Characterization of the *Vibrio parahaemolyticus* Na⁺/Glucose Cotransporter

A BACTERIAL MEMBER OF THE SODIUM/GLUCOSE TRANSPORTER (SGLT) FAMILY*

Zhiyi Xie, Eric Turk, and Ernest M. Wright‡

From the Department of Physiology, UCLA School of Medicine, Los Angeles, California 90095-1751

The *Vibrio parahaemolyticus* sodium/glucose transporter (vSGLT) is a bacterial member of the SGLT gene family. Wild-type and mutant vSGLT proteins were expressed in *Escherichia coli*, and transport activity was measured in intact cells and plasma membrane vesicles. Two cysteine-less vSGLT proteins exhibited sugar transport rates comparable with that of the wild-type protein. Six residues in two regions of vSGLT known to be of functional importance in SGLT1 were replaced individually with cysteine in the cysteine-less protein. Characterization of these single cysteine-substituted vSGLT proteins showed that two residues (Gly-151 and Gln-428) are essential for transport function, whereas the other four residues (Leu-147, Leu-149, Ala-423, and Gln-425) are not. 2-Aminoethylmethanethiosulfonate (MTSEA) blocked Na⁺/glucose transport by only the transporter bearing a cysteine at position 425 (Q425C). MTSEA inhibition was reversed by dithiotothreitol and blocked by the presence of both Na⁺ and D-glucose, indicating that conformational changes of the vSGLT protein are involved in Na⁺/glucose transport. A split version of vSGLT was generated by co-expression of the N-terminal (N7) and C-terminal (C7) halves of the transporter. The split vSGLT maintained Na⁺-dependent glucose transport activity. Chemical cross-linking of split vSGLT, with a cysteine in each N7 and C7 fragment, suggested that hydrophilic loops between helices 4 and 5 and between helices 10 and 11 are within 8 Å of each other. We conclude that the mechanism of Na⁺/glucose transport by vSGLT is similar to mammalian SGLTs and that further studies on vSGLT will provide novel insight to the structure and function of this class of cotransporters.

Sodium cotransporters belong to a superfamily of membrane proteins responsible for the uphill transport of substrates coupled to the downhill transport of Na⁺ (1). The intestinal brush border Na⁺/glucose cotransporter (SGLT1) functions in the absorption of dietary glucose and galactose as well as salt and water (2). SGLT1 was the first member of a large gene family to be cloned (3), and currently there are more than 55 members of this family in bacteria, yeast, invertebrates, and vertebrates.

A bacterial member of the SGLT1 family is the Na⁺/glucose symporter (SgIS) of *Vibrio parahaemolyticus* (4, 5). The amino acid sequence of the protein has 31% identity and 75% similarity with the human SGLT1 (5). Owing to its close functional and evolutionary relationships with mammalian SGLT1, we will refer to the *Vibrio SgIS* gene product as vSGLT. There is evidence to infer that members of the Na⁺/glucose cotransporter family share a similar transport mechanism (6–9). A secondary structure model of vSGLT has been proposed based on sequence comparisons with other family members whose secondary structure models have been supported by experimental analysis (3, 10–12). In this model (Fig. 1), vSGLT is composed of 14 transmembrane spans, with the hydrophilic N terminus located in the periplasmic space. The ultimate C-terminal hydrophobic domain forms the 14th transmembrane span with the C terminus at the periplasmic surface of the membrane.

Although our knowledge of SGLT1 function has improved in the last decade, elucidation of Na⁺/glucose cotransporter structure has been slow, in part due to the lack of purified functional protein. Some progress has been made with site-directed mutagenesis, but many mutant proteins are not targeted properly to the plasma membrane in eukaryotic expression systems (13–17). Bacteria, in contrast, directly integrate membrane proteins into the plasma membrane. Furthermore, techniques have been developed to isolate purified functional bacterial membrane proteins from *Escherichia coli* and to study their structure. For example, the tertiary structure of the lactose permease, the H⁺/lactose cotransporter, has been determined by indirect methods (18). We therefore anticipate that studies of a bacterial Na⁺/glucose cotransporter should be quite fruitful.

