

SODIUM-COUPLED TRANSPORTERS FOR KREBS CYCLE INTERMEDIATES

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KEY WORDS: succinate, citrate, α -ketoglutarate, sodium, citric acid cycle intermediates

ABSTRACT

Krebs cycle intermediates such as succinate, citrate, and α -ketoglutarate are transferred across plasma membranes of cells by secondary active transporters that couple the downhill movement of sodium to the concentrative uptake of substrate. Several transporters have been identified in isolated membrane vesicles and cells based on their functional properties, suggesting the existence of at least three or more Na^+ /dicarboxylate cotransporter proteins in a given species. Recently, several cDNAs, called NaDC-1, coding for the low-affinity Na^+ /dicarboxylate cotransporters have been isolated from rabbit, human, and rat kidney. The Na^+ /dicarboxylate cotransporters are part of a distinct gene family that includes the renal and intestinal Na^+ /sulfate cotransporters. Other members of this family include a Na^+ - and Li^+ -dependent dicarboxylate transporter from *Xenopus* intestine and a putative Na^+ /dicarboxylate cotransporter from rat intestine. The current model of secondary structure in NaDC-1 contains 11 transmembrane domains and an extracellular N-glycosylated carboxy terminus.

INTRODUCTION

The metabolic intermediates of the Krebs or citric acid cycle constitute a group of organic anions that include dicarboxylates such as succinate and α -ketoglutarate and tricarboxylates such as citrate. The Krebs cycle intermediates are transported across plasma membranes of cells by secondary active transporters that couple the downhill movement of sodium to the concentrative

uptake of substrate. Tricarboxylates are often carried in protonated form, and therefore, the transporters are referred to as Na^+ /dicarboxylate cotransporters. These transporters have broad substrate specificities and carry a wide range of di- and tricarboxylic acids. The primary distribution of Na^+ /dicarboxylate cotransporters is in the epithelial cells of the renal proximal tubule and small intestine. However, these transporters are also found in organs such as colon, liver, placenta, and brain.

The sodium-coupled transport of Krebs cycle intermediates in the kidney has been reviewed previously by Wright (71), Hamm (21), and Murer et al (38). This review discusses the different classes of Na^+ /dicarboxylate cotransporters that have been characterized in kidney as well as other organs. This review also provides an update on the molecular advances in the field. Several of the low-affinity Na^+ /dicarboxylate cotransporter cDNAs have now been isolated. The Na^+ /dicarboxylate cotransporters, called NaDC, are related in sequence and probably also in structure to the Na^+ /sulfate cotransporter, NaSi-1. The Na^+ /dicarboxylate and Na^+ /sulfate cotransporters belong to a distinct gene family that is not related to any of the other known families of transport proteins.

FUNCTIONAL CHARACTERIZATION OF SODIUM-COUPLED DICARBOXYLATE TRANSPORTERS

Functional studies in isolated cells and membrane vesicles have identified several categories of sodium-coupled transporters for Krebs cycle intermediates (Table 1). The transporters differ in substrate affinity and selectivity, in sensitivity to inhibition by lithium, and in tissue distribution and species distribution. In addition to providing information on the physiological role of these transporters, the identification of functional differences between transport pathways may be a useful predictor of the number of different transport proteins that are likely to exist.

Low-Affinity Transporters for Krebs Cycle Intermediates

The best characterized of the Na^+ /dicarboxylate cotransporters is the low-affinity transporter found on the apical membranes of proximal tubule cells in rabbit kidney (Table 1). This transporter has been studied in isolated, perfused tubules and in brush border membrane vesicles. The primary function of this pathway is to reabsorb Krebs cycle intermediates from the tubular filtrate.

SUBSTRATE SPECIFICITY The substrate specificity of the low-affinity transport pathway is very broad (63, 74). The transporter accepts a wide variety of di- and tricarboxylates, including succinate, citrate, and α -ketoglutarate. The

Table 1 Functional properties of Na⁺/dicarboxylate cotransporters

| K_m (succinate or α KG) | Lithium inhibition | Electrogenic | Species | Organ/cell type | Reference |
|----------------------------------|--------------------|--------------|-------------|-------------------------------|------------|
| 0.5–1 mM | Yes | Yes | Rabbit | Renal cortex bbm ¹ | 29, 76 |
| | | | Rat | Small intestine bbm | 67 |
| 0.4 mM | No | Yes | Rat | Colon bbm | 67 |
| 0.5 (citrate) | n.d. | n.d. | Opossum | OK cell line | 30 |
| 100–175 μ M | Yes | Yes | Rat | Renal cortex bbm | 14, 58 |
| | | | Pig | Intestinal bbm | 69 |
| 5–30 μ M | Yes | Yes | Rat, rabbit | Renal cortex blm | 10, 14, 76 |
| | | | Human | Placenta bbm | 18, 40 |
| | | | Rat | Liver blm | 37, 77 |
| 2 μ M | n.d. | n.d. | Rat, mouse | Brain synaptosomes | 56, 57 |
| 25 μ M | n.d. | No | Chick | Intestinal cells | 28 |

¹Abbreviations: bbm, brush border membranes; blm, basolateral membranes; n.d., not determined.

preferred structure consists of a four-carbon, terminal carboxylic acid with the carboxylate groups in *trans* configuration (74). The transporter has a high affinity for succinate, with a K_m of approximately 0.5–1 mM, and for this reason, succinate is often used as a test substrate. The kinetic properties of Na⁺/succinate cotransport are asymmetrical: The succinate K_m measured from influx experiments was approximately the same as that measured in efflux experiments, but the V_{max} for influx was threefold faster than for efflux (22).

