Functional Reconstitution of SdcS, a Na⁺-Coupled Dicarboxylate Carrier Protein from *Staphylococcus aureus*[▽]

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In *Staphylococcus aureus*, the transport of dicarboxylates is mediated in part by the Na⁺-linked carrier protein SdcS. This transporter is a member of the divalent-anion/Na⁺ symporter (DASS) family, a group that includes the mammalian Na⁺/dicarboxylate cotransporters NaDC1 and NaDC3. In earlier work, we cloned and expressed SdcS in *Escherichia coli* and found it to have transport properties similar to those of its eukaryotic counterparts (J. A. Hall and A. M. Pajor, J. Bacteriol. 187:5189–5194, 2005). Here, we report the partial purification and subsequent reconstitution of functional SdcS into liposomes. These proteoliposomes exhibited succinate counterflow activity, as well as Na⁺ electrochemical-gradient-driven transport. Examination of substrate specificity indicated that the minimal requirement necessary for transport was a four-carbon terminal dicarboxylate backbone and that productive substrate-transporter interaction was sensitive to substitutions at the substrate C-2 and C-3 positions. Further analysis established that SdcS facilitates an electroneutral symport reaction having a 2:1 cation/dicarboxylate ratio. This study represents the first characterization of a reconstituted Na⁺-coupled DASS family member, thus providing an effective method to evaluate functional, as well as structural, aspects of DASS transporters in a system free of the complexities and constraints associated with native membrane environments.

The divalent-anion/Na⁺ symporter (DASS) family is an evolutionarily related collection of secondary active-transport systems having representation in all three kingdoms of life (23, 28, 30, 33). Members of this group (also referred to as the SLC13 gene family in the human gene nomenclature) couple the movement of Na⁺ down its electrochemical gradient to the accumulation of a variety of dicarboxylates and inorganic anions. In higher organisms, such action provides a means to regulate the extracellular concentrations of these substrates, and in certain instances, transporter malfunction can lead to a variety of disorders that include growth retardation, life span alteration, and the development of kidney stones (9, 14, 20, 23, 28, 30, 36). However, despite their physiological importance, a detailed understanding of the DASS family members has been hampered by the inability to analyze their mechanistic and structural properties in a system not restricted by the complexities of their native environments.

The reconstitution of purified transport proteins has served as a valuable technique in the elucidation of their structures and functions (1, 7, 35, 41). We previously cloned and functionally expressed SdcS, a Na⁺-coupled dicarboxylate transporter from *Staphylococcus aureus*, and found it to exhibit many of the traits characteristic of mammalian DASS transporters (12). In work described here, we have partially purified and reconstituted this carrier protein into proteoliposomes in order to examine its transport properties in a cell-free system. Our analysis of reconstituted SdcS not only confirmed its

whole-cell transport features, but also expanded upon the substrate and energy requirements of the carrier protein. Taken together, these findings indicate that SdcS, when in the presence of an inward-directed Na⁺ electrochemical gradient, facilitates the electroneutral symport of two cations and a single dicarboxylate.

MATERIALS AND METHODS

Strains and plasmids. Strain BL21 [F[−] ompT hsdS_B(r_B[−] m_B[−]) gal dcm] (Novagen) served as host for the expression vector pQE-80L/SdcS (12). This plasmid encodes SdcS with an N-terminal MRGS(H)₆GS amino acid extension and places SdcS expression under the control of a T5 promoter/lac operator element.

Purification and reconstitution. Histidine-tagged SdcS was purified by nickel-nitrilotriacetic acid metal affinity chromatography. All steps were performed at 4°C unless otherwise stated. Overnight cultures were diluted 200-fold into 400 ml LB broth containing 100 µg/ml ampicillin and grown at 37°C to an optical density at 660 nm of 1.0 to 1.2. To induce SdcS expression, isopropyl-β-D-galactopyranoside was added to 0.5 mM, and cells were harvested after 2 h. The cells were then washed twice with 20 ml 100 mM KP_i, pH 7, and resuspended in the same buffer containing 1 mM phenylmethylsulfonyl fluoride and 20 µg/ml DNase I. The cells were disrupted by two passages through a cold French pressure cell (16,000 lb/in²), and after removal of unbroken cells and cell debris by two low-speed centrifugations (5,000 × g; 10 min), the membrane vesicles were pelleted (150,000 × g; 1 h) and resuspended in 8 ml buffer A (90 mM KP_i, 10 mM NaP_i, 20% glycerol, 5 mM β-mercaptoethanol, 0.2% *Escherichia coli* phospholipid, 25 mM succinate, 1.25% n-dodecyl-β-maltoside, 1 mM phenylmethylsulfonyl fluoride, 200 mM NaCl, pH 8). After incubation for 1 h on ice, insoluble materials were removed by centrifugation (150,000 × g; 1 h), and the supernatant was added to a nickel-nitrilotriacetic acid-agarose slurry (QIAGEN) preequilibrated in buffer A. This mixture was incubated for 2 h with gentle shaking, after which the resin was loaded onto a Poly-Prep column (Bio-Rad) and washed six times with 2 ml buffer A adjusted to pH 7 and containing 0.25% *E. coli* phospholipid and 75 mM imidazole. Histidine-tagged SdcS was then eluted from the resin by adding 400 µl of buffer A, pH 7, containing 0.25% *E. coli* phospholipid and 250 mM imidazole instead of 200 mM NaCl. The purified material was stored at −80°C until use.