As a first step in the characterization of structure and function of vSGLT and its comparison with SGLT1, we have analyzed the transport activity of vSGLT expressed in *E. coli*. Taking advantage of the fact that native vSGLT contains only one cysteine residue (Cys-411), we have also generated and analyzed two cysteine-less, six single cysteine-substituted, and nine double cysteine-substituted vSGLT proteins to assess the feasibility of studying vSGLT structural/functional relations by cysteine-scanning mutagenesis (19). In addition, inhibition of transport by 2-aminoethylmethanethiosulfonate (MTSEA) has provided evidence for substrate-induced conformational transitions of vSGLT. Finally, we have expressed functional vSGLT in two fragments (N7 and C7) and estimated the distance between two cysteine-substituted residues by cross-linking. These results demonstrate that site-directed thiol cross-linking of split vSGLT can be used to determine the helical packing of the protein.
EXPERIMENTAL PROCEDURES

Growth of Cells, Membrane Vesicle, and Cell Lyase Preparation—E. coli strain JM1100 (ptsG ptsM ptsF mg1 galP) (20) was generously provided by Dr. P. J. F. Henderson (University of Leeds, Leeds, UK). JM1100 cells transformed with described plasmids were grown at 37 °C in modified Tanaoka medium (21) (Na+ salts were replaced with K+ salts) supplemented with 1% tryptone, 100 μg/ml thymine, 90 μg/ml L-histidine, and 20 μg/ml ampicillin. Cells were harvested at A600 - 0.9–1.2 for sugar and Na+ transport assays and for preparation of crude cell lysates and membrane vesicles.

Right-side-out membrane vesicles were prepared (22, 23). Crude cell lysates were prepared as described by concentrating E. coli cells 10-fold by centrifugation and suspension in 20 mM Tris-HCl (pH 7.5), 5 mM EDTA. Lysozyme (to 50 μg/ml) was then added, and the cell suspension was incubated at room temperature sonicated before fractionation.

Sugar Transport Assays—[3H]-[14C]glucose, [3H]-[14C]galactose, and methyl-[3H]-[14C]glucopyranose were purchased from Amersham Pharmacia Biotech. Assay buffers for sugar transport into intact cells consisted of 0.2 mM MOPS-Tris (pH 7.5), 10 mM MgSO4, 20 mM Na-lactate acid, 0.1 mM sucrose, 0.1 mM [3H]glucose (200 μCi/ml), and 15 mM NaCl or 15 mM choline chloride as indicated. Cells were washed three times in a choline chloride assay buffer without sugar and suspended in the same buffer to about 1–2 mg of cell protein/ml. The transport assay was initiated by mixing 50 μl of assay buffer and 10 μl of cell suspension at 22 °C, stopped after 1 min by adding 3 ml of ice-cold buffer containing 0.4 mM potassium phosphate (KP, pH 7.4) and 10 mM MgSO4, and filtered rapidly through a nitrocellulose membrane (0.45 μm). The filters were washed twice with the same buffer, and radioactivity trapped on the filters quantitated by liquid scintillation counting.

Right-side-out membrane vesicles for glucose transport assays were suspended to 5 mg/ml in a buffer containing 100 mM KP, (pH 7.4) and 10 mM MgSO4 Assay buffers contained 10 mM MgSO4, 0.1 mM glucose, 0.02 mM [3H]glucose (200 μCi/ml), 0–50 mM NaCl and adjusted with 100–50 mM KP, (pH 7.4) to a final (Na+ plus K+) concentration of 100 mM. The assay procedure was the same as that for the sugar transport assay except that the buffer containing 100 mM KP, (pH 7.4) and 10 mM MgSO4 was used as wash buffer.

Na+ Transport Assay—Cells were washed twice with a buffer consisting of 0.2 mM MOPS-tetramethylammonium hydroxide (TMMA) (pH 7.5) and 10 mM MgSO4, and suspended in the same buffer to 20–30 mg of cell protein/ml. Transport of Na+ was measured using a Na+–selective electrode (Microelectodes, Inc, Bedford, NH) in a 1-ml glass vessel continually purged with nitrogen (24). Fifty microliters of cell suspension was mixed in the vessel with 450 μl of anaerobic sugar solution (100 mM KPi (pH 7.4) and 10 mM MgSO4, and suspended in the same buffer to about 1–2 mg of cell protein/ml. The transport assay was initiated by mixing 50 μl of assay buffer and 10 μl of cell suspension at 22 °C, stopped after 1 min by adding 3 ml of ice-cold buffer containing 0.4 mM potassium phosphate (KP, pH 7.4) and 10 mM MgSO4, and filtered rapidly through a nitrocellulose membrane (0.45 μm). The filters were washed twice with the same buffer, and radioactivity trapped on the filters quantitated by liquid scintillation counting.

