Role of cationic amino acids in the Na⁺/dicarboxylate co-transporter NaDC-1

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The role of cationic amino acids in the Na⁺/dicarboxylate co-transporter NaDC-1 was investigated by site-directed mutagenesis and subsequent expression of mutant transporters in *Xenopus* oocytes. Of the ten residues chosen for mutagenesis, eight (Lys-34, Lys-107, Arg-108, Lys-333, Lys-390, Arg-368, Lys-414 and Arg-541) were found to be non-essential for function or targeting. Only two conserved residues, Lys-84 (at the cytoplasmic end of helix 3) and Arg-349 (at the extracellular end of helix 7), were found to be important for transport. Both mutant transporters were expressed at the plasma membrane. The mutation of Lys-84 to Ala resulted in an increased K_m for

succinate of 1.8 mM, compared with 0.3 mM in the wild-type NaDC-1. The R349A mutant had Na⁺ and citrate kinetics that were similar to those of the wild type. However, succinate handling in the R349A mutant was altered, with evidence of inhibition at high succinate concentrations. In conclusion, charge neutralization of Lys-84 and Arg-349 in NaDC-1 affects succinate handling, suggesting that these residues might have roles in substrate binding.

Key words: citrate, mutagenesis, sodium, succinate, *Xenopus* oocytes.

INTRODUCTION

The Na⁺/dicarboxylate co-transporter NaDC-1 is found on the apical membranes of epithelial cells of the mammalian renal proximal tubule and the small intestine [1]. In the kidney, NaDC-1 is important in regulating the urinary concentrations of citrate, which chelates Ca²⁺ and prevents the formation of stones. NaDC-1 has a broad substrate selectivity for dicarboxylates, such as succinate or 2-oxoglutarate, and tricarboxylates in protonated form, such as citrate²⁻. The transport cycle involves the ordered binding of four charged molecules, three Na⁺ ions and one bivalent anion substrate, with the net movement of one positive charge per reaction cycle [1].

NaDC-1 belongs to a distinct gene family of Na⁺-coupled transport proteins that carry either dicarboxylates or sulphate. NaDC-1, the low-affinity Na⁺/dicarboxylate co-transporter, has been cloned from rabbit, human and rat [2–4]. The gene family also includes NaDC-2, from *Xenopus* intestine, which transports Na⁺ or Li⁺ [5], and the high-affinity Na⁺/dicarboxylate co-transporters, NaDC-3, from rat and flounder [5–8]. The Na⁺/dicarboxylate co-transporters are related in sequence to the Na⁺/sulphate co-transporters NaSi-1 and SUT-1 [9,10].

Relatively little is known about the structures of substrate-binding sites and cation-binding sites in transport proteins. However, there is some evidence that cationic amino acids might be important in the binding of substrates. For example, arginine residues are thought to participate in substrate binding in the lactose permease of *Escherichia coli* [11], in the glutamate transporter, GLAST-1 [12], and in halorhodopsin [13]. Furthermore, succinate binding to aspartate carbamoyltransferase involves interactions with one lysine and two arginine residues [14]. We therefore investigated the roles of lysine and arginine residues in NaDC-1 by site-directed mutagenesis. Of the 10 residues mutated, eight were found to be non-essential because their

replacement with alanine did not alter the functional properties of NaDC-1. However, the mutation of Lys-84 or Arg-349 to alanine affected transport activity. The K84A mutant had an increased K_m for succinate of 1.8 mM. The R349A mutant had altered succinate handling but normal Na⁺ and citrate kinetics. At high succinate concentrations the R349A mutant showed evidence of inhibition, probably due to extracellular succinate. We conclude that some arginine and lysine residues in NaDC-1 are important for substrate binding.

EXPERIMENTAL

Construction of mutant transporter cDNA species

Mutants of the rabbit NaDC-1 were prepared with the oligonucleotide-directed technique of Kunkel [15] with reagents from the Muta-Gene Kit (Bio-Rad). Mutations were verified by sequencing. The mutant nomenclature consists of the single-letter amino acid notation of the mutated residue and its location followed by the amino acid replacement at that position (for example, K84A is a mutant of NaDC-1 containing an alanine replacement of Lys-84).

cRNA transcription *in vitro*

The wild-type and mutant NaDC-1 cDNA species were linearized with *Xba*I for use as a template. cRNA was transcribed *in vitro* with the T7 mMessage mMachine (Ambion). The cRNA species were suspended in water at a final concentration of 1 µg/µl.

