Functional Characterization of a Na\(^+\)-Coupled Dicarboxylate Carrier Protein from Staphylococcus aureus

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We have cloned and functionally characterized a Na\(^+\)-coupled dicarboxylate transporter, SdcS, from Staphylococcus aureus. This carrier protein is a member of the divalent anion/Na\(^+\) symporter (DASS) family and shares significant sequence homology with the mammalian Na\(^+\)/dicarboxylate cotransporters NaDC-1 and NaDC-3. Analysis of SdcS function indicates transport properties consistent with those of its eukaryotic counterparts. Thus, SdcS facilitates the transport of the dicarboxylates fumarate, malate, and succinate across the cytoplasmic membrane in a Na\(^+\)-dependent manner. Furthermore, kinetic work predicts an ordered reaction sequence with Na\(^+\) \((K_{\text{Na}} \approx 2.7 \text{ mM})\) binding before dicarboxylate \((K_{\text{S}} \approx 4.5 \mu\text{M}}\). Because this transporter and its mammalian homologs are functionally similar, we suggest that SdcS may serve as a useful model for DASS family structural analysis.

Na\(^+\)-coupled transport is a common method of substrate uptake in which the movement of Na\(^+\) down its electrochemical gradient facilitates the accumulation of small solutes within the cell (14, 32, 37, 39). Mammalian members of the divalent anion/Na\(^+\) symporter (DASS) family mediate the transport of dicarboxylates and inorganic anions across the cellular membrane in such a Na\(^+\)-dependent manner (24, 27, 28, 31). Functionally characterized proteins of this group include the Na\(^+\)/dicarboxylate cotransporters NaDC-1 and NaDC-3 (5, 16, 26), as well as the Na\(^+\)-coupled SO\(_4\) transporters NaSi-1 and SUT-1 (9, 23). While study of these proteins has led to a detailed understanding of their transport mechanism, information concerning their structure remains limited.

To address this problem, we sought to identify a bacterial homolog of these mammalian transporters that can serve as a model for their structural analysis. The DASS family (also referred to as the SLC13 gene family in the human gene nomenclature) is a collection of evolutionarily related transport proteins that spans all three kingdoms of life, with representatives from prokaryotes comprising the majority of its membership (31). However, to date no bacterial member of this family has been shown to be mechanistically similar to its mammalian counterparts. Here, we report the cloning and functional expression of SdcS, a Staphylococcus aureus DASS transporter that shares sequence similarity with a number of mammalian transporters Na\(^+\)/dicarboxylate cotransporters and its Na\(^+\)-coupled DASS family structural analysis.

**MATERIALS AND METHODS**

**Bacterial strains.** Escherichia coli strain XL1-Blue (recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F proAB lacZAM15 Tn10]) (Strategene) was used for all cloning steps. E. coli strain BL21 [FompT lacSgAPT+ mna+] gol dcm (Novagen) served as host for tests of expression and function of plasmid-encoded SdcS.

**Cloning.** The sdcS gene (GenBank accession number BA000017; locus SAV7196) was amplified from S. aureus genomic DNA (ATCC 700699D) by PCR and cloned into plasmid pQE-80L (bâlacF) (Qiagen) via restriction sites (BamHI and HindIII) created with the primers 5'-CGATGATCCATGGCTTATTCAATCAACATC-3' and 5'-CGCTAAGCTTACTATTTCAATGGCAGTTGGTTG-3'. This recombinant plasmid (pQE-80LsdcS) encodes SdcS with an N-terminal MRGS(H)\(_9\)GS amino acid extension and places SdcS expression under control of a T5 promoter/lac operon element. To construct an IPTG (isopropyl-\(\beta\)-d-thiogalactopyranoside)-inducible C-terminal MRGS(H)\(_9\)GS-tagged SdcS, a pair of primers (5'-CATGGATTTCAATCAACATC-3' and 5'-AGCTTATGGATTTCAATCAACATC-3') was amplified using the MRGS(H)\(_9\)GS encoding plasmid (pQE-80L). A BamHI- and HindIII-compatible restriction site (underlined) was added to each primer. The resulting plasmid was digested with NcoI and BamHI, ligated to the sdcS gene amplified as described above using the primers 5'-GGGAATTCTTACCCATCAATCAACATC3' and 5'-GGATGATTTCAATCAACATC3' and sequenced (Molecular Biology Core Facility, University of Texas Southwestern Medical Center, Dallas, TX). The resulting plasmid was transformed into Staphylococcus aureus strain BL21 housing plasmid pQE-80L.