Concentration of Plasmids Expressing N- and C-terminal Seven Transmembrane Helices (N, and C) of vSGLT—Plasmid pVibA was constructed by ligation of three fragments: 1) the promoter region (HindIII-Ndel fragment), generated from the plasmid pYAT271A by PCR using primer Vib-M1 (which introduces a Ndel site at the first Met codon) and an upstream primer (HindIII-Ndel fragment), obtained from the plasmid pB2VN; and 2) the pACYC vector (KpnI-HindIII fragment), which was derived from the plasmid pC6 (generously provided by Dr. H. R. Kaback) (25). Plasmid pVibA was generated by swapping a 1-kb fragment from pVibA into the pYAT271A. pVibA and pVibB contained a full-length vSGLT gene under its endogenous promoter and an Ndel site at the first Met codon. The difference between these two constructs is that pVibA is in a pACYC vector (chloramphenicol-resistant), whereas pVibB is in a pBR322 vector (ampicillin-resistant). E. coli JM1100 cells harboring pVibA and pVibB showed Na+/glucose transport activity indistinguishable from cells harboring pYAT271A.

Construction of Plasmids Expressing N- and C-terminal Seven Transmembrane Helices (N, and C) of vSGLT—Plasmid pVibA was constructed by ligation of three fragments: 1) the promoter region (HindIII-Ndel fragment), generated from the plasmid pYAT271A by PCR using primer Vib-M1 (which introduces a Ndel site at the first Met codon) and an upstream primer (HindIII-Ndel fragment), obtained from the plasmid pB2VN; and 2) the pACYC vector (KpnI-HindIII fragment), which was derived from the plasmid pC6 (generously provided by Dr. H. R. Kaback) (25). Plasmid pVibA was generated by swapping a 1-kb fragment from pVibA into the pYAT271A. Thus, both pVibA and pVibB contain a full-length vSGLT gene under its endogenous promoter and an Ndel site at the first Met codon. The difference between these two constructs is that pVibA is in a pACYC vector (chloramphenicol-resistant), whereas pVibB is in a pBR322 vector (ampicillin-resistant). E. coli JM1100 cells harboring pVibA and pVibB showed Na+/glucose transport activity indistinguishable from cells harboring pYAT271A.

An Ndel-HindIII fragment containing the DNA sequence for the N-terminal seven-transmembrane helices (N, amino acids 1–279) was generated by PCR using the primer Vib-SV2, which introduces a stop codon and a HindIII site after the residue Ser-279, and an upstream non-mutagenic primer. This fragment (residues Met-1–Ser-279) was ligated into the Ndel-HindIII fragment containing the DNA sequence for the C-terminal seven-transmembrane helices (C, amino acids 280–549) was generated by PCR using the primer Vib-SV1, which introduces a Met site before the residue Val-280, and a downstream non-mutagenic primer. This fragment (residues Met–Val-280–Trp-543) was then introduced into the plasmid pVibA to replace the Ndel-HindIII (residues Met-1–Ser-525) to generate the plasmid pC7. An Ndel-HindIII fragment containing the DNA sequence for the C-terminal seven-transmembrane helices (C, amino acids 280–549) was generated by PCR using the primer Vib-SV1, which introduces a Met site before the residue Val-280, and a downstream non-mutagenic primer. This fragment (residues Met–Val-280–Trp-543) was then introduced into the plasmid pVibA to replace the Ndel-HindIII (residues Met-1–Ser-525) to generate the plasmid pC7. DNA fragments in these plasmids that came from PCR reactions were sequenced to confirm that there was no other mutation introduced. Plasmids carrying the cysteine-less (pC7C111) and the single cysteine mutant (pN7L147C, pN7L149C, pN7G151C, pC7A423C, pC7Q425C, pC7Q428C) were generated by DNA fragment swapping.

Expression of Split vSGLT and Disulfide Cross-linking—E. coli strain JM1100 was transformed with both pN7 and pC7, each encoding a vSGLT fragment, with or without a given cysteine mutation. Cultures were grown at 37 °C in the same medium described above with addition of chloramphenicol (34 μg/ml). Cells were harvested by centrifugation, washed once in 50 mM KP, (pH 7.5), once in a buffer containing 20 mM Tris-HCl (pH 7.5), 5 mM EDTA, and 5 mM dithiothreitol, and suspended in the same buffer. Lysozyme was added to a final concentration of 50 μg/ml, and the suspension was incubated at 22 °C for 30 min. Crude cell membranes were prepared by sonication followed by low speed centrifugation (1400 × g × 2 min) to remove intact cells and an ultracentrifugation (350,000 g × 15 min) to collect membranes. Membranes were washed and resuspended in 20 mM Tris-HCl (pH 7.0) for use. Cross-linking was carried out by adding 0.5 mM bismaleimidohexane (BMH), 1,4-bismaleimidobutane (BMB), or bismaleimidoethane (BMOE) (Pierce) or iodine to membrane preparations and incubated at 22 °C for 2 h. Reactions with BMH, BMB, and BMOE were terminated by adding 5 mM dithiothreitol. Reactions with iodine were terminated by adding 10 mM N-ethylmaleimide. Samples were then subjected to Western blot analysis.

