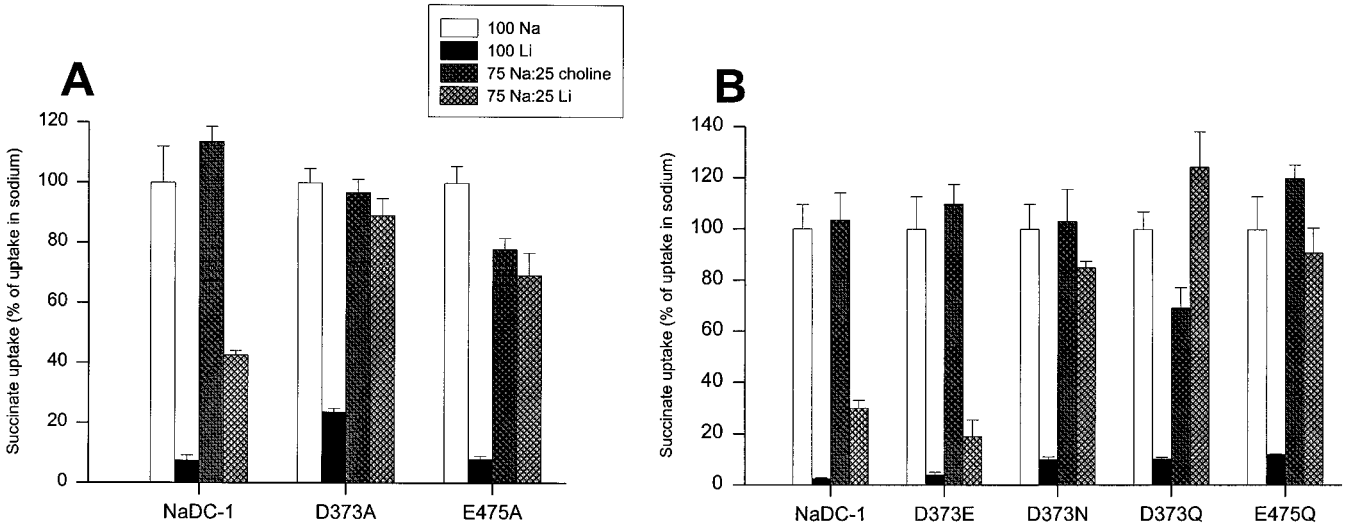

FIGURE 5: Lithium interaction with wild-type NaDC-1 and Asp-373 and Glu-475 mutant transporters: (A) NaDC-1 and alanine-substituted mutant transporters; and (B) NaDC-1 and mutants with other substitutions at positions 373 and 475. Transport of 500 μM succinate was measured for 15 min in transport buffer containing 100 mM Na^+, 100 mM Li^+, 75 mM Na^+ + 25 mM choline, or 75 mM Na^+ + 25 mM Li^+. The data are expressed as a percentage of the uptake in 100% sodium, and each data point represents the mean ±sem, $n = 4-7$ oocytes.