The low-affinity Na⁺/dicarboxylate cotransporter is characterized by an insensitivity of dicarboxylate transport to changes in pH (19, 75). In contrast, the transport of tricarboxylates such as citrate is strongly stimulated by acidic pH, in parallel with the concentrations of protonated citrate in the medium (7, 19, 75). Therefore the preferred substrates of the transporter appear to be divalent anions, which suggests that the affinity for protonated citrate is very high. As an example, in rabbit renal brush border membrane vesicles the apparent K_m for total citrate was calculated as 0.4 mM, but when the concentrations of protonated citrate were taken into account, the K_m was approximately 15 μ M (3). High concentrations of trivalent citrate appear to inhibit transport of protonated citrate (3).

CATIONS The transport of Krebs cycle intermediates is coupled to the movement of sodium. In rabbit renal brush border membrane vesicles, the replacement of sodium by other cations, with the exception of lithium (see below), abolished transport (72). Sodium acts as an essential activator of dicarboxylate transport. It is thought that three sodium ions bind first to the transporter and

trigger a conformational change that results in an increased substrate affinity (23, 73). Transport kinetics measured at different sodium concentrations showed that the K_m for succinate became larger as the sodium concentration decreased (73). Direct measurements of sodium and succinate transport in brush border membrane vesicles showed that three sodium ions are coupled to the transport of succinate (23). The coupling coefficient was independent of succinate and sodium concentrations.

LITHIUM Treatment of human patients with therapeutic doses of lithium salts led to a rapid increase in the renal excretion of α -ketoglutarate and glutarate (6). This response was also seen in rats (5). In rabbit renal brush border membrane vesicles, the effects of lithium were shown to be a result of competition with sodium in the Na^+ /dicarboxylate cotransporter (72). The inhibition by millimolar concentrations of lithium (K_i 1.2 mM) occurs in the presence of 100 mM sodium, suggesting that at least one of the cation binding sites has a high affinity for lithium (72). Lithium can replace sodium in the transport of succinate, but succinate transport occurs at a greatly reduced rate and the K_m for succinate in Li^+ is very high, about 30 mM (72). This result indicates that the binding of lithium to the Na^+ /dicarboxylate cotransporter does not produce the optimal conformation for succinate binding.

ELECTROGENICITY Sodium-coupled transport of dicarboxylates in rabbit renal brush border membranes is an electrogenic process. The coupled transport of succinate and sodium was sensitive to changes in membrane potential (73), and the transport of succinate also depolarized the membrane potential in vesicles (54). The predominant effect of voltage on transport in vesicles was on the K_m for succinate; the K_m became smaller when measured at more negative membrane potentials (73). Interestingly, there is one report of electrogenic Na^+ /succinate cotransport in outer cortical membrane vesicles that predicts a coupling stoichiometry of only 2:1 based on static-head experiments (17).

DISTRIBUTION AND FUNCTION The primary function of the Na^+ /dicarboxylate cotransporter on the apical membrane of renal proximal tubule cells is to recover Krebs cycle intermediates from the tubular filtrate. These transported substrates are transferred to the mitochondria and metabolized. Estimates of citrate contribution to proximal tubule oxygen consumption are as high as 10% (21). Recently, it was shown that the cytosolic enzyme, ATP citrate lyase, is abundant in kidney and plays an important role in citrate metabolism, suggesting that transported citrate could be used in cholesterol and lipid biosynthesis (35).

The renal Na^+ /dicarboxylate cotransporter is involved in regulating urinary citrate concentrations. Citrate prevents urinary stone formation by chelating

calcium. Approximately 50% of patients with kidney stones exhibit hypocitraturia (49), but there is no direct evidence that a defect in the transporter is involved in the hypocitraturia that results in stone formation. Hypocitraturia is often accompanied by acidosis, and approximately half of the hypocitraturia seen in acidosis can be attributed to increased activity of ATP citrate lyase (35).

Low-affinity Na^+ /dicarboxylate cotransport with properties similar to that of the renal transporter has been characterized in isolated brush border membranes from the rat small intestine (67) (Table 1). Sodium-coupled transport of succinate, citrate, and α -ketoglutarate has also been reported in everted sacs of hamster small intestine (8) and in brush border membrane vesicles of calves (68). The small intestine Na^+ /dicarboxylate cotransporter functions to absorb dicarboxylates from the diet, as well as to recover citrate that is secreted in pancreatic and gastric juice (32, 52). There is evidence for net transepithelial transfer of citrate in hamster intestine (8). The efflux of di- and tricarboxylates across the intestinal basolateral membrane appears to be mediated by an anion exchanger (70). Evidence exists for intestinal absorption of citrate in humans: Oral administration of citrate results in increased serum citrate concentrations within 30 min (53a).

Functional differences observed between low-affinity sodium-dicarboxylate cotransporters suggest that different transport proteins may exist (Table 1). The brush border membrane of rat colon contains a low-affinity transporter that is not sensitive to inhibition by lithium (Table 1), whereas the same study showed that the transporter in small intestine is sensitive to lithium (67). Therefore, different proteins are likely to mediate uptake of dicarboxylates in the colon and small intestine (67). Another sodium-dependent transporter for Krebs cycle intermediates has been reported in OK cells (opossum kidney) (30) (Table 1). The OK cell transporter has a relatively low-affinity for citrate, which may be carried in trivalent form, and an unusual substrate specificity: It does not appear to handle succinate. It is not known whether the OK cell transporter is found in native opossum kidney membranes or whether transporters with these properties are found in other organs or species. The differences in transport properties suggest that there are at least three different sodium-coupled transport proteins that carry di- and tricarboxylates with low-affinity.