Purified SdcS was reconstituted into proteoliposomes by detergent dilution. Briefly, in a final volume of 370 µl (or multiple thereof), 1 µg of protein and 82 µl bath-sonicated *E. coli* phospholipid (45 mg/ml) were mixed for 20 min on ice in a buffer, pH 7, containing 100 mM KP_i, 1 mM dithiothreitol, and 1.5%

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FIG. 1. Purification of SdcS. The protein compositions of fractions obtained during the purification of SdcS were determined by SDS-PAGE. Lane 1, membrane vesicles (25 μ g protein); lane 2, crude detergent extract (25 μ g protein); lane 3, Ni^{2+} -agarose flowthrough fraction (25 μ g protein); lane 4, Ni^{2+} -agarose first wash fraction (25 μ g protein); lane 5, Ni^{2+} -agarose 250 mM imidazole eluted SdcS purified fraction (2.5 μ g protein); lane 6, Western blot of purified SdcS fraction (2.5 μ g protein) probed with antibody reactive to the SdcS N-terminal histidine tag. The positions of molecular mass standards are indicated.

octyl- β -D-glucoside. Proteoliposomes were formed by 40-fold dilution into a chilled P_i -based loading buffer and isolated by centrifugation ($150,000 \times g$; 1 h) at 4°C . After removal of the supernatant, the proteoliposome pellet was resuspended in a chilled P_i -based resuspension buffer and stored on ice prior to being assayed. The loading and resuspension buffers varied, and their compositions are described in the individual experiments.

Transport assays. SdcS-mediated transport activity was assayed by diluting reconstituted proteoliposomes 25- to 50-fold into appropriate assay buffers equilibrated at room temperature and containing, unless otherwise stated, 100 μM labeled succinate. At the indicated times, aliquots were removed for filtration on Millipore filters (0.22- μm pore size; type GSTF), rinsed twice with 5 ml chilled wash buffer (100 mM KPi , pH 7, or 100 mM KPi , 250 mM KCl, pH 7), and counted by liquid scintillation using Econo-Safe (Research Products International Corp.) as a scintillant.

Apparent kinetic constants (K_m and maximum rate of transport [$V_{\max}$]) for SdcS-mediated succinate transport were determined by fitting initial transport rates (1 min) to the Michaelis-Menten equation $\{v = (V_{\max}[S])/(K_m + [S])\}$, where $[S]$ is substrate concentration, using nonlinear regression analysis. The apparent half-saturation constant ($K_{0.5}$) values for Na^+ and Li^+ were estimated by fitting initial cation-dependent succinate transport rates (1 min) to the Hill equation ($v = (V_{\max}[S]^n)/(K_{0.5}^n + [S]^n)$, where n represents the Hill coefficient) using nonlinear regression analysis.

SDS-PAGE and immunoblot analysis. Proteins, resuspended in sample buffer and loaded without being preheated, were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (11%) (21) and then either visualized by GelCode Blue Stain Reagent (Pierce) staining or transferred to nitrocellulose for immunoblot analysis. The latter procedure was carried out using a mouse monoclonal antibody reactive to the SdcS N-terminal RGS(H) $_4$ epitope (QIAGEN). To evaluate expression, Western blots were developed by the chemiluminescence method (SuperSignal West Pico Chemiluminescent Substrate; Pierce) using a horseradish peroxidase-conjugated anti-mouse immunoglobulin G antibody (Jackson ImmunoResearch Laboratories, Inc.).

Protein determination. The protein contents of membrane vesicles and detergent extracts were assayed using the method of Brown et al. (4). A modification of the method of Schaffner and Weissmann (37) was used to determine the amounts of protein incorporated into proteoliposomes.

Chemicals. [^{14}C]succinate (44 mCi/mmol; >99% pure) was from Moravsek Biochemicals. *E. coli* phospholipid was obtained from Avanti Polar Lipids, Inc. (Alabaster, AL), and both *n*-dodecyl- β -maltoside and octyl- β -D-glucoside were from Calbiochem.