**Transport assays.** Overnight cultures were diluted 200-fold into Luria-Bertani broth (containing 100 \(\mu\)g/ml ampicillin and 150 \(\mu\)M IPTG). Cells were grown at 37°C to a density of \(5 \times 10^8\) to \(1 \times 10^9\) cells/ml (approximately 3 h), harvested by centrifugation, and then washed twice and resuspended in assay buffer (50 mM MOPS [morpholinepropanesulfonic acid], 5 mM NaCl, 95 mM choline chloride, pH 7 [pH adjusted with Tris base]) at an optical density at 600 nm of 1.4, equivalent to about \(1 \times 10^9\) to \(2 \times 10^9\) cells/ml. After equilibration at room temperature, tests of substrate transport were initiated by adding a 1:20 volume of labeled substrate to a final concentration of 100 \(\mu\)M. At indicated times, aliquots were removed for a final concentration of 40-100 \(\mu\)M pore size, type HAWP, rinsed twice with 5 ml wash buffer (50 mM MOPS-Tris, 100 mM choline chloride, pH 7), and counted by liquid scintillation using Elico-Safe (Research Products International Corp.) as scintillant. Unless otherwise indicated, SdcS transport activity is reported after subtracting background uptake values from strain BL21 housing pQE-80L.
di- and tricarboxylates as well as inorganic oxyanions such as broad anionic substrate specificity, transporting a wide array of under aerobic growth conditions such as that used here (6, 13). With the assumption that Na\(^{+}/H\(^{+}\)) were from Moravek Biochemicals. Calculated from a replot of the apparent Km (V max/[S])/(Km + [S]), where n represents the Hill coefficient) using nonlinear regression analysis. True Km (for succinate) and Kd (for Na\(^{+}\)) constants were calculated from a replot of the apparent Km of succinate as a function of Na\(^{+}\), with the assumption that Na\(^{+}\) binding to the SdcS active site precedes dicarboxylate binding (35).

**Immunoblot analysis.** Cells prepared for transport were resuspended in sample buffer, loaded without preheating, and separated by sodium dodecyl sulfate-polyacrylamide (11%) gel electrophoresis (20). Protein was transferred to nitrocellulose and probed with a mouse monoclonal antibody reactive to the SdcS N- and C-terminal RGS(H)4 epitope tags (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 (Amersham Biosciences). Unlabeled substrates and \([\text{14C}]\)fumarate (6.3 mCi/mmol) were from Sigma Chemical Company, \([\text{35S}]\)Na\(_2\)SO\(_4\) (1,500 Ci/mmol) and \([\text{14C}]\)malate (52 mCi/mmol) were obtained from PerkinElmer Life Sciences and Amersham Biosciences, respectively. \([\text{14C}]\)citrate (55 mCi/mmol) and \([\text{14C}]\)succinate (44 mCi/mmol) were from Moravek Biochemicals.

**RESULTS**

Cloning and functional expression of SdcS. In the present study our objective was to identify and characterize a bacterial homolog of the mammalian members of the DASS family. To do this, a protein-versus-protein similarity search was performed with FASTA3 (default settings) from the European Bioinformatics Institute (http://www.ebi.ac.uk/fasta33/) using the low-affinity Na\(^{+}/\)dicarboxylate cotransporter from human kidney, NaDC-1, as query (25). This search resulted in the identification of the putative \(SAV1916\) gene from \(S.\) aureus (strain Mu50), whose protein product is approximately 35% identical in sequence to that of human NaDC-1.