Intermediate-Affinity Transporters for Krebs Cycle Intermediates

The Na^+ /dicarboxylate cotransporters found on renal and intestinal brush border membranes exhibit functional differences between species (Table 1). Apical membrane transporters have been reported with intermediate substrate affinities, with K_m s for succinate or α -ketoglutarate around 100 μM . For example, rat kidney contains a sodium-dependent transport pathway with substrate specificity

similar to the one found in rabbit kidney (58). The rat transporter has an apparent K_m for methylsuccinate of $130 \mu\text{M}$ (58) and a K_m for α -ketoglutarate of $158 \mu\text{M}$ (14). Aside from the difference in substrate affinity, the apical membrane transporters in rat and rabbit are very similar in their sodium dependence and sensitivity to inhibition by millimolar concentrations of lithium, K_i 1.8 mM (58). In addition, the insensitivity of dicarboxylate transport to pH and the stimulation of tricarboxylate transport at acidic pH is similar in both rats and rabbits.

Intestinal brush border membrane vesicles from pig also express an intermediate affinity Na^+ /dicarboxylate cotransporter with a K_m for succinate of $175 \mu\text{M}$ (69). The transport of both fumarate and citrate was sensitive to changes in membrane potential, and there was a strong stimulation of citrate uptake at pH 5.5. This study is consistent with electrogenic transport of divalent citrate in pig intestine and supports a coupling stoichiometry of $3 \text{ Na}^+ : 1$ divalent anion substrate (69). Interestingly, another study of succinate transport in pig intestine reported very low rates of sodium-coupled transport and what appears to be a very large diffusive component (36). It is possible that the differences between studies are a result of dietary regulation of Na^+ /dicarboxylate cotransport in the pig intestine. To my knowledge no studies have examined the dietary regulation of intestinal dicarboxylate transport, but it is well established that many nutrients regulate the activity of their transporters in the intestine (16). The dietary induction of transporters could also explain why Na^+ /succinate transport in brush border membrane vesicles from rabbit small intestine was measured in some studies (44) but not in others (61).

Note that the conditions used for uptake experiments can contribute to differences in apparent substrate affinities. If initial rates of transport are not used at all substrate concentrations in kinetic studies, the transport rates measured at higher substrate concentrations can be underestimates of the true rates (73). The calculated V_{max} from those data would be lower than the true V_{max} , and the K_m (or the concentration that produces $1/2 V_{\text{max}}$) would appear to be smaller than the true K_m . For example, the K_m for succinate transport in rabbit renal brush border membrane vesicles was found to be $110 \mu\text{M}$ when 15-s uptakes were used (74), whereas the K_m for succinate measured with 1-s uptakes was 0.7 mM (73). Although the succinate K_m reported for rat kidney is consistently lower than that reported for rabbit, more studies are necessary to rule out the possibility that the intermediate affinity transporters are in fact a subset of the low-affinity transporters.

High-Affinity Transporters for Krebs Cycle Intermediates

The functional properties of the high-affinity transporters appear to exhibit a number of differences compared with the low- and intermediate-affinity

transporters (Table 1). Of high-affinity transporters, the best characterized are those found on the basolateral membranes of kidney proximal tubule cells. However, high-affinity transporters have also been reported on other cells and organs, including liver, placenta, and brain.

SUBSTRATES The high-affinity Na^+ /dicarboxylate cotransporters that have been functionally characterized have K_m s for succinate or α -ketoglutarate between 5 and 33 μM . Comparison of transport in brush border and basolateral membranes from the same organs has shown that the high-affinity transporter has a much lower transport capacity than the transporter on the apical membrane (14, 76). Although the high-affinity and low-affinity transport pathways have many substrates in common, some differences appear in substrate specificity. Citrate appears to be carried by the low-affinity transporter but is not a good substrate of the high-affinity pathway. The apparent K_m for citrate in rabbit renal basolateral membrane vesicles is around 2.5 mM (25). The transport of citrate is also relatively insensitive to pH changes, suggesting that trivalent citrate is the transported species (76). The pH dependence of succinate transport in the high-affinity pathway has a pH optimum at 7.5 (10, 76). The high-affinity Na^+ /dicarboxylate transporter interacts with substrates such as dimethylsuccinate, meso-2,3-dimercaptosuccinate, and *cis*-aconitate (63), which do not inhibit the low-affinity transporter.

CATIONS The high-affinity Na^+ /dicarboxylate cotransporters also couple multiple sodium ions to the transport of dicarboxylates, and transport is thought to be electrogenic. The transport of dicarboxylates but not of citrate appears to be sensitive to changes in membrane potential (10, 14, 76), which also supports the idea that citrate is carried in trivalent form.

There may be differences between the high-affinity transporters in their sensitivity to inhibition by lithium. The Na^+ /dicarboxylate cotransporters in rat kidney basolateral membranes (10, 14) and rat liver basolateral membranes (37, 77) are sensitive to inhibition by millimolar concentrations of lithium. However, Na^+ /dicarboxylate cotransport in human placenta is much less sensitive, with an apparent IC_{50} for Li^+ of around 10 mM (18), which could indicate some species or tissue differences in the transporter proteins.

TISSUE DISTRIBUTION AND FUNCTION High-affinity Na^+ /dicarboxylate cotransport has been characterized in the basolateral membranes of renal proximal tubules of rat and rabbit (10, 14, 76), rat liver sinusoidal basolateral membranes (37, 77), human placental brush border membranes (18, 40), mouse and rat brain synaptosomes (56, 57), and isolated chick intestinal cells (28) (Table 1).