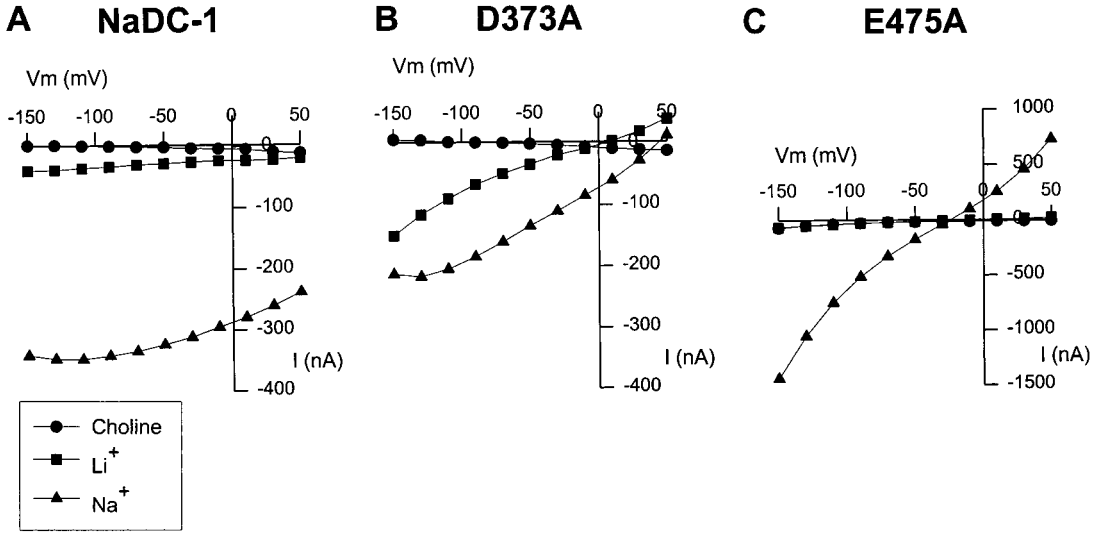

FIGURE 6: Voltage dependence of substrate-induced steady-state currents mediated by wild-type (A), and D373A (B), and E475A (C) mutant NaDC-1 transporters expressed in *Xenopus* oocytes. The curves represent the differences between currents measured in the presence and absence of 10 mM succinate, in transport buffer containing 100 mM choline, 100 mM Li^+, or 100 mM Na^+. The experiments shown in this figure were done with oocytes from the same frog.

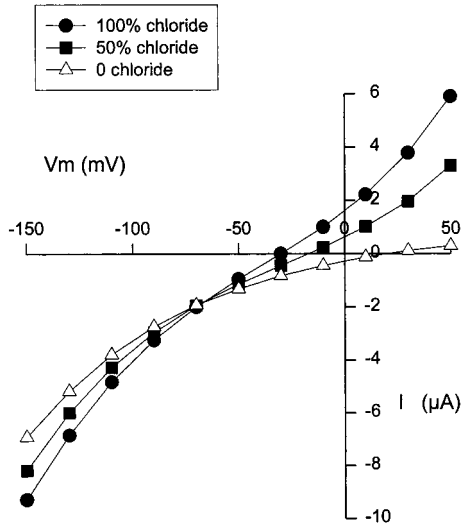

FIGURE 7: Chloride dependence of substrate-induced currents in E475A. Two-electrode voltage clamp of an oocyte expressing the E475A mutant was performed as described in Experimental Procedures. The substrate-induced currents (10 mM succinate) were measured in sodium buffer containing chloride or gluconate salts. The 50% chloride solution was made using equal volumes of chloride- and gluconate-containing transport buffer.

E475A mutant were very different from the others (Figure 6C). The currents seen in E475A exhibited a steep voltage dependence and a reversal of the current at approximately −30 mV. Furthermore, no substrate-dependent currents were observed in the E475A mutant when the sodium was replaced by lithium or choline (Figure 6C).

The chloride sensitivity of mutant E475A was tested by measuring substrate-dependent currents in the presence of 100% and 50% chloride and in the absence of chloride. As shown in Figure 7, the outward current was abolished when the chloride was replaced by gluconate, and there was a slight decrease in the inward current seen at negative potentials. There was no change in the currents seen in NaDC-1 in the presence or absence of chloride (not shown). The results indicate that the outward current seen in E475A is mediated by the movement of anions, such as chloride.