Xenopus oocytes

Female *Xenopus laevis* were obtained from Nasco or Xenopus I. Stage V and VI oocytes were dissected and treated with collagenase as described previously [2]. Oocytes were injected with 50 nl

Abbreviations used: NaDC-1, Na⁺/dicarboxylate co-transporter 1; TMD, transmembrane domain.

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Figure 1 Secondary-structure model of NaDC-1, showing the locations of the arginine and lysine residues mutated in this study

The N-glycosylation site at the C-terminus is shown by a Y.

of cRNA on the following day. The oocytes were cultured at 18 °C in Barth's medium supplemented with 5% (v/v) heat-inactivated horse serum, 2.5 mM pyruvate and 50 mg/ml gentamicin. Culture dishes and medium were changed daily.

Transport experiments

The uptake of [14 C]succinate (DuPont-NEN) and [14 C]citrate (Moravsek Biochemicals and NEN) was measured in groups of five oocytes between 4 and 6 days after cRNA injection, as described [2]. After a rinse with choline buffer [100 mM choline chloride/2 mM KCl/1 mM MgCl₂/1 mM CaCl₂/10 mM Hepes/Tris (pH 7.5)], transport was initiated with the appropriate substrate in Na⁺ transport buffer (as above but with 100 mM NaCl in place of choline chloride). Transport was ended with the addition of ice-cold choline buffer, followed by the removal of extracellular radioactivity with three additional washes with cold choline buffer. Each oocyte was transferred to a separate scintillation vial and dissolved in 0.25 ml of 10% (w/v) SDS. Scintillation cocktail was added and radioactivity was counted. Counts in control uninjected oocytes were subtracted from counts in cRNA-injected oocytes. Kinetic constants were calculated by non-linear regression to the Michaelis–Menten and Hill equations, with SigmaPlot 5.0 software (Jandel Scientific). Statistical analysis was performed with SigmaStat (Jandel Scientific).

Electrophysiology

Currents in the R349A mutant were measured with the two-electrode voltage clamp method, as described previously [16]. The pulse protocol consisted 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 difference between steady-state currents measured

in the presence and the absence of substrate is the substrate-dependent current [16].

Immunodetection of cell-surface-expressed transporters

Cell-surface biotinylation and Western blotting were used to determine relative protein expression, as described previously [17]. The oocytes were labelled with the membrane-impermeant biotin reagent, sulfo-NHS-LC-biotin (Pierce), and the biotinylated proteins were precipitated with ImmunoPure immobilized streptavidin beads (Pierce). The proteins were blotted to nitrocellulose membranes and probed with an anti-(NaDC-1) antibody [17] applied at 1:5000 dilution for 2 h followed by incubation with horseradish-peroxidase-linked anti-rabbit Ig (Amersham) at a 1:5000 dilution for 1 h. Antibody binding to NaDC-1 was detected with the Supersignal CL-HRP substrate system (Pierce).

RESULTS

Site-directed mutagenesis of cationic amino acids in rabbit NaDC-1 was undertaken to determine the role of these amino acids in function. Figure 1 shows the secondary-structure model of NaDC-1 and the locations of the 10 residues mutated in this study. In the initial round of mutagenesis, the cationic amino acids were replaced by alanine. Most of the amino acids chosen for mutagenesis are conserved residues. Lys-34, Arg-108 and Arg-349 are found in all members of the family related to NaDC-1, whereas Lys-84, Lys-107, Lys-333, Lys-390 and Lys-414 are conserved in the NaDC-1 orthologues. Two arginine residues, Arg-368 and Arg-541, found only in the rabbit NaDC-1, were chosen for mutagenesis on the basis of their proximity to transmembrane domains 8 and 11, which were shown in a previous study to affect differences in substrate specificity [18,19]. Of the ten residues mutated, only Lys-333 is predicted to be in a transmembrane domain, whereas Lys-34, Lys-84 Arg-349 and

Table 1 Succinate transport in mutant NaDC-1 transporters expressed in *Xenopus* oocytes

Transport of 100 μ M succinate was measured for 15 min; uptakes are expressed as a percentage of transport measured in the wild-type control from the same batch of oocytes. The number of separate experiments, n , is shown in the last column. Results are means $\pm$ S.E.M. ($n = 3-5$) or mean $\pm$ range ($n = 2$). *Significant difference from wild type ($P < 0.01$).