RESULTS

Purification and reconstitution of functional SdcS. In previous work, we cloned SdcS, an Na^+ -coupled dicarboxylate carrier protein from *S. aureus*, and functionally characterized its function in an *E. coli* whole-cell system (12). To confirm and

FIG. 2. Succinate counterflow in proteoliposome vesicles. Purified SdcS was reconstituted into proteoliposomes loaded with either 100 mM KPi , pH 7 (○); 100 mM KPi , 20 mM succinate, pH 7 (●); 50 mM KPi , 50 mM NaPi , pH 7 (□); or 50 mM KPi , 50 mM NaPi , 20 mM succinate, pH 7 (■). After collection, the proteoliposomes were resuspended in 100 mM KPi , pH 7 (circles), or 50 mM KPi , 50 mM NaPi , pH 7 (squares), and then diluted 25-fold in the same buffer containing 100 μM [^{14}C]succinate. Transport was assayed as described in Materials and Methods. The data are from three independent trials and are shown as means $\pm$ standard errors.

extend these findings, as well as to circumvent the complexities associated with evaluating SdcS-mediated transport in whole cells, we sought to purify and reconstitute this protein. To do this, histidine-tagged SdcS was solubilized and subsequently subjected to Ni^{2+} -agarose affinity chromatography. This procedure removed >99% of the original membrane protein and resulted in a purified protein fraction estimated to be 75 to 85% SdcS (Fig. 1).

Partially purified SdcS was reconstituted into proteoliposomes, and transport function was assayed by following succinate entrance counterflow activity. The counterflow phenomenon arises when the exit of radiolabeled substrate transported into proteoliposomes is competitively inhibited by high concentrations of preloaded nonradioactive substrate, resulting in the accumulation of radiolabeled substrate (40, 42). Since the conformational changes of a substrate-loaded carrier are often faster than those of an unloaded carrier, preloading with unlabeled substrate in many cases also results in an increased rate of reorientation from the internal to the external membrane face and thus stimulation of radiolabeled-substrate uptake. As illustrated in Fig. 2, preloaded SdcS-containing proteoliposomes accumulated succinate to levels 10-fold (Na^+ present) to 20-fold (Na^+ absent) above equilibrium. In the absence of such preloading, transport activity was low, consistent with the equilibration of radiolabeled substrate across the proteoliposome membrane. That succinate accumulation occurred in the absence of Na^+ suggested to us that SdcS either catalyzes cation-independent counterflow or utilizes H^+ as a cosubstrate. To test the latter possibility, the rate of succinate transport was assayed in the presence of an inward-directed H^+ electrochemical gradient (internal, pH 7; external, pH 6). Such a condition resulted in a transport rate comparable to that reported for the diffusion of succinate across a proteinless phospholipid membrane, where a similar pH difference was imposed (data not shown) (15, 17). While this finding does not rule out a role for H^+ in SdcS-mediated transport, it does

FIG. 3. Ion gradient-driven succinate transport. Purified SdcS was reconstituted into proteoliposome vesicles loaded with 100 mM K^+ , pH 7, collected by centrifugation, and then resuspended in the same buffer. Proteoliposomes were then diluted 50-fold into 100 mM K^+ , pH 7 ($\blacklozenge$ and $\diamond$), or 100 mM Na^+ , pH 7 ($\blacksquare$ and $\square$), containing 100 μM [^{14}C]succinate and assayed for transport as described in Materials and Methods. Nigericin at 1 μM (open symbols) or an equal volume of ethanol vehicle (closed symbols) was added at the time of dilution. The values shown are means $\pm$ standard errors for three independent experiments.

imply, as discussed below, that this transporter can facilitate dicarboxylate exchange in the absence of a coupling cation.

Na^+ electrochemical-gradient-driven dicarboxylate transport. Having established that SdcS could be functionally reconstituted, our next set of experiments were designed to identify ionic forces that could drive dicarboxylate transport. Because both the rate and extent of succinate counterflow activity were enhanced in the presence of Na^+ (Fig. 2), SdcS transport activity was monitored under conditions that generated an inward-directed Na^+ electrochemical gradient ($\Delta p\text{Na}^+$). The dilution of proteoliposomes loaded with 100 mM K^+ , pH 7, into 100 mM Na^+ , pH 7, resulted in the accumulation of succinate (Fig. 3). In the absence of this gradient, no significant transport was observed. For instance, dilution of proteoliposomes loaded with 100 mM K^+ , pH 7, into the same medium did not stimulate SdcS-mediated succinate uptake. Likewise, the dissipation of $\Delta p\text{Na}^+$ by the addition of the ionophore nigericin, which catalyzes electroneutral exchange of both Na^+ and H^+ for K^+ , abolished SdcS-mediated succinate transport (Fig. 3). These observations are consistent with the idea that a $\Delta p\text{Na}^+$, and not simply the presence of Na^+ , is required to facilitate dicarboxylate uptake.