DISCUSSION

The goal of the present study was to identify amino acid residues in the Na^+/dicarboxylate cotransporter, NaDC-1, that are important for cation and substrate binding. A number of recent studies have suggested that conserved acidic amino acid residues within transmembrane regions may be important for the activity of cation transporting proteins (*12*−*15*). Accordingly, the functional consequences of individually replacing several acidic amino acid residues in NaDC-1 were investigated. Of the six amino acids tested, four (Asp-52, Glu-74, Glu-101, and Glu-332) were found to be unimportant for transport activity. The key findings of this study are that Asp-373 and Glu-475 in transmembrane domains M8 and M9, respectively, of NaDC-1 may play roles directly or indirectly in cation binding and selectivity, and that remarkably, these residues are also involved in determining the K_m for substrate.

Analysis of mutant NaDC-1 transporters with replacements of Asp-373 suggest that the negatively charged oxygen of the carboxylate side chain at this position is a determinant of the apparent affinity for sodium. Removal of the carboxylate by replacement of Asp-373 with alanine, asparagine, or glutamine resulted in transporters with reduced sodium affinities. Repositioning of the carboxylate in the D373E mutant resulted in an increased apparent sodium affinity. In addition, neutral substitutions of Asp-373 resulted in an altered cation selectivity, with increased transport seen in the presence of lithium. Therefore, it is likely that Asp-373 plays a role in the topological organization of one or more of the cation-binding sites, that is, in holding side chains that directly interact with the cations in place. Similar "second shell" residues forming hydrogen bond networks around metal ion-binding sites have been identified in a number of metal-binding (*20*) and metal-containing proteins (*21*, *22*).

Our present studies indicate that Glu-475 may play a more critical role in cation binding by NaDC-1. Replacement of Glu-475 yielded transporters with dramatically diminished transport activity. The transport defect in the E475A mutant

transporter appeared to be poor sodium binding. This mutant also had an altered cation selectivity, exhibiting a reduced inhibition by lithium, suggesting that the high-affinity lithium-binding site had been altered. In contrast, substitution of Glu-475 by glutamine resulted in a transporter with an increased apparent affinity for sodium, although the V_{max} was still low. This suggests that either the carbonyl oxygen of the Glu-475 side chain alone is important for transport or both oxygens of the carboxylate group are important and can be substituted by the carbonyl oxygen and amide nitrogen atoms of the glutamine residue. Although it does not appear likely that Glu-475 is a direct ligand of sodium, this residue does influence cation binding in NaDC-1.

Sodium acts as an essential activator of transport in NaDC-1, with the binding of sodium triggering a conformational change that increases the affinity for substrate (*1*). Lithium occupation of one of the three sodium-binding sites causes inhibition of transport by producing a less than optimal binding site for substrate. The apparent K_m for succinate is at least 30-fold higher in lithium than in sodium (*9*, *10*). Recent studies of the Na^+/glucose cotransporter, SGLT1 (*23*), and the melibiose permease of *E. coli* (*12*) show that the conformation of the substrate-binding site depends on the transported cation. The Asp-373 and Glu-475 mutants in this study exhibited altered interactions with lithium. The D373A mutant had increased succinate transport (both uptakes and substrate-evoked currents) in lithium, and there was no inhibition by lithium in the presence of sodium. Therefore, alanine substitution of Asp-373 results in a transporter in which lithium binding produces a more optimal conformational change of the substrate-binding site. Alternately, the substrate-binding site in D373A could already have a more optimal configuration before lithium binding, so that the binding of lithium results in a more effective transport stimulation. In contrast, the other mutants with neutral substitutions of Asp-373 and Glu-475 had similar proportional rates of transport in lithium compared with wild-type NaDC-1 (3−10% of transport in sodium) and reduced sensitivity to inhibition by lithium, suggesting less effective competition between lithium and sodium at the inhibitory binding site.