One of the primary functions of the high-affinity transporter on the renal basolateral membrane appears to be in organic anion secretion (53). Organic

anions, such as *p*-aminohippurate (PAH), enter the cells of the proximal tubule on an organic anion/dicarboxylate exchanger located on the basolateral membrane (53). The dicarboxylates preferred by this exchanger include glutarate and α -ketoglutarate; succinate and citrate are not substrates of the exchanger. Glutarate and α -ketoglutarate are accumulated in proximal tubule cells by the Na^+ /dicarboxylate cotransporters on the apical (13) and basolateral membranes. The organic anion/dicarboxylate exchanger couples the efflux of dicarboxylates down their electrochemical gradients to the concentrative uptake of organic anions into the cells. The functional coupling between Na^+ /dicarboxylate cotransporters and organic anion secretory pathways may also operate in other organs such as liver (77).

The Na^+ /dicarboxylate cotransporter in liver may play a role in regulating the synthesis of glutamine (62). The high-affinity Na^+ /dicarboxylate cotransporter in rat liver is found on the basolateral membranes of a subset ($\sim 7\%$) of hepatocytes located in perivenous regions (62). The perivenous hepatocytes express glutamine synthetase, and the α -ketoglutarate transported into these hepatocytes is quickly converted to glutamine (62). In brain, the transport of α -ketoglutarate into synaptic terminals is thought to be the first step in replenishing pools of neurotransmitters such as glutamate (56). Synaptosome membranes contain at least one high-affinity Na^+ -dependent α -ketoglutarate transport pathway (56, 57). This transport pathway is inhibited by glutamate and aspartate, which do not inhibit other high-affinity transporters, suggesting that the isoform found in brain is a different protein than the renal isoform. Chick intestinal cells also contain a high-affinity Na^+ /dicarboxylate cotransporter, with a K_m for succinate of $25 \mu\text{M}$ (28). The chick intestinal transporter does not appear to be sensitive to membrane potential and may couple only 2 Na^+ to the transport of each succinate. Because the experiments were done with dissociated enterocytes, it is not known whether this transporter is on the apical or basolateral membrane.

REGULATION OF SODIUM-DICARBOXYLATE COTRANSPORTERS

Relatively little information is available about the regulation of Na^+ /dicarboxylate cotransporters. All studies to date have dealt with the effects of chronic conditions on transport in the kidney. For example, studies with perfused proximal tubules and isolated brush border membranes from fasted rats have shown increased transport of α -ketoglutarate (58) and citrate (66). After three-day starvation, the K_m for α -ketoglutarate remained unchanged but the V_{max} doubled (58). Interestingly, no corresponding change appeared in basolateral Na^+ /dicarboxylate cotransport with starvation (64). Transport of citrate is also

increased under conditions of metabolic acidosis (24) and chronic K^+ depletion, which is also mediated by an increased V_{\max} with no change in K_m (31). There are no reports of regulation of Na^+ /dicarboxylate transport in other organs or short-term changes induced by hormones, although parathyroid hormone may affect citrate transport in human kidney (12).

MOLECULAR STUDIES OF SODIUM-DICARBOXYLATE COTRANSPORTERS

Cloning and Characterization of NaDC-1 Family Members

The cDNA coding for the rabbit renal low-affinity Na^+ /dicarboxylate cotransporter, NaDC-1, was isolated by expression cloning in *Xenopus* oocytes (41). The primary amino acid sequence of NaDC-1 contains 593 amino acids and has a predicted mass of 66 kDa. The amino acid sequence of NaDC-1 is 43% identical to that of the renal and intestinal Na^+ /sulfate cotransporter NaSi-1 (34, 39). The transporters in this gene family are not related to any of the other transporter superfamilies.

Functionally, NaDC-1 corresponds to the low-affinity Na^+ /dicarboxylate cotransporter of the brush border membrane of the renal proximal tubule (71). NaDC-1 expressed in *Xenopus* oocytes has an apparent K_m for succinate of 0.5 mM and a K_m for citrate of about 0.9 mM (45). NaDC-1 has also been expressed in COS-7 cells and exhibits similar functional properties, including a K_m for succinate of 0.5 mM (48). The substrate specificity of NaDC-1 resembles that of the rabbit low-affinity Na^+ /dicarboxylate cotransporter (71); succinate transport in NaDC-1 was inhibited by a range of di- and tricarboxylates but not by monocarboxylates such as lactate. Transport by NaDC-1 was also insensitive to inhibition by dimethylsuccinate, a test substrate of the high-affinity transporter (63). As seen previously in brush border membrane vesicles (75), the transport of succinate by NaDC-1 was insensitive to changes in pH, whereas the transport of citrate was stimulated at acidic pH values (45). The primary effect of pH on citrate transport in NaDC-1 is on K_m with no change in V_{\max} , consistent with the idea that NaDC-1 prefers protonated citrate as a substrate (45). NaDC-1 is inhibited by furosemide, IC_{50} 1.5 mM, and flufenamate, IC_{50} 250 μ M, but it is not affected by anion transport inhibitors such as bumetanide, probenecid, or 4,4'-diisothiocyano stilbene-2,2'-disulfonic acid (DIDS) (45).

The cation specificity of NaDC-1 is also similar to that of rabbit renal brush border membrane vesicles. Sodium activation curves with NaDC-1 are sigmoidal, with apparent Hill coefficients of 1.6–2.9 (41, 48), indicating that multiple sodium ions are involved in the transport of succinate. The half-saturation constant for sodium in NaDC-1 is approximately 50 mM (41). The transport of

succinate is dependent on the presence of sodium, and replacement of sodium by cations such as choline results in the reduction of activity to background levels (41). Replacement of sodium by lithium, however, results in a low rate of transport, approximately 5% of that seen in sodium (46). NaDC-1 is also sensitive to inhibition by lithium. In the presence of saturating concentrations of sodium, millimolar concentrations of lithium inhibit transport by NaDC-1, with an apparent K_i of 1.8 mM (45), similar to the K_i reported in renal membranes (72).