SdcS electrical character and transport stoichiometry. While functionally characterized mammalian members of the DASS family are electrogenic—transporting Na^+ and dicarboxylate with a ratio of 3:1—previous work with SdcS in whole cells suggested that this transporter coupled the uptake of a single divalent carboxylate to two Na^+ ions. To confirm this coupling stoichiometry, we examined the electrical characteristics of SdcS-catalyzed transport in the presence of a membrane potential ($\Delta\Psi$). Our first set of experiments sought to determine whether the presence of $\Delta\Psi$, in the absence of a $\Delta p\text{Na}^+$, was sufficient to drive dicarboxylate transport. Proteoliposomes, loaded with and then diluted into buffers suitable for the generation of a K^+ gradient, were assayed for succinate uptake in the presence and absence of valinomycin. The addition of this K^+ -conducting ionophore had no effect on succi-

FIG. 4. Electrical character of SdcS transport. The effect of a $\Delta\Psi$ of internally negative (A) or internally positive (B) polarity on SdcS-mediated succinate transport (100 μM) was assayed as described in Materials and Methods. (A) ($\bullet$ and $\circ$) Proteoliposomes loaded and resuspended in 50 mM K^+ , 50 mM *N*-methyl-D-glucamine (NMG)- P_i , pH 7, and then diluted (50-fold) into 50 mM Na^+ , 50 mM NMG- P_i , pH 7; ($\blacksquare$ and $\square$) proteoliposomes loaded and resuspended in 50 mM K^+ , 50 mM Na^+ , pH 7, and then diluted in 50 mM Na^+ , 50 mM NMG- P_i , pH 7. (B) ($\bullet$ and $\circ$) Proteoliposomes loaded and resuspended in 100 mM NMG- P_i , pH 7, and then diluted into 50 mM Na^+ , 50 mM K^+ , pH 7; ($\blacksquare$ and $\square$) proteoliposomes loaded and resuspended in 50 mM Na^+ , 50 mM NMG- P_i , pH 7, and then diluted in 50 mM Na^+ , 50 mM K^+ , pH 7. Valinomycin at 2.5 μM (open symbols) or an equal volume of ethanol vehicle (closed circles) was added at the time of dilution. The data are reported as means $\pm$ standard errors for three independent experiments.

nate transport, irrespective of whether an internally negative ($\text{K}^+_{\text{in}} > \text{K}^+_{\text{out}}$) (Fig. 4A) or positive (Fig. 4B) ($\text{K}^+_{\text{out}} > \text{K}^+_{\text{in}}$) $\Delta\Psi$ was established (K^+_{in} and K^+_{out} are K^+ concentrations inside and outside the proteoliposomes, respectively). These findings indicate that $\Delta\Psi$ alone is unable to facilitate SdcS-mediated substrate uptake and suggest that the transport reaction is electrically neutral.

We extended our study of the electrical properties of SdcS by analyzing the effect that $\Delta\Psi$ had on succinate uptake in the presence of a $\Delta p\text{Na}^+$. Proteoliposomes were assayed as described above, with the exception that the buffering system was designed so that an inward-directed $\Delta p\text{Na}^+$ was generated. Valinomycin was then used to establish a $\Delta\Psi$ of internally negative (Fig. 4A) or internally positive (Fig. 4B) polarity. In the first experiment, the imposition of a $\Delta\Psi$ (inside negative) caused an initial acceleration in succinate transport relative to proteoliposomes not treated with valinomycin (Fig. 4A). This stimulatory effect was not maintained over time—an observation one would not predict for an electrogenic transporter tested under these conditions but rather for a carrier protein mediating electroneutral transport. In support of this conclusion is the finding that the establishment of an internally positive $\Delta\Psi$ had no marked influence on the rate of succinate accumulation (Fig. 4B), a result consistent with an electroneutral symport reaction with a Na^+ /dicarboxylate ratio of 2:1. An attempt was made to confirm this transport stoichiometry by comparing [^{14}C]succinate and $^{22}\text{Na}^+$ rates of uptake. However, direct demonstration of dicarboxylate-dependent Na^+ transport was not possible due to the high background counts associated with $^{22}\text{Na}^+$ uptake in the absence of succinate (data not shown).

FIG. 5. Kinetics of SdcS-mediated transport. Initial rates (1 min) of succinate transport in proteoliposomes were determined as outlined in Materials and Methods. (A) Proteoliposomes were loaded and resuspended with 100 mM KPi , 250 mM KCl, pH 7. Concentrated stocks were then diluted 25-fold in the same buffer (background transport) or 100 mM KPi , 100 mM NaCl, 150 mM KCl, pH 7, containing [^{14}C]succinate. The concentration range of radiolabeled succinate used to estimate kinetic constants was 0.2 to 200 μM . (B) Proteoliposome vesicles were prepared as for panel A and then diluted 25-fold in assay buffer containing 100 mM KPi , 100 μM [^{14}C]succinate, pH 7, plus the indicated concentrations of NaCl. Chloride salt (NaCl plus KCl) isotonicity was maintained at 250 mM by the addition of the necessary amounts of KCl. The data are presented as a curve fit of the means $\pm$ standard errors of three independent experiments. Kinetic constants for SdcS under the above-mentioned conditions were determined for each of three independent trials and are reported in the text as means $\pm$ standard errors.