Western Blot Analysis—An aliquot of crude cell lysate or membrane preparation was subjected to an 8% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. vSGLT was detected using a polyclonal antibody (#8792 (26)) raised to a peptide fragment (STLFTMIDYTKIRKKASEK) of rabbit SGLT1. There is a close match of eight amino acid residues between vSGLT (STLFTMDY) used to raise the antibody and vSGLT (TIFTMDY) in this region residues in the C1 fragment of the split vSGLT protein. Goat-anti-rabbit IgG peroxidase conjugate (Calbiochem) was used as the secondary antibody. Immunoblots were developed by SuperSignal chemiluminescence (Pierce).

Protein Determination—Protein concentration was determined by using the BCA protein assay (Pierce) with bovine serum albumin as a standard.

Labeling by 2-Aminomethylmethanethiosulfonate—The MTSEA was purchased from Toronto Research Biochemicals (Downview, Ontario, Canada) and prepared at 1–100 mM in H2O immediately before use.

The initial rates of 95 μM sugar uptake were measured in cells incubated in buffers with and without 12.5 mM NaCl. Na⁺-dependent 1-min uptake experiments are presented as the mean of triplicate estimates of uptakes (± S.E.) in a single experiment and are given in nmol/min/mg of cell protein. Na⁺ uptake into cells was measured using a Na⁺-selective electrode. The cells were incubated in buffer containing 25 μM NaCl and are reported as the initial rate of Na⁺ uptake after the addition of sugar (final concentration 5 mM). Each experiment was repeated on at least three separate batches of cells transfected with either plasmid pYAT271A carrying the gene for the vSGLT transporter or with the empty vector. No Na⁺/sugar cotransport was observed in the control experiments. These experiments confirm the results obtained earlier by Tsuchiya and co-workers (4, 5). See "Experimental Procedures" for further experimental details.

<table>
<thead>
<tr>
<th>Sugar transport</th>
<th>Na⁺ transport</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol/min/mg protein</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>56 ± 2</td>
</tr>
<tr>
<td>D-Galactose</td>
<td>25 ± 1</td>
</tr>
<tr>
<td>α-MDG</td>
<td>&lt;0.4</td>
</tr>
<tr>
<td>α-MDG</td>
<td>1.7</td>
</tr>
<tr>
<td>n-Fucose</td>
<td>6.5</td>
</tr>
<tr>
<td>D-Fucose</td>
<td>2.6</td>
</tr>
<tr>
<td>D-Galactose</td>
<td>25 ± 1</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>56 ± 2</td>
</tr>
<tr>
<td>n-Fucose</td>
<td>1.7</td>
</tr>
</tbody>
</table>

Cells or vesicles were treated with MTSEA and then washed twice with wash buffer before glucose transport assay. For assays including diithiothreitol, vesicles were washed twice with wash buffer between each treatment and before suspending for transport assays.

RESULTS

Substrate Specificity of vSGLT—To determine the sugar specificity of Na⁺/glucose cotransporter of V. parahaemolyticus, plasmid pYAT271A (5), carrying the sgIS gene with its endogenous promoter, was introduced into E. coli strain JM100, which is deficient in glucose and galactose transport systems (20). Sugar transport assays were carried out in buffers containing 12.5 mM Na⁺ and 95 μM glucose, galactose, or α-MDG. Na⁺-dependent sugar transport was the difference between sugar transport in Na⁺- and in choline buffers. JM1100 cells harboring the empty vector pBR322 showed a very low endogenous total Na⁺-glucose transport (1.0 ± 0.1 nmol/min/mg of protein). However, JM1100 cells harboring the vSGLT expressing construct, pYAT271A (Table I), showed a high rate of Na⁺-dependent Na⁺-glucose transport (56 nmol/min/mg of protein). The initial Na⁺-glucose transport rate in Na⁺ ranging from 20 to 80 nmol/min/mg of protein in seven independent experiments. D-Galactose was also transported, but α-MDG was not.