Mutant	Uptake (% of wild type)	n
K34A	100 $\pm$ 24	3
K84A	25 $\pm$ 6*	4
K107A	109 $\pm$ 25	3
R108A	78 $\pm$ 14	5
K333A	96 $\pm$ 6	2
R349A	9 $\pm$ 6*	2
R368A	81 $\pm$ 2	2
K390A	138 $\pm$ 55	2
K414A	96 $\pm$ 17	3
R541A	150 $\pm$ 70	2

Arg-541 are predicted to be at the interface between the membrane and the extramembranous regions; the rest are in the intracellular or extracellular loops (Figure 1).

The mutant transporters were expressed in *Xenopus* oocytes and succinate transport was measured. Seven of the mutants (K34A, K107A, K333A, R368A, K390A, K414A and R541A) exhibited more than 80 % of the wild-type activity (Table 1). These mutants also had succinate K_m values between 176 and 577 μ M, similar to that of wild-type NaDC-1 (Table 2) [2]. One mutant, R108A, exhibited variable expression, with transport activities between 42 % and 120 % of wild-type (Tables 1 and 2), and cell-surface biotinylation experiments showed that the activity of R108A paralleled the amount of cell-surface protein (results not shown). In contrast, the K84A and R349A mutants had transport rates that were much lower than the activity of wild-type NaDC-1 (Table 1); they were therefore selected for further study.

The cell-surface expression of the K84A and R349A mutants was assessed by cell-surface biotinylation with the membrane-impermeant reagent, sulfo-NHS-LC-biotin, followed by Western blotting (Figure 2). NaDC-1 expressed in oocytes typically appears as two protein bands of 74 and 55 kDa, representing differently glycosylated forms of the transporter [17,20]; control, uninjected oocytes do not show any immunoreactivity [17]. The 55 kDa band is not visible in the wild-type NaDC-1 sample shown in Figure 2 but it was evident after a longer exposure of the film (result not shown). Both K84A and R349A were expressed at the plasma membrane and seemed to be more abundant than the wild-type NaDC-1 (Figure 2). The decreased activity of these mutants was therefore not due to a decrease in cell-surface expression of the transporter proteins.

Mutation of Lys-84 to alanine affected the apparent substrate affinity. The K84A mutant had a K_m for succinate of 2.0 mM (Figure 3A), compared with 0.3–0.5 mM in the wild-type NaDC-1 [20]. In four separate experiments with K84A, the mean K_m for succinate was 1.8 ± 0.2 mM and V_{max} was 3.1 ± 0.7 nmol/h per oocyte (mean $\pm$ S.E.M.). This V_{max} is lower than that of the wild-type transporter (Table 2) [20]. In contrast, the Na⁺ activation kinetics with the K84A mutant (Figure 3B) were similar to that of the wild type [20]. In the experiment shown in Figure 3(B), the apparent K_{Na} in the K84A mutant was 26 mM; the Hill coefficient, h , was 1.5. In three experiments, the mean K_{Na} in K84A was 34 ± 9 mM and the mean h was 1.5 ± 0.2 (mean $\pm$ S.E.M.).

Table 2 Succinate kinetics in mutant transporters with wild-type activity

The errors represent standard errors of the fit to the Michaelis–Menten equation. (Note that the R108A mutant had variable expression. In this experiment the cell-surface protein was approx. 30 % of wild type.)