A surprising finding of this study was the changed voltage dependence of the E475A mutant. Unlike the wild-type NaDC-1, E475A exhibited a very steep current−voltage relationship in the presence of substrate, and outward currents were observed at potentials more positive than −30 mV. However, the outward currents were only seen in the presence of sodium and substrate, but not when sodium was replaced by other cations, suggesting that these currents occur when the transporter is functioning. We have never observed outward currents in oocytes expressing wild-type NaDC-1 (*10*), and transport of succinate is not affected by the removal of Cl^- (Pajor, unpublished observations). In addition, replacement of chloride by gluconate abolished the outward currents seen in the E475A mutant. Our results with the E475A mutant resemble the chloride channel activity induced by mutation of Glu-404 in the Na^+/glutamate transporter, GLT-1 (EAAT2) (*24*). Several members of the EAAT-related family exhibit chloride channel activity, although GLT-1 does not (*25*). However, the GLT-1-E404D mutant that is thought to be defective in K^+ coupling also exhibits chloride channel activity (*24*). Although NaDC-1 is unrelated in sequence to the EAAT family of transporters, there may be similarities in transport mechanism. By analogy with GLT-1, the results of our present study suggest that NaDC-1 may also have the potential for chloride channel activity that is normally not observed, but is uncovered in the E475A mutant. However, this hypothesis will need to be tested thoroughly to eliminate the possibility that the outward currents are due to activation of channels in the oocyte membrane (*26*).

In addition to effects on cation binding, the substitutions of Asp-373 and Glu-475 affected the K_m for succinate, which appeared to be distinct from the effects on cation affinity. Our previous studies have shown that the substrate recognition site in NaDC-1 is located in the carboxy-terminal half of the protein (*27*). Therefore, an explanation for the effects on succinate K_m is that the carboxylate side chains of Asp-373 and Glu-475 are situated at or near the substrate-binding site, and could possibly play a role in defining the contours of the substrate-binding pocket. However, an alternate explanation that remains to be tested is that these residues affect the rate constants of one or more steps in the reaction cycle, which could include the cation-induced conformational change of the protein, translocation of the loaded carrier, release of cations and succinate inside the cell, or reorientation of the empty carrier. It is also possible that the mutations could induce long-range conformational effects that cause changes in substrate kinetics. In this paper we report the Michaelis constant (K_m) for succinate transport which is related to the actual succinate-binding site affinity in a complex manner involving all of the rate constants for the transport cycle (see, for example, ref *28*). Therefore, it is possible that the observed differences in K_m values could be caused by changes in the translocation rate constant rather than changes in the actual succinate-binding site. In the Na^+/glucose cotransporter, SGLT1, Asp-176 was found to determine the rate constants for the reorientation of the ligand-binding sites in the unloaded transporter (*29*).

Of the six acidic amino acid residues mutated in NaDC-1, alanine replacements of four (Asp-52, Glu-74, Glu-101, and Glu-332) had no effect on transport function. Therefore, it is possible to mutate single charged residues within loops and even transmembrane helices without interfering with the transporter's function. These findings confer a degree of specificity to the several other mutations that do alter the function of NaDC-1 by indicating that these transport defects are not likely the result of an overall destabilization of the transporter. It should be noted, however, that the insertion of charged amino acid residues into the membrane, a region of low dielectric, is energetically unfavorable (*30*). Charged residues in the membrane typically occur as ion pairs (*31*), or lining an aqueous cavity or pore where they very often perform a catalytic function (*14*). Therefore, Asp-373 and Glu-475 are likely to be found in an aqueous environment, possibly in the translocation pathway of NaDC-1.

In conclusion, Asp-52, Glu-74, Glu-101, and Glu-332 are unimportant for the transport function of NaDC-1. The conserved aspartic acid at position 373 is most likely involved in the topological organization of one or more of the cation-binding sites. In contrast, Glu-475 is involved in cation binding to NaDC-1. Remarkably, in addition to their roles in cation binding, Asp-373 and Glu-475 appear to be determinants of the K_m for succinate. We propose that at

least one of the cation-binding sites and the substrate-binding site are close together or overlapping in the carboxy-terminal portion of NaDC-1, and thus transmembrane domains M8 and M9 are candidate structures for the formation of the translocation pathway.

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