Transport kinetics. As shown in Fig. 3, the imposition of an inward-directed ΔpNa^+ is sufficient to stimulate the uptake of dicarboxylate into proteoliposomes. This condition was used to assay the kinetic parameters of the SdcS transport reaction. In the presence of saturating concentrations of Na^+ , the apparent affinity (K_m) of succinate was $12 \pm 1.9 \mu\text{M}$ —a value in good agreement with that obtained from studies with whole cells—and the V_{max} was $1.8 \pm 0.27 \mu\text{mol/mg protein/min}$ (Fig. 5A). The relationship between the cation concentration and SdcS transport activity was also examined. Na^+ promoted succinate uptake in a concentration-dependent manner, with $K_{0.5}$ and V_{max} values of $12 \pm 0.80 \text{ mM}$ and $2.2 \pm 0.20 \mu\text{mol/mg protein/min}$, respectively (Fig. 5B). Li^+ also stimulated succinate transport, having a $K_{0.5}$ at a minimum 10-fold greater than that found for Na^+ (data not shown). However, because succinate transport was not saturated at the highest Li^+ concentration tested—200 mM—we were unable to calculate a $K_{0.5}$ for this

FIG. 6. SdcS substrate specificity. Rates of succinate transport were measured in the presence of various test inhibitors. Proteoliposomes, loaded and resuspended with 100 mM KPi , 250 mM KCl, pH 7, were diluted 25-fold into a buffer containing 100 mM KPi , 100 mM NaCl, 150 mM KCl, 100 μM [^{14}C]succinate, and 2 mM test substrate. αKG , α -ketoglutarate; DMS, dimethylsuccinate. Succinate transport was assayed as described in Materials and Methods after a 5-min incubation. Data from three independent experiments were normalized to transport in the absence of any test inhibitor ($2.1 \pm 0.09 \mu\text{mol/mg protein}$) and are reported as means plus standard errors.

cation. Further analysis of Na^+ -dependent succinate transport, using data employed to produce the transport curve illustrated in Fig. 5B, generated Hill coefficients ranging from 1.1 to 1.2. These findings were taken to reflect the presence of two co-operative cation binding sites in the SdcS substrate binding pocket, an interpretation that reinforces our work with valinomycin (Fig. 4), which suggests an electroneutral coupling stoichiometry of two cations per dicarboxylate transported.

Substrate specificity of SdcS. DASS family members exhibit broad substrate specificity, facilitating the transport of a wide array of divalent carboxylates and inorganic oxyanions (23, 28, 30, 33). We showed previously that SdcS recognizes three dicarboxylates—fumarate, malate, and succinate—which are transported in their dianionic forms (12). Here, we undertook a more comprehensive study of SdcS substrate preference by assaying the abilities of various test substrates to inhibit succinate transport. This analysis confirmed fumarate, malate, and succinate as high-affinity substrates (Fig. 6), suggesting that a productive interaction of substrate with SdcS requires, at a minimum, a compound of four-carbon length with carboxylate moieties at its C-1 and C-4 positions. Deviations from this structural template, via either removal of one carboxylate or the lengthening/shortening of the carbon backbone between carboxylates, resulted in failure to inhibit succinate transport (Fig. 6). Furthermore, the introduction of certain functional groups at the substrate C-2 or C-3 position—hydroxyls at both sites (e.g., tartarate), a keto moiety at C-2 (e.g., oxaloacetate), and the presence of a methyl group(s) (e.g., methylated succinate derivatives)—had little or no inhibitory effect on SdcS-mediated succinate transport, indicating that SdcS is sensitive to substitutions at these two locations. While compounds having these alterations are likely poor SdcS substrates, in some instances (e.g., glutarate, adipate, and methyl- and dimethylsuccinates) they are recognized by other DASS transporters.

Such substrate preference differences within the DASS family are well documented (5, 8, 24, 25, 31) and imply that the binding pockets of these transporters have diverged sufficiently to accommodate compounds that deviate slightly from the substrate template structure.

DISCUSSION

Owing to their physiological significance, the best-characterized members of the DASS family are those from mammalian species. This group includes the Na^+ /dicarboxylate symporters NaDC1 and NaDC3 (8, 18, 29), as well as the Na^+ -coupled SO_4^{2-} transporters NaS1 and NaS2 (11, 22). The study of these eukaryotic transporters has allowed considerable advances to be made in our understanding of this group of proteins. However, information pertaining to the DASS family transport mechanism and structure remains limited. As a first step in addressing this problem, we previously identified SdcS, a bacterial member of this family that shares 33% and 37% sequence identity with human NaDC1 and NaDC3, respectively (12). SdcS expression and functional evaluation in *E. coli* whole cells indicated transport properties typical of its eukaryotic counterparts, suggesting that the protein could serve as a paradigm for DASS family analysis. In the present work, we have extended our study by reconstituting partially purified SdcS into proteoliposomes. Such a system has a number of advantages, including the absence of complexities/interferences associated with the native membrane environment, greatly reduced metabolic conversion of substrate, and control over the compositions of both the internal and external environments (1, 7, 35, 41). Together, these benefits allow a more accurate description of SdcS, and hence the DASS family, to be obtained.