Experiment	Mutant	K_m (μ M)	V_{max} (pmol/h per oocyte)
1	Wild type	285 $\pm$ 65	5368 $\pm$ 375
	K34A	437 $\pm$ 112	7972 $\pm$ 728
	K107A	176 $\pm$ 42	4344 $\pm$ 268
	R108A	216 $\pm$ 29	1842 $\pm$ 68
2	K333A	321 $\pm$ 51	11048 $\pm$ 432
	R368A	577 $\pm$ 71	14745 $\pm$ 538
	K390A	466 $\pm$ 70	14939 $\pm$ 619
3	K414A	524 $\pm$ 56	9361 $\pm$ 377
4	R541A	408 $\pm$ 144	10810 $\pm$ 1011

Figure 2 Western blot of NaDC-1 transporters expressed at the oocyte cell surface

Xenopus oocytes were biotinylated with the membrane-impermeant reagent sulfo-NHS-LC-biotin. The biotinylated proteins were precipitated with streptavidin–agarose beads, separated by SDS/PAGE and transferred to nitrocellulose. The blots were probed with anti-(NaDC-1) antibody at 1:10000 dilution followed by secondary antibody at 1:5000 dilution. Some sample lanes have been removed between the K84A and R349A sample lanes. The lane containing samples from control, uninjected oocytes is labelled 'Un'.

The R349A mutant had unusual succinate kinetics compared with the other mutants (Figure 4A). As the succinate concentration increased, the transport rate in R349A first increased, reached a peak at approx. 250 μ M and then decreased, which resembled the kinetics of substrate or product inhibition [21]. The succinate kinetic experiment with the R349A mutant was also performed with the two-electrode voltage clamp technique to measure substrate-dependent currents at more rapid time points (100 ms) under voltage-clamp conditions (Figure 4B). The apparent inhibition at high substrate concentrations was still evident. Furthermore, the amount of inhibition estimated from the ratio of the current measured at the 10 mM succinate concentration and the peak current at 250 μ M succinate concentration was independent of voltage. For example, the current measured at 10 mM succinate was 61 % of the maximum current at -150 mV, 67 % at -70 mV and 69 % at -30 mV

Figure 3 Kinetics of transport in the K84A mutant expressed in *Xenopus* oocytes

(A) Succinate kinetics. Succinate concentrations were between 25 μ M and 5 mM; 5 min uptakes were measured. The K_m for succinate is 2.2 ± 0.5 mM; V_{max} is 3030 ± 314 pmol/h per oocyte. (B) Na^+ activation of succinate transport. Uptakes of 100 μ M ^3H -succinate were measured for 5 min in concentrations of Na^+ between 0 and 100 mM (Na^+ replaced by choline). The kinetic constants are: K_{Na} 26 ± 7 mM, V_{max} 145 ± 19 pmol/h per oocyte, Hill coefficient 1.5 ± 0.4 (the error represents the standard error of the regression). Error bars show S.E.M. ($n = 5$).

Figure 4 Succinate kinetics in the R349A mutant expressed in *Xenopus* oocytes

(A) Succinate kinetics measured with radiotracer uptakes. Uptakes were measured for 5 min. Error bars show S.E.M. (B) Succinate kinetics measured with a two-electrode voltage clamp with 100 ms voltage pulses. Steady-state substrate-dependent inward currents were measured at each succinate concentration. The currents at three voltages are shown: $\bullet$, -70 mV; $\square$, -110 mV; $\blacktriangle$, -150 mV.

(Figure 4B). This result suggests a voltage-independent inhibitory binding step for succinate.

Interestingly, although the R349A mutant seemed to have altered handling of succinate, the kinetics of citrate and Na^+ in this mutant seemed similar to those of the wild type. The kinetics of citrate uptake in R349A are hyperbolic, with an apparent K_m of 2.2 mM (Figure 5A). In three separate experiments, the mean K_m for citrate was 1.5 ± 0.4 mM (mean $\pm$ S.E.M.). For comparison, the K_m for citrate in wild-type NaDC-1 is 0.9 mM [20]. Figure 5(B) shows the result of an experiment on Na^+ activation of succinate transport in oocytes expressing the R349A mutant. In this experiment, the K_{Na} was 46 mM and the apparent Hill coefficient, h , was 1.8. In three separate experiments, the mean K_{Na} was 44 ± 6 mM and the mean h was 2.2 ± 0.5 (mean $\pm$ S.E.M.).