The \(SAV1916\) gene, renamed \(sdcS\) (for sodium/dicarboxylate symporter), encodes a hypothetical protein of 520 residues having a molecular mass of 57.2 kDa. To test whether SdcS is functionally expressed, we cloned \(sdcS\) into plasmid pQE-80L and monitored its expression and transport activity in \(E.\) coli. As illustrated in Fig. 1A, N-terminal histidine-tagged SdcS is expressed only in IPTG-induced cells housing plasmid pQE-80L/SdcS, resulting in a protein band corresponding to 45 to 50 kDa. Placement of the histidine tag at the C terminus of SdcS identified a protein band of the same molecular mass, indicating that the fast migration of N-terminal histidine-tagged SdcS on sodium dodecyl sulfate-polyacrylamide gels was not due to premature termination of translation or proteolytic cleavage of the C-terminal region of this protein (data not shown). Rather, the increased mobility of SdcS is likely due to its hydrophobic nature which, as is frequently exhibited by membrane transport proteins, results in an apparent molecular mass lower than that predicted from its protein sequence (18, 34). Parallel experiments designed to evaluate transport activity demonstrated that only the SdcS-expressing strain rapidly accumulated succinate (Fig. 1B). Uptake in this strain was followed by a slow fall to the equilibrium level, an observation that we attribute to both the leakage and metabolism of internalized substrate. That succinate transport, albeit slow, also occurred in the absence of SdcS is likely due to the action of the endogenous \(E.\) coli dicarboxylate transporter DetA. This transporter catalyzes H\(^{+}/\)dicarboxylate cotransport and is expressed at a low level under aerobic growth conditions such as that used here (6, 13).

**Dicarboxylate specificity.** Members of the DASS family have broad anionic substrate specificity, transporting a wide array of di- and tricarboxylates as well as inorganic oxyanions such as sulfate. Analysis of SdcS substrate preference tested directly by using radiolabeled citrate, sulfate, fumarate, malate, and succinate indicated that only the latter three were transported appreciably (Fig. 2 and data not shown). Further study found that other diatomic carboxylates—aspartate, glutamate, glutarate, \(\alpha\)-ketoglutarate, and maleate—did not inhibit succinate transport when present at 1 mM, suggesting that these com-
pounds are, at best, poor SdcS substrates (data not shown). Ligands transported by SdcS exhibit similar kinetic profiles, with nearly equivalent maximal velocities, and $K_m$ values between 5 and 15 $\mu$M (Fig. 2; Table 1). To determine which charged form(s) of carboxylate SdcS was transported, we evaluated succinate uptake at external pH values from pH 5.0 to 7.5, a range that spans the $pK_a$ of this substrate ($pK_a = 5.6$). We noted considerable changes in both kinetic parameters ($K_m$ and $V_{max}$) over this pH range (Fig. 3). In particular, as assay pH fell below the $pK_a$ of succinate, the affinity of this substrate for SdcS decreased quickly, showing a nearly threefold drop from its value of 5.3 $\mu$M at pH 5.5 to near 15 $\mu$M at pH 5.0. This observation provides a strong indication that SdcS recognizes and transports only the divalent form of its natural substrates.

**Cation specificity.** Because members of the DASS family are $Na^+$-coupled transporters, the cation requirements and selectivity of SdcS were examined. We found that at low salt concentrations (5 mM) only $Na^+$ stimulated succinate transport (Fig. 4A). By contrast, at high concentrations of cation (100 mM) $Li^+$ promoted uptake of succinate whereas $Na^+$ had an inhibitory effect (Fig. 4B). We also determined what effect, if any, the addition of various salts had upon $Na^+$-dicarboxylate transport. Interestingly, the presence of either choline chloride, KCl, or $K_2SO_4$ in the assay medium increased $Na^+$-dependent succinate uptake roughly two- to threefold (Fig. 4C), indicating that high medium osmolarity may enhance SdcS transport activity. The inclusion of both $Na^+$ and $Li^+$ in the assay medium did not stimulate succinate transport but, instead, completely inhibited it. This result, coupled with the finding that these cations promote dicarboxylate uptake when present individually, leads us to suggest that all cation binding sites must be occupied by the same cationic species for transport to occur.