The mRNA coding for NaDC-1 is found in kidney cortex and small intestine (41), a distribution similar to that of low-affinity Na^+ /dicarboxylate cotransport activity. However, after long exposure of Northern blots probed at high stringency with NaDC-1 cDNA, hybridization signals also appear in liver, lung, and adrenal, suggesting either low abundance of NaDC-1 or related messages in those organs (41). More recently, antibodies have been prepared against a fusion protein consisting of 60 amino acids from NaDC-1 and glutathione-*S*-transferase (44). The antibodies were originally raised in chickens (44) but were also raised in rabbits (46) because of background problems with the chicken antibodies. NaDC-1 protein is found in kidney and intestine brush border membrane vesicles (44). The antibodies recognized a glycosylated protein of approximately 63 kDa in rabbit renal brush border membranes and two proteins of 57 and 115 kDa in rabbit intestinal brush border membranes (44). Recent studies have verified that chronic metabolic acidosis in rats leads to an increase in NaDC-1 protein, up to 5.6-fold higher than controls (1). Chronic metabolic acidosis also resulted in an increased amount of NaDC-1 message in the kidney, up to 3.5-fold, which was correlated with the decrease in serum HCO_3^- (1).

The cDNA coding for the human ortholog of NaDC-1, called hNaDC-1, was isolated by hybridization with the rabbit NaDC-1 cDNA (42). The amino acid sequence of hNaDC-1 is 78% identical to NaDC-1 and 47% identical to NaSi-1 (42). Functionally, hNaDC-1 also appears to correspond to a low-affinity transporter, with a K_m for succinate of about 0.4 mM (42). In general, the functional properties of NaDC-1 and hNaDC-1 are similar: Both transporters have a relatively low affinity for succinate and glutarate, both are sodium dependent, and both exhibit similar responses to pH (45). However, the two transporters have some interesting differences. The K_m for citrate is about eightfold higher in hNaDC-1 (7 mM) than in NaDC-1 (0.9 mM) (45). Humans appear to have a higher fractional excretion of citrate in the urine—between 10 and 35% of the filtered load—compared with species, such as dog or rat, that excrete about 2–7% of the filtered load (59). It is possible that the differences in citrate K_m could contribute to the species differences in citrate handling.

The human Na^+ /dicarboxylate cotransporter, hNaDC-1, appears to have a low cation affinity, with an apparent K_{Na} of around 80 mM compared with 50 mM for NaDC-1 (45). The Hill coefficient is around 2.1, again consistent

with multiple sodium ions being coupled to the movement of substrate. Human patients receiving therapeutic doses of lithium exhibit increased excretion of glutarate and α -ketoglutarate, indicating that the Na^+ /dicarboxylate cotransporter in human kidney is sensitive to inhibition by lithium, similar to the rabbit transporter (6). In contrast, hNaDC-1 expressed in oocytes is insensitive to inhibition by lithium, with an apparent K_i of 10 mM (45). The reason for the apparent difference is not known. It is possible that either multiple isoforms of hNaDC-1 exist in the apical membrane of kidney proximal tubule, or the conditions used in the oocyte experiments, such as membrane potential or cation concentrations, affect lithium sensitivity.

The mRNA coding for hNaDC-1 was found in kidney and small intestine, suggesting that the same protein is expressed in both organs (42). In addition, the gene coding for hNaDC-1 was found on chromosome 17 using mouse-hamster somatic hybrid cell lines, but it has not been localized further (42).

The cDNA coding for the rat renal Na^+ /dicarboxylate cotransporter, rNaDC-1, was recently isolated by hybridization screening (55). The amino acid sequence of rNaDC-1 is 73% identical to that of NaDC-1 (Table 2). The rat transporter is sodium dependent and sensitive to inhibition by lithium, and it carries a variety of di- and tricarboxylic acids in divalent anion form (12a, 54a). The rNaDC-1 protein is found on the apical membranes of proximal tubules, predominantly from S2 and S3 segments (54a). The cDNA coding for rNaDC-1 was independently cloned by a second group and given the name SDCT1 (12a). In situ hybridization experiments identified rNaDC-1 (SDCT1) mRNA in the kidney, small intestine, lung, liver, and epididymis (12a).

A cDNA related to NaDC-1, called RI-19, was isolated from a rat intestinal cDNA library (27). The carboxy terminus of NaDC-1 contains approximately 60 amino acids with about 80% sequence identity with rat intestinal mucin (41). Khatri and colleagues used homology screening with mucin cDNA to isolate the RI-19 cDNA from rat intestine (27). RI-19 has not been functionally

Table 2 Properties of cloned Na^+ /dicarboxylate and Na^+ /sulfate cotransporters

| Name | Source | % identity with NaDC-1 | K_m succinate | Lithium inhibition | Electrogenic | Genbank number | Reference |
|---------|--------------------------|------------------------|-----------------|--------------------|------------------|----------------|------------|
| NaDC-1 | Rabbit kidney | 100 | 0.5 mM | Yes | Yes | U12186 | 41, 45 |
| hNaDC-1 | Human kidney | 78 | 0.8 mM | No | Yes ¹ | U26029 | 42, 45 |
| rNaDC-1 | Rat kidney | 73 | n.d. | n.d. | n.d. | AB001321 | 55 |
| RI-19 | Rat intestine | 60 | n.d. | n.d. | n.d. | U51153 | 27 |
| NaDC-2 | <i>Xenopus</i> intestine | 62 | 0.3 mM | No | Yes ¹ | U87318 | 2 |
| NaSi-1 | Rat kidney, intestine | 43 | — | No | Yes | L19102 | 11, 34, 39 |