Our proteoliposome analysis is largely consistent with data gathered from transport studies in whole cells (12); SdcS functions as an Na^+ -dependent transporter that facilitates the electroneutral symport of two cations for a single four-carbon dicarboxylate. Work in this reconstituted system does highlight aspects of the SdcS transport mechanism that were not identified and/or were subject to misinterpretation in intact *E. coli* cells. For example, while whole-cell studies implied cation-dependent transport, assays using proteoliposomes differentiate between the role of cations as activator and driving force. Whereas the presence of an inward-directed ΔpNa^+ stimulates SdcS transport activity, the establishment of equimolar Na^+ concentrations in the internal and external milieu—via either dissipation of a gradient (Fig. 3) or experimental design (Fig. 4)—does not stimulate the rate of dicarboxylate uptake. Also, unlike work done with intact cells, SdcS-containing proteoliposomes did not display cation-mediated inhibition of succinate transport at Na^+ concentrations over 10 mM (compare Fig. 5 to Fig. 5 in reference 12). This inhibitory behavior, rather than being a property unique to SdcS, has been found for a number of Na^+ -dependent cotransporters (6, 13, 32, 34, 38, 39) and appears to be a complication resulting from the preincubation of whole cells with buffer with a high Na^+ content. Such an assay condition can produce a “*trans* effect” in which the intracellular accumulation of Na^+ stalls transport via dissipation of the Na^+ motive force. The failure to expel Na^+ in a timely fashion is likely due to inadequate expression and/or

poor activity of NhaA and NhaB, the two Na^+/H^+ antiporters of *E. coli* tasked with maintaining the inward-directed ΔpNa^+ across the cytoplasmic membrane (26).

The finding that SdcS may catalyze facilitated diffusion of dicarboxylate in the absence of a cationic cosubstrate was unexpected (Fig. 2). Mammalian members of the DASS family couple substrate transport to the movement of Na^+ down its electrochemical gradient (23, 28, 30, 33), and analysis of SdcS function in a whole-cell system indicated an ordered reaction sequence with Na^+ binding before dicarboxylate (12). Although we can offer no definitive reason why SdcS exhibits Na^+ -independent counterflow activity at the present time, we suggest that this behavior may result from a combination of the high external concentrations of succinate used in the counterflow assay and a less than optimal coupling between Na^+ and dicarboxylate. In cases (MelB, LacY, and LacS) where entrance counterflow has been carried out at saturating external substrate concentrations, sugar exchange proceeds without the dissociation/association of the coupling cation (2, 3, 10, 16). Furthermore, it has been shown that systems known to exhibit an ordered binding sequence— Na^+ or H^+ first—can associate, and possibly transport, the cosubstrate in the absence of cation if the second substrate is present at saturating concentration (27, 44). Such unordered binding/transport can also arise in the absence of high substrate concentrations if symport is not tightly coupled. For instance, mutations in LacY and MelB that alter the coupling of cosubstrate flows allow H^+ - and/or Na^+ -independent sugar translocation (19, 32, 43).

The work presented here represents the first characterization of a Na^+ -coupled DASS family member in a reconstituted system and provides a starting point at which to begin structural analysis of this family of transporters. In addition to exhibiting many of the functional properties characteristic of mammalian DASS transporters, transmembrane prediction algorithms suggest that SdcS and the human Na^+ /dicarboxylate cotransporters NaDC1 and NaDC3 have similar topologies (12). While the accuracy of this supposition still needs to be evaluated in detail, initial tests using PhoA and LacZ reporter fusions to map the topology of the N-terminal half of SdcS identify the same transmembrane segments predicted bioinformatically (J. A. Hall and A. M. Pajor, unpublished data). Continued topological study, together with the ability to overexpress, purify, and reconstitute functional SdcS, will allow significant gains to be made in the understanding of DASS transporter architecture.

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REFERENCES

1. Banerjee, R. K., and A. G. Datta. 1983. Proteoliposome as the model for the study of membrane-bound enzymes and transport proteins. *Mol. Cell Biochem.* **50**:3–15.
2. Bassilana, M., T. Pourcher, and G. Leblanc. 1987. Facilitated diffusion properties of melibiose permease in *Escherichia coli* membrane vesicles. Release of co-substrates is rate limiting for permease cycling. *J. Biol. Chem.* **262**:16865–16870.
3. Bassilana, M., T. Pourcher, and G. Leblanc. 1988. Melibiose permease of *Escherichia coli*. Characteristics of co-substrates release during facilitated diffusion reactions. *J. Biol. Chem.* **263**:9663–9667.
4. Brown, R. E., K. L. Jarvis, and K. J. Hyland. 1989. Protein measurement using bicinchoninic acid: elimination of interfering substances. *Anal. Biochem.* **180**:136–139.