Our next tests addressed the relationship between cation concentration and SdcS transport activity. Initial work (Fig. 4) suggested that both $Na^+$ and $Li^+$ stimulated dicarboxylate uptake in a concentration-dependent manner. Further analysis confirmed this supposition. Thus, the $K_{0.5}$ for $Na^+$ was 1 to 2 mM, while that for $Li^+$ was 20-fold greater (Table 1). Also, the maximal SdcS dicarboxylate transport activity attained with $Li^+$ was only one-half that found with $Na^+$ (Fig. 5; Table 1). This lowered affinity for $Li^+$ and the reduced transport of dicarboxylates in its presence are characteristics of several vertebrate DASS family members (2, 29, 41). The sigmoidal nature of cation-dependent transport (Fig. 5) indicated cooper-

![FIG. 2. Kinetics of SdcS-mediated dicarboxylate transport. Initial rates of fumarate (○), malate (■), and succinate (●) transport were estimated as described in Materials and Methods. Data from three independent trials are shown as means ± standard errors. $K_m$ and $V_{max}$ values for SdcS under these conditions are shown in Table 1.](image)

**TABLE 1. Transport properties of SdcS**

<table>
<thead>
<tr>
<th>Substrate pair</th>
<th>$K_m$ (mM)</th>
<th>$V_{max}$ (nmol/mg protein/min)</th>
<th>$K_{0.5}$ (mM)</th>
<th>$V_{max}$ (nmol/mg protein/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$Na^+$ /fumarate</td>
<td>15 ± 0.54</td>
<td>16 ± 1.7</td>
<td>1.5 ± 0.02</td>
<td>11 ± 0.84</td>
</tr>
<tr>
<td>$Na^+$ /malate</td>
<td>8.1 ± 0.40</td>
<td>12 ± 1.0</td>
<td>1.2 ± 0.01</td>
<td>14 ± 0.93</td>
</tr>
<tr>
<td>$Na^+$ /succinate</td>
<td>6.6 ± 0.75</td>
<td>17 ± 2.0</td>
<td>1.0 ± 0.04</td>
<td>15 ± 3.1</td>
</tr>
<tr>
<td>$Li^+$ /succinate</td>
<td>ND*</td>
<td>ND</td>
<td>23 ± 0.60</td>
<td>7.2 ± 0.52</td>
</tr>
</tbody>
</table>

a Rates of transport were measured as described in Materials and Methods, and kinetic constants were determined by fitting initial transport rates to the Michaelis-Menten equation. $K_m$ and $V_{max}$ values are means ± standard errors for three independent experiments. The concentration range of dicarboxylate used to approximate kinetic constants was 1 to 200 $\mu$M; $Na^+$ concentration was fixed at 5 mM.

b As in footnote a, except that kinetic constants were determined by fitting transport rates to the Hill equation. $K_{0.5}$ and $V_{max}$ values are means ± standard errors for three independent experiments. The $Na^+ /Li^+$ concentration range used to approximate kinetic constants was 0.5 to 7.5 mM; dicarboxylate concentration was fixed at 100 $\mu$M. Isotonic conditions were maintained at 100 mM by supplementation with choline chloride.

c ND, not determined.
ative binding between SdcS cation binding sites, and Hill coefficients for various cation (Li⁺ and Na⁺)/dicarboxylate (fumarate, malate, and succinate) pairings ranged from 2.5 to 3.5. However, unlike other DASS transporters, SdcS exhibited strong cation-mediated inhibition, with Na⁺-dependent succinate transport having a $K_i$ for Na⁺ of 26 ± 3.4 mM (Fig. 5). The closeness of the $K_{0.5}$ and $K_i$ of Na⁺ prevents SdcS from attaining maximal transport activity ($V_{max}$) (8), resulting in an overestimation of the Hill coefficient. Thus, it is likely that two to three Na⁺ (or Li⁺) cations along with a single dicarboxylate must occupy the SdcS active site for transport to occur. Work with permeabilized whole cells indicated that valinomycin had no effect on SdcS transport activity, suggesting an electroneutral symport reaction with a Na⁺/dicarboxylate ratio of 2:1 (data not shown).