¹AM Pajor, unpublished observations.

characterized. The predicted amino acid sequence of RI-19 is 60% identical in sequence to NaDC-1 (27). However, there appear to be several frame shifts in the sequence, and by adding or deleting a total of five nucleotides, the sequence identity with NaDC-1 rises to 73% (AM Pajor, unpublished observations). This suggests either that RI-19 represents a nonfunctional pseudogene or that the published sequence contains errors. At the nucleotide level, RI-19 is 98.5% identical to rNaDC-1.

A cDNA called NaDC-2, coding for the intestinal Na^+ /dicarboxylate cotransporter from the African clawed frog, *Xenopus laevis*, was isolated by functional expression in *Xenopus* oocytes (2). The amino acid sequence of NaDC-2 is approximately 63% identical to the sequence of NaDC-1. NaDC-2 message was found only in intestine and not in any other organ such as kidney and liver (2). When expressed in *Xenopus* oocytes, NaDC-2 transports succinate, citrate, and glutarate, and transport was not inhibited by dimethylsuccinate or sulfate (2). NaDC-2 exhibits an unusual cation selectivity compared with NaDC-1. Replacement of sodium by lithium results in little or no decrease in transport rate in NaDC-2 (2). The K_m for succinate measured in sodium is approximately 0.3 mM and the K_m for succinate measured in lithium is 0.7 mM. The apparent sodium and lithium affinities are the same (K_{Na} or $K_{\text{Li}} \sim 45$ mM), and the apparent Hill coefficients are around 1.3 in either sodium or lithium.

Relatively little information exists about the high-affinity Na^+ /dicarboxylate cotransporters. A high-affinity Na^+ /dicarboxylate cotransporter was expressed in *Xenopus* oocytes microinjected with a 2–3 kb size fraction of mRNA from rat kidney (60). The high-affinity Na^+ /dicarboxylate cotransporter NaDC-3 has recently been isolated from rat placenta (V Ganapathy, personal communication). NaDC-3 is 42% identical in amino acid sequence to NaDC-1, and it exhibits properties of a high-affinity transporter, including a K_m for succinate of $2 \mu\text{M}$ and interaction with dimethylsuccinate. The tissue distribution of NaDC-3 mRNA in placenta, kidney, liver, and brain also resembles the distribution of high-affinity Na^+ /dicarboxylate transport.

Genbank and other databases have a number of sequences related to the NaDC-1 gene family. The function of these proteins from organisms such as *Caenorhabditis elegans* and yeast have not been determined. However, a distinct family of transporters in bacteria, called the 2-hydroxy-carboxylate carriers, includes sodium- and proton-coupled dicarboxylate transporters (65). The members of this family are unrelated in either primary sequence or predicted secondary structure to NaDC-1 or NaSi-1. Despite the lack of sequence similarity, the Na^+ /citrate cotransporter of *Klebsiella pneumoniae*, called CitS, exhibits interesting functional parallels with NaDC-1. CitS also appears to carry citrate as a divalent anion, and lithium can substitute for sodium but with much reduced affinity (33). The coupling stoichiometry is thought to be $2 \text{Na}^+ : 1 \text{H}^+ : 1 \text{citrate}^{2-}$, and therefore transport by CitS should be electrogenic (33).

Structure of NaDC-1-Related Family Members

Relatively little information is available regarding the structure of transporters related to NaDC-1, but members of the same gene family will likely have strong similarities in their structures. Initial hydrophobicity analysis of NaDC-1 using the Kyte-Doolittle hydropathy scale resulted in a predicted secondary structure containing 8 transmembrane domains (34, 41). This model has since been revised (Figure 1) after reanalysis using the Rao-Argos buried helix parameter scale, which predicts that NaDC-1 contains 11–12 hydrophobic regions long enough to span the membrane as α -helices (44).

The sequence of NaDC-1 contains two consensus sites for N-glycosylation, at Asn-160, in a large polar region, and Asn-578 near the carboxy terminus (44). However, mutagenesis experiments showed that Asn-578 is the site that is glycosylated, which places the carboxy terminus of NaDC-1 on the outside of the cell (44) (Figure 1). Interestingly, all members of the family related to NaDC-1 contain one or two consensus sequences for N-glycosylation located close to the carboxy terminus. Some of the family members, including NaSi-1