5. Burckhardt, B. C., J. Lorenz, C. Kobbe, and G. Burckhardt. 2005. Substrate specificity of the human renal sodium dicarboxylate cotransporter, hNaDC-3, under voltage-clamp conditions. *Am. J. Physiol. Renal Physiol.* **288**:F792–F799.
6. Chen, C. C., T. Tsuchiya, Y. Yamane, J. M. Wood, and T. H. Wilson. 1985. Na^+ (Li^+)-proline cotransport in *Escherichia coli*. *J. Membr. Biol.* **84**:157–164.
7. Chen, C. C., and T. H. Wilson. 1986. Solubilization and functional reconstitution of the proline transport system of *Escherichia coli*. *J. Biol. Chem.* **261**:2599–2604.
8. Chen, X., H. Tsukaguchi, X. Z. Chen, U. V. Berger, and M. A. Hediger. 1999. Molecular and functional analysis of SDCT2, a novel rat sodium-dependent dicarboxylate transporter. *J. Clin. Invest.* **103**:1159–1168.
9. Dawson, P. A., L. Beck, and D. Markovich. 2003. Hyposulfatemia, growth retardation, reduced fertility, and seizures in mice lacking a functional NaSi-1 gene. *Proc. Natl. Acad. Sci. USA* **100**:13704–13709.
10. Foucaud, C., and B. Poolman. 1992. Lactose transport system of *Streptococcus thermophilus*. Functional reconstitution of the protein and characterization of the kinetic mechanism of transport. *J. Biol. Chem.* **267**:22087–22094.
11. Girard, J. P., E. S. Baekkevold, J. Feliu, P. Brandtzaeg, and F. Amalric. 1999. Molecular cloning and functional analysis of SUT-1, a sulfate transporter from human high endothelial venules. *Proc. Natl. Acad. Sci. USA* **96**:12772–12777.
12. Hall, J. A., and A. M. Pajor. 2005. Functional characterization of a Na^+ -coupled dicarboxylate carrier protein from *Staphylococcus aureus*. *J. Bacteriol.* **187**:5189–5194.
13. Hama, H., T. Shimamoto, M. Tsuda, and T. Tsuchiya. 1987. Properties of a Na^+ -coupled serine-threonine transport system in *Escherichia coli*. *Biochim. Biophys. Acta* **905**:231–239.
14. Inoue, K., Y. J. Fei, W. Huang, L. Zhuang, Z. Chen, and V. Ganapathy. 2002. Functional identity of *Drosophila melanogaster* Indy as a cation-independent, electroneutral transporter for tricarboxylic acid-cycle intermediates. *Biochem. J.* **367**:313–319.
15. Janausch, I. G., O. B. Kim, and G. Unden. 2001. DctA- and Dcu-independent transport of succinate in *Escherichia coli*: contribution of diffusion and of alternate carriers. *Arch. Microbiol.* **176**:224–230.
16. Kaczorowski, G. J., and H. R. Kaback. 1979. Mechanism of lactose translocation in membrane vesicles from *Escherichia coli*. 1. Effect of pH on efflux, exchange, and counterflow. *Biochemistry* **18**:3691–3697.
17. Kaim, G., and P. Dimroth. 1999. ATP synthesis by F-type ATP synthase is obligatorily dependent on the transmembrane voltage. *EMBO J.* **18**:4118–4127.
18. Kekuda, R., H. Wang, W. Huang, A. M. Pajor, F. H. Leibach, L. D. Devoe, P. D. Prasad, and V. Ganapathy. 1999. Primary structure and functional characteristics of a mammalian sodium-coupled high affinity dicarboxylate transporter. *J. Biol. Chem.* **274**:3422–3429.
19. King, S. C., and T. H. Wilson. 1990. Towards an understanding of the structural basis of 'forbidden' transport pathways in the *Escherichia coli* lactose carrier: mutations probing the energy barriers to uncoupled transport. *Mol. Microbiol.* **4**:1433–1438.
20. Knauf, F., B. Rogina, Z. Jiang, P. S. Aronson, and S. L. Helfand. 2002. Functional characterization and immunolocalization of the transporter encoded by the life-extending gene *Indy*. *Proc. Natl. Acad. Sci. USA* **99**:14315–14319.
21. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**:680–685.
22. Markovich, D., J. Forgo, G. Stange, J. Biber, and H. Murer. 1993. Expression cloning of rat renal $\text{Na}^+/\text{SO}_4^{2-}$ cotransport. *Proc. Natl. Acad. Sci. USA* **90**:8073–8077.
23. Markovich, D., and H. Murer. 2004. The SLC13 gene family of sodium sulphate/carboxylate cotransporters. *Pflügers Arch.* **447**:594–602.