Additional substitutions were made at position 349 in NaDC-1. Replacement of the arginine residue at position 349 with the anionic glutamic acid resulted in a mutant transporter (R349E)

that was not found on the plasma membrane and thus had no transport activity (Figure 6). Replacements with the neutral amino acids isoleucine (R349I) and glutamine (R349Q) produced mutants with very low transport activity, less than 5% of the activity of the wild-type NaDC-1 (Figure 6B). However, the R349I and R349Q proteins were expressed at the cell surface; their abundance was approximately half that of the wild-type transporter, on the basis of scans of the immunoblots (Figure 6A). In contrast, the replacement of lysine at position 349 resulted in a transporter with high transport activity and high cell-surface expression, indicating that the charge at position 349 is important for function and expression. The kinetics of the R349K mutant were difficult to assess. In one experiment, the kinetics seemed hyperbolic, with a K_m for succinate of 700 μ M, but in two other experiments, the kinetics showed some inhibition at high succinate concentrations, although not as severe as the R349A mutant.

Figure 5 Citrate and Na⁺ activation kinetics in the R349A mutant

(A) Kinetics of citrate uptake in R349A. K_m 2.2 ± 0.8 mM, V_{max} 3012 ± 460 pmol/h per oocyte. (B) Na⁺ activation of succinate transport. Uptakes of $100 \mu\text{M}$ succinate were measured for 5 min in Na⁺ between 0 and 100 mM. K_{Na} 46 ± 5 mM, V_{max} 468 ± 36 pmol/h per oocyte, Hill coefficient 1.8 ± 0.2 (the error represents the standard error of the regression). Results are means $\pm$ S.E.M. for $n = 5$ oocytes.

Figure 6 Effect of additional substitutions at position 349

(A) Western blot of wild-type (WT) and mutant NaDC-1 transporters expressed at the oocyte cell surface. *Xenopus* oocytes were biotinylated with the membrane-impermeant reagent sulphonyl-NHS-LS-biotin (as described in the legend to Figure 2). (B) Transport of $50 \mu\text{M}$ (open bars) or 1 mM (filled bars) succinate in the same batch of oocytes from the same frog as those used for the Western blot (A). Uptakes were measured for 5 min in Na⁺-containing buffer.

DISCUSSION

The goal of the present study was to identify cationic amino acids in NaDC-1 that are involved in transport. Previous studies have shown that arginine residues can participate in substrate binding

in the lactose permease of *E. coli* [11], in the glutamate transporter, GLAST-1 [12] and in halorhodopsin [13]. In addition, succinate binding to aspartate carbamoyltransferase involves interactions with lysine and arginine residues [14]. We therefore investigated the roles of lysine and arginine residues in NaDC-1 by site-directed mutagenesis. Most of the cationic amino acids mutated in this study could be replaced without loss of function or change in trafficking properties. Furthermore, none of the mutations completely abolished transport, similarly to our previous study of conserved anionic amino acids in NaDC-1 [18], suggesting that intermembrane salt bridges are not important in the function of NaDC-1.

Neutralization of Lys-84 by replacing it with alanine resulted in a transporter with an increased K_m and a decreased V_{max} relative to the wild-type NaDC-1. The mutant transporter did not show any decrease in cell-surface abundance. Lys-84 is found at the cytoplasmic surface of transmembrane domain (TMD) 3. However, our previous studies have identified residues or regions of the C-terminal half of the transporter that are important in substrate recognition and binding. Chimeras between NaDC-1 and the Na⁺/sulphate co-transporter NaSi-1 have shown that substrate selectivity is determined by residues in TMDs 5–11 [22]. Residues that determine differences in citrate and Na⁺ affinities are located in TMDs 7, 10 and 11 [19]. Finally, residues that are involved in Na⁺ and substrate binding are located in TMDs 8 and 9 [18]. It is therefore likely that mutation of Lys-84 affects one or more rate constants for translocation of the loaded or unloaded carrier, which could be reflected in both the K_m and V_{max} [23,24].