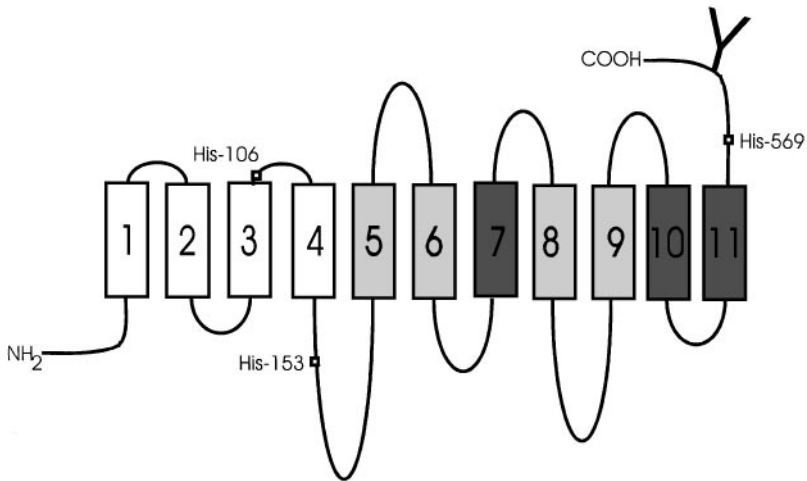


Figure 1 Secondary structure model of NaDC-1. The secondary structure of NaDC-1 contains 11 putative transmembrane domains, shown by the *numbered rectangles*. The amino terminus is on the cytoplasmic side of the membrane, and the carboxy terminus is on the outside of the cell. **Y** designates the N-glycosylation site at Asn-576. The *shaded* transmembrane domains represent regions of the protein important in substrate binding. Transmembrane domains 5–11 contain the substrate recognition domain (46). The *darker shaded* transmembrane domains, 7, 10, and 11 contain residues that determine affinity for citrate (26). Transmembrane domains 8 and 9 contain acidic residues that may interact with sodium (20). The histidines at positions 153 and 569 bind diethylpyrocarbonate, which inhibits transport; histidine-106 is involved in protein stability or trafficking (47).

and rNaDC-1, contain additional N-glycosylation sequences around amino acids 140–250. The human hNaDC-1 has both of its N-glycosylation sites at the carboxy terminus, and the size increase after in vitro translation experiments suggests that both sites are glycosylated (42). Aside from the extracellular carboxy terminus, the rest of the secondary structure model in NaDC-1 and the rest of the family remain to be tested in detail.

The role of N-glycosylation in NaDC-1 appears to be in protein trafficking or stability, which also seems to be the case in other transport proteins (44). A mutant of NaDC-1 lacking N-glycosylation sites, called GM2, exhibited a decrease in transport activity of about 50% (44), which was paralleled by a 65% reduction in the abundance of GM2 protein at the plasma membrane (46).

Structure-Function Studies of Na⁺/Dicarboxylate Cotransporters

Structure-function studies of membrane transport proteins are somewhat limited by the lack of good structural information about integral membrane proteins. Two general approaches have been taken in studies of structure-function relationships in transporters: site-directed mutagenesis and chimera formation. In the absence of structural information that might lead to hypotheses about locations of binding sites, we have used site-directed mutagenesis based on previous studies with amino acid selective reagents and suggestions of amino acids that might be involved in coordinating cation binding. In addition, the differences in function between the members of the NaDC-1 family have been exploited by formation of chimeric transporters.

Previous studies using the histidine-selective reagent diethylpyrocarbonate (DEPC) suggested that a histidine might be found at or near the substrate binding site in the rabbit renal low-affinity Na⁺/dicarboxylate cotransporter (4). In order to test this hypothesis, each of the 11 histidines in NaDC-1 was mutated to alanine, and the mutant transporters were expressed in *Xenopus* oocytes (47). Replacement of 10 of the 11 histidines by alanine had no effect on the function of NaDC-1. However, replacement of His-106 by alanine resulted in a 98% decrease in transport activity. The H106A mutant had no change in the K_m for succinate but a large decrease in V_{max} . The abundance of H106A protein at the plasma membrane was correspondingly reduced, although there was very little decrease in the total amount of H106A protein in the oocyte. Therefore histidine does not appear to be required for transport in NaDC-1, although His-106 is likely to be involved in trafficking or stability. The DEPC binding sites in NaDC-1 were identified as histidines 153 and 569, and the inhibition of transport by DEPC binding at these sites probably occurs by steric hindrance (47).

A second experimental approach, the formation of chimeric transporters, was used to identify the substrate recognition domains in the Na⁺/dicarboxylate and Na⁺/sulfate cotransporters (46). The amino acid sequence of NaDC-1 is 43%

identical to that of the Na⁺/sulfate cotransporter, NaSi-1 (34). The two transporters have very distinct substrate specificities. NaDC-1 does not transport sulfate, and succinate transport is not inhibited by sulfate. Similarly, NaSi-1 does not interact with succinate. The technique of homologous recombination (9) was used to prepare chimeras between NaDC-1 and NaSi-1. Only one functional chimera resulted, probably because of the difference in sequence between the two transporters. Chimera SiDC-4 contained the first four transmembrane domains from NaSi-1 and the last seven transmembrane domains from NaDC-1. The substrate specificity of SiDC-4 was identical to that of the parent NaDC-1, suggesting that the substrate recognition domain is found in the carboxy terminal two-thirds of the protein (Figure 1). Residues affecting substrate affinity and inhibition by furosemide and flufenamate were found in the amino terminal portion of the protein. Finally, it appears that residues involved in cation binding may be found in both the amino- and carboxy-terminal portions of the transporters (34).

A similar chimera formation strategy has been taken to identify the domains that determine the differences in substrate affinity between the human and rabbit NaDC-1. The rabbit NaDC-1 has a K_m for citrate of 0.9 mM, whereas the human hNaDC-1 has a K_m of about 7 mM (42). Chimeras formed between NaDC-1 and hNaDC-1 confirmed that the carboxy-terminal half of the protein, transmembrane domains 7–11, contains residues that determine the differences in citrate affinity (26) (Figure 1). The differences in citrate affinity appear to be the result of interactions between residues in three transmembrane domains, 7, 10, and 11. A chimera with substitutions of all three transmembrane domains (7, 10, and 11) had the same K_m for citrate as the donor of the three transmembrane domains, whereas substitution of one or two of these transmembrane domains resulted in only a partial change in affinity (26).