24. Oshiro, N., S. C. King, and A. M. Pajor. 2006. Transmembrane helices 3 and 4 are involved in substrate recognition by the $\text{Na}^+/\text{dicarboxylate}$ cotransporter, NaDC1. *Biochemistry* **45**:2302–2310.
25. Oshiro, N., and A. M. Pajor. 2006. Ala-504 is a determinant of substrate binding affinity in the mouse $\text{Na}^+/\text{dicarboxylate}$ cotransporter. *Biochim. Biophys. Acta* **1758**:781–788.
26. Padan, E., and S. Schuldiner. 1994. Molecular physiology of the Na^+/H^+ antiporter in *Escherichia coli*. *J. Exp. Biol.* **196**:443–456.
27. Page, M. G. P., and I. C. West. 1982. Alternative-substrate inhibition and the kinetic mechanism of the β -galactoside/proton symport of *Escherichia coli*. *Biochem. J.* **204**:681–688.
28. Pajor, A. M. 2006. Molecular properties of the SLC13 family of dicarboxylate and sulfate transporters. *Pflügers Arch.* **451**:597–605.
29. Pajor, A. M. 1995. Sequence and functional characterization of a renal sodium/dicarboxylate cotransporter. *J. Biol. Chem.* **270**:5779–5785.
30. Pajor, A. M. 1999. Sodium-coupled transporters for Krebs cycle intermediates. *Annu. Rev. Physiol.* **61**:663–682.
31. Pajor, A. M., R. Gangula, and X. Yao. 2001. Cloning and functional characterization of a high-affinity $\text{Na}^+/\text{dicarboxylate}$ cotransporter from mouse brain. *Am. J. Physiol. Cell Physiol.* **280**:C1215–C1223.
32. Poolman, B., and W. N. Konings. 1993. Secondary solute transport in bacteria. *Biochim. Biophys. Acta* **1183**:5–39.
33. Prakash, S., G. Cooper, S. Singhi, and M. H. Saier, Jr. 2003. The ion transporter superfamily. *Biochim. Biophys. Acta* **1618**:79–92.
34. Quick, M., H. Yano, N. R. Goldberg, L. Duan, T. Beuming, L. Shi, H. Weinstein, and J. A. Javitch. 2006. State-dependent conformations of the translocation pathway in the tyrosine transporter Tytl, a novel neurotransmitter:sodium symporter from *Fusobacterium nucleatum*. *J. Biol. Chem.* **281**:26444–26454.
35. Rigaud, J. L., B. Pitard, and D. Levy. 1995. Reconstitution of membrane proteins into liposomes: application to energy-transducing membrane proteins. *Biochim. Biophys. Acta* **1231**:223–246.
36. Rogina, B., R. A. Reenan, S. P. Nilsen, and S. L. Helfand. 2000. Extended life-span conferred by cotransporter gene mutations in *Drosophila*. *Science* **290**:2137–2140.
37. Schaffner, W., and C. Weissmann. 1973. A rapid, sensitive, and specific method for the determination of protein in dilute solution. *Anal. Biochem.* **56**:502–514.
38. Speelmans, G., B. Poolman, and W. N. Konings. 1993. Amino acid transport in the thermophilic anaerobe *Clostridium fervidus* is driven by an electrochemical sodium gradient. *J. Bacteriol.* **175**:2060–2066.
39. Stewart, L. M. D., and I. R. Booth. 1983. Na^+ involvement in proline transport in *Escherichia coli*. *FEMS Microbiol. Lett.* **19**:161–164.
40. Tsuchiya, T., K. Ottina, Y. Moriyama, M. J. Newman, and T. H. Wilson. 1982. Solubilization and reconstitution of the melibiose carrier from a plasmid-carrying strain of *Escherichia coli*. *J. Biol. Chem.* **257**:5125–5128.
41. Varadhachary, A., and P. C. Maloney. 1990. A rapid method for reconstitution of bacterial membrane proteins. *Mol. Microbiol.* **4**:1407–1411.
42. Wong, P. T., and T. H. Wilson. 1970. Counterflow of galactosides in *Escherichia coli*. *Biochim. Biophys. Acta* **196**:336–350.
43. Zani, M. L., T. Pourcher, and G. Leblanc. 1993. Mutagenesis of acidic residues in putative membrane-spanning segments of the melibiose permease of *Escherichia coli*. II. Effect on cationic selectivity and coupling properties. *J. Biol. Chem.* **268**:3216–3221.
44. Zhou, A., A. Wozniak, K. Meyer-Lipp, M. Nietschke, H. Jung, and K. Fendler. 2004. Charge translocation during cosubstrate binding in the $\text{Na}^+/\text{proline}$ transporter of *E. coli*. *J. Mol. Biol.* **343**:931–942.