The arginine residue at position 349 is conserved in all members of the family related to NaDC-1, including the sulphate transporters NaSi-1 and SUT-1 [9,10]. Mutation of Arg-349 to the neutral alanine resulted in a transporter with apparently normal citrate and Na⁺ handling but unusual succinate kinetics. At high concentrations of succinate, the transport activity by R349A was inhibited. Because the inhibition profiles were similar in uptake experiments (5 min measurements) and two-electrode voltage clamp experiments (100 ms measurements), it is likely that the

inhibition occurs at the extracellular face of the transporter. In addition, the inhibition seems to be independent of voltage, which is similar to our previous results suggesting that substrate binding in NaDC-1 is likely to be voltage-independent [16]. The difference between citrate and succinate handling in this mutant also suggests that Arg-349 might be important in substrate recognition. The charge at position 349 seems to be required for normal activity of NaDC-1 because charge neutralization resulted in decreased activity and the unusual succinate kinetics. Reversal of the charge at position 349 was not tolerated; the R349E mutant was not present on the plasma membrane. However, the replacement of Arg-349 by lysine resulted in high activity and high protein expression. We propose that Arg-349 is involved in substrate binding, particularly in the outward-facing orientation. It is likely that the substrate-binding site in NaDC-1 contains several residues that interact with the substrate through hydrogen bonds, similarly to the phosphate and sulphate transport receptors of *E. coli* [25,26]. In the *E. coli* receptor, phosphate binding occurs within a deep cleft and involves interactions, predominantly through hydrogen bonds, with at least 12 amino acids, including an arginine residue. The surface electrostatic potential of the phosphate transport receptor is negatively charged but the binding of phosphate is insensitive to changes in charge interaction, primarily because of the interactions with so many residues [25,26]. Similarly, two-electrode voltage clamp studies with NaDC-1 indicate that substrate binding is insensitive to changes in voltage at negative membrane potentials [16]. Therefore mutagenesis of a single residue would be expected to have varying effects on substrate binding, which could be direct or indirect, depending on the number of residues that interact with the substrate. Furthermore, charge neutralization would not have as great an effect on substrate binding if the substrate-binding site were composed of interactions with many amino acids, in comparison with direct salt interaction with relatively few residues. In contrast, the crystal structure of succinate bound to the enzyme aspartate carbamoyl transferase shows that the carboxylates of succinate interact with two arginine residues, forming salt pairs [14]. It is likely that charge neutralization of either of these arginine residues would have profound effects on substrate binding.

Interestingly, none of the residues mutated in this study was found to be absolutely required for expression of NaDC-1 at the plasma membrane. This was somewhat surprising because arginine and lysine residues are often found in trafficking signals that mediate protein sorting through different membrane compartments in cells [27–29]. For example, the Arg-Lys-Arg motif in the K_{ATP} channel mediates retention in the endoplasmic reticulum; mutations in this motif result in escape of the proteins to the plasma membrane [29]. Mutagenesis of Arg-427 to alanine in the Na^+ /glucose co-transporter results in the accumulation of the transporter just below the plasma membrane [30]. In our study, the arginine residues at positions 108 and 349 seemed to have some effects on protein expression. Arg-108 might have a minor role in protein trafficking because the amount of expression of R108A was highly variable. It is possible that the R108A mutation could cause a slight change in protein structure, which could alter the position of more important trafficking signals. Our previous study showed that the nearby His-106 is important in protein trafficking in NaDC-1 [17]. Arg-349 in NaDC-1 might function in determining the amount of protein that reaches the plasma membrane, rather than forming part of a protein-sorting motif. The R349A and R349K mutants seemed to be more abundant than the wild-type NaDC-1, whereas charge reversal in the R349E mutant resulted in a protein that was absent from the plasma membrane.

In conclusion, this study has identified only two out of ten cationic residues in NaDC-1 that seem to be functionally important. Eight residues in NaDC-1 (Lys-34, Lys-107, Arg-108, Lys-333, Lys-390, Arg-368, Lys-414 and Arg-541) could be replaced without loss of function or impairment in protein trafficking. In contrast, mutations of Lys-84, at the cytoplasmic face of TMD 3, and Arg-349, at the extracellular surface of TMD 7, resulted in transporters with altered function. Lys-84 is likely to be important in the substrate translocation or reorientation of the carrier, whereas Arg-349 might be a potential substrate-binding or co-ordinating residue.

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