Transport kinetics of SdcS. The requirement of Na⁺ (or Li⁺) for transport function implies that SdcS operates as a symporter. As such, the true affinity of one substrate will be observed only when its cosubstrate is present at saturating concentrations. With this in mind, we generated a series of transport curves in which the concentration of one substrate was varied while the other was held constant (Fig. 6). This experiment not only documented the stimulatory and inhibitory properties of Na⁺ upon succinate transport (Fig. 6A) but indicated that the $K_{0.5}$ of Na⁺ for SdcS decreases as succinate concentration increases (Fig. 6B). Furthermore, at subinhibitory Na⁺ concentrations, the apparent affinity of succinate (100 μM decreasing to 6.1 μM at 7.5 mM Na⁺), but not the maximal velocity (17 to 20 nmol/mg protein/min), was variable. This finding was interpreted as reflecting an ordered bireactant system in which Na⁺ binds before succinate (35), and a replot of these data gave true $K_m$ values for Na⁺ and succinate of 2.7 ± 0.14 mM and 4.5 ± 0.20 μM, respectively (Fig. 6C). In agreement with work cited above, the finding that linearization of the replot was obtained only when the “Na⁺” term was raised to the second power reflects an apparent coupling of at least two Na⁺ ions for each succinate transported.

**DISCUSSION**

The primary function of proteins of the DASS family is the transport of divalent carboxylates and oxyanions. In multicellular organisms, these transporters provide a mechanism to regulate the extracellular concentration of their respective substrates, and in certain instances their dysfunction can lead to a
The presence of Na\(^+\) (or Li\(^+\)) is essential to the function of both SdcS and its mammalian homologs. However, our work highlights two differences in how these transporters couple the cation to dicarboxylate uptake. First, among these cotransporters, only with SdcS does Na\(^+\) have closely linked stimulatory and inhibitory activities (Fig. 5); other DASS family members have \(K_{\text{app}}\) activation values that range from 10 to 50 mM (5, 16, 23, 26) but show no dicarboxylate transport inhibition at Na\(^+\) concentrations below 100 mM. While we can offer no definitive answer for this unusual behavior at the present time, this finding does suggest that SdcS activity in \(S.\) aureus is tightly regulated by the extracellular Na\(^+\) concentration. Second, while functionally characterized NaDC-1 and NaDC-3 orthologs are electrogenic (24, 27, 28)—coupling three Na\(^+\) ions to the transport of a single divalent carboxylate—our preliminary work regarding the electrical character of SdcS indicates that this transporter facilitates the electroneutral transport of Na\(^+\) and dicarboxylate with a stoichiometry of 2:1. Such differences in coupling stoichiometry are not unusual in functionally (and evolutionarily) related transporters. For instance, the human Na\(^+\)/glucose transporter (hSGLT1) and the \(E.\) coli Na\(^+\)/proline transporter (PutP), both members of the Na\(^+\)/substrate symporter family (15, 40), catalyze uptake with a Na\(^+\):substrate ratio of 2:1 and 1:1, respectively (4, 38). In spite of this and other dissimilarities, these two proteins, as well as other Na\(^+\)/substrate symporter family members, share a common structural theme (15, 40).

Structural information gleaned from the study of bacterial transporters has often been applied to the analysis of their eukaryotic homologs. For instance, the solved structures of the glycerol 3-phosphate antiporter (GlpT) and lactose permease (LacY) of \(E.\) coli and the oxalate/formate exchange protein (OxIT) from \(Oxalobacter\ formigenes\) provide a framework for the architecture of eukaryotic members of the major facilitator superfamily (1, 10, 11). Similarly, structure and function stud-
ies of eukaryotic ATP-binding cassette transporters have been guided by the known structures of the MshA lipid and BtuCD vitamin B_{12} transporters of \textit{E. coli} (3, 22). Within the DASS family, to our knowledge only two prokaryotic proteins have been cloned and functionally characterized, SdcS and the \textit{E. coli} citrate carrier CitT (30). Of these two proteins, only SdcS has a transport mechanism similar to that of its mammalian counterparts; whereas CitT catalyzes dicarboxylate exchange. SdcS and its eukaryotic homologs function as Na\textsuperscript{+}-dependent cotransporters. Comparison of the SdcS sequence with those of mammalian DASS transporters such as human NaDC-1 and human NaDC-3 showed that these proteins share significant amino acid identity (~35\% for human NaDC-1; ~33\% for human NaDC-3), with conserved residues distributed across their entire lengths. This similarity extends to the structural level where, using membrane topology prediction algorithms, we have recently found that in many cases putative SdcS transmembrane segments are predicted to be present in regions where conserved residues are clustered. Taken together, these findings suggest that SdcS can serve as a bacterial paradigm for the functional and structural properties of its eukaryotic counterparts.

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**REFERENCES**