In an effort to identify individual residues involved in substrate or cation binding, conserved anionic amino acids in NaDC-1 were mutated to alanines (20). Of the amino acids mutated, only Asp-373 and Glu-475 appear to be required for transport function. Asp-373 is in transmembrane domain 8, and Glu-475 is in transmembrane domain 9 (Figure 1). Replacement of these amino acids resulted in changes in cation selectivity and affinity, as well as substrate affinity. The results suggest that these acidic residues contribute to the structure of the cation binding sites or participate in the coordination of cations (20).

Electrophysiological Analysis of NaDC-1 Function

Electrophysiological methods have been used to study a number of cloned electrogenic transporters expressed in *Xenopus* oocytes or cultured cells. The approach was first used to study currents generated by the Na⁺/glucose cotransporter, SGLT1 (50, 51). The use of electrophysiological measurements provides some advantages over measurements of radiolabeled uptakes. It is

possible to make rapid measurements of transport activity, and the ability to control membrane potential provides more precise information about the kinetics of transport, which is particularly important when interpreting the effects of mutations on function.

NaDC-1 is an electrogenic transporter, with a transport cycle that moves one net positive charge across the membrane. In voltage-clamped oocytes expressing NaDC-1, superfusion with substrates such as succinate, citrate or methylsuccinate resulted in inward currents as large as $3 \mu\text{A}$ (43). The production of currents in the presence of tricarboxylate substrates supports the idea that divalent anions are the preferred substrates of NaDC-1. At -50 mV , the $K_{0.5}^{\text{succinate}}$ was $180 \mu\text{M}$ and the $K_{0.5}^{\text{Na}^+}$ was 19 mM . The Hill coefficient for sodium was 2.3, which is consistent with the predicted transport stoichiometry of $3\text{Na}^+:1$ divalent anion substrate. Although Na^+ is the preferred cation, Li^+ was also able to support transport, although much less effectively (the $K_{0.5}^{\text{succinate}}$ in Li^+ was 3 mM). However, in the presence of sodium, lithium binds with high affinity to one of the cation binding sites, resulting in transport inhibition (43).

Electrophysiological characterization of the rat ortholog, rNaDC-1 (SDCT1), has revealed some interesting differences with NaDC-1. Both NaDC-1 and rNaDC-1 (SDCT1) exhibit voltage-dependent steps in translocation but voltage independence of substrate binding (43, 12a). Unlike NaDC-1, however, rNaDC-1 (SDCT1) does not exhibit inward currents in the presence of lithium, although lithium is a potent inhibitor of transport (12a). Under voltage-clamp conditions, the $K_{0.5}$ for succinate in rNaDC-1 (SDCT1) is around $25 \mu\text{M}$ (12a, 54a) compared with $180 \mu\text{M}$ for NaDC-1, which verifies previous suggestions that the rat transporter has an intermediate substrate affinity. The stoichiometry of transport in rNaDC-1 (SDCT1) is three sodium ions for each succinate or citrate molecule transported, determined from the simultaneous measurement of currents and radiotracer uptakes under voltage-clamp conditions (12a, 54a).

The renal Na^+ /sulfate transporter, NaSi-1, is also electrogenic. Earlier radiotracer transport studies with NaSi-1 suggested that transport was electroneutral, based on an apparent Hill coefficient for sodium of about 1.8 (34). However, the presence of substrates such as sulfate, thiosulfate, and selenate induced inward currents in oocytes expressing NaSi-1 (11). Under voltage-clamp conditions, the Hill coefficient for sodium is 2.8, which indicates a coupling stoichiometry in NaSi-1 similar to that of NaDC-1: $3 \text{ Na}^+:1 \text{ sulfate}^{2-}$, resulting in the net movement of one positive charge across the membrane (11). In preliminary studies, we have also observed substrate-induced currents in oocytes expressing hNaDC-1 and NaDC-2 (AM Pajor, unpublished observations), which have Hill coefficients for sodium of 2.1 (hNaDC-1) and 1.3 (NaDC-2). The results suggest that the coupling stoichiometry in hNaDC-1 and NaDC-2 is also likely to be $3 \text{ Na}^+:1 \text{ succinate}^{2-}$. The differences in Hill coefficients could be due to the

effects of membrane potential on transport in nonvoltage-clamped oocytes, or there may be differences in the extent of cooperativity between cation binding sites in these transporters.

CONCLUSIONS AND FUTURE DIRECTIONS

The characterization of sodium-coupled dicarboxylate transporters in isolated cells and membrane vesicles has identified transport pathways with functional differences such as substrate affinity, substrate specificity, and sensitivity to inhibition by lithium. The functional studies suggest that a number of different isoforms of Na⁺/dicarboxylate cotransporters are likely to be found in a given organism, and there may be species differences in transporters as well. The recent cloning of cDNAs coding for Na⁺/dicarboxylate cotransporters shows that a family of related proteins exists, with differences in kinetics and other functional properties. Cloning of additional members of the family will allow the identification and functional characterization of the different transporter isoforms and the determination of their tissue distribution, and will also provide a more complete picture of the handling of Krebs cycle intermediates. Although there is some information on regions of the proteins that may be involved in specific functions, a well-tested model of protein structure of the NaDC-1-related family is needed. The use of mutants and chimeric transporters, in the context of a good model of protein structure, should help to identify substrate and cation binding sites and will provide information on the mechanism of transport. There is also relatively little information on the regulation of the transporters; increased availability of such information would be greatly facilitated by the development of a cell culture model. The combination of molecular and functional approaches will provide powerful tools in future studies of the NaDC-1-related family of transporters.

ACKNOWLEDGMENTS

Thanks to Drs. Stephen Wright and Bruce Hirayama for discussions. The author's work in this area is supported by National Institutes of Health grants DK46269 and DK02429.

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