Sugar-induced Na⁺ transport into JM1100 cells expressing vSGLT was measured using a Na⁺-selective electrode. D-Glucose, D-galactose, and D-fucose all induced Na⁺ uptake, but α-MDG did not (Table I). No D-glucose- or D-galactose-induced Na⁺ transport was detected in JM1100 cells harboring empty vector pBR322.

Phlorizin, rhamnatin, and deoxyrhapontin are potent inhibitors of mammalian SGLT1 (27). Glucose transport by vSGLT was inhibited 27% by 1 mM phlorizin and 14% by 1 mM rhamnatin, but there was no inhibition by 0.1 mM deoxyrhapontin.

Cysteine-less vSGLT Is Functionally Similar to the Wild-type Protein—The endogenous cysteine (Cys-411) in the 10th transmembrane helix (Fig. 1) was removed to make the C-less protein. Since most of the SGLT family members contain an isoleucine or a valine residue at this position (3), these two amino acids were chosen to replace Cys-411. As shown in Table II, the Na⁺/glucose transport activities of C-less vSGLT proteins are similar to that of the wild-type protein. C411I, which showed higher glucose transport activity than C411V, was chosen for further analysis.

Characterization of Cysteine-substituted vSGLT—The connecting loop between transmembrane helices 4 and 5 is thought to be involved in the Na⁺ binding and voltage-sensing properties of rabbit SGLT1 (28, 29). Previous work has also shown that residue Gln-457, in the putative sugar translocation domain of human SGLT1, is involved in the conformational changes that are responsible for the coupling of Na⁺ and sugar transport (30). To elucidate the function of the homologous regions of vSGLT in Na⁺/glucose cotransport by chemical modification, several residues were independently mutated to cysteines. Cysteine residues at L147C, L149C, and G151C reside in the connecting loop of helices 4 and 5, and cysteine residues A423C, Q425C, and Q428C reside in the analogous vicinity of SGLT1 Q457. All cysteine-substituted vSGLTs were made on the C-less (C411I) background, and their transport capabilities are compared in Table II. Two single cysteine-substituted vSGLTs, L149C and A423C, retained Na⁺/glucose transport activity comparable with that of C-less vSGLT, whereas activities of L147C and Q428C were about 50% and 25% that of the C-less vSGLT. G151C and Q428C were inactive, suggesting that these two residues play important roles in vSGLT function. Glucose-induced Na⁺ transport activities of single cysteine-substituted vSGLT were also determined. Patterns of Na⁺ transport similar to that of glucose transport were observed for all cysteine-substituted vSGLT.

To determine whether the loss of transport activity shown by some cysteine-substituted vSGLT was due to deficient protein accumulation, vSGLT was analyzed using Western blots. An antibody raised to a peptide fragment of rabbit SGLT1 was found to recognize vSGLT (see “Experimental Procedures”). The antibody recognized a 46-kDa band from crude cell lysates of cells expressing wild-type vSGLT (see Fig. 3A) but not from cells bearing the empty vector pBR322. The actual molecular mass of vSGLT is 59 kDa (31), but hydrophobic membrane proteins commonly run at a lower apparent molecular mass position in SDS-polyacrylamide gel electrophoresis (32). Although the Na⁺/glucose transport activity of cysteine-substituted vSGLTs varied dramatically, there were no significant differences in the amount of the protein in the plasma membrane. Thus, the substitution affected the vSGLT transport activity.

Glucose transport activities of intact cells carrying double cysteine substitutions were determined, and results are shown in Table II. The glucose transport activity of each double cysteine-substituted mutant was comparable with the synergistic activity of each corresponding single mutant. There was no increase of activity by pairing of two low activity single mutations (e.g. G151C and Q428C) or loss of activity by pairing of two high activity single mutations.

MTSEA Inhibition—Methanethiosulfonate (MTS) reagents (33) have been used successfully in studying SGLT1 Q457C to demonstrate that conformational changes accompany the coupling of Na⁺ and sugar transport (30). The MTS reagent MT-
SEA was applied to cells expressing single cysteine-substituted vSGLT protein to probe the involvement of specific residues and conformational changes in the transport process. Interestingly, MTSEA (1 mM) treatment of cells carrying the C-less vSGLT (Cys411I) for 1 min increased Na⁺/glucose transport activity slightly (28%, 60 ± 1 versus 47 ± 1 nmol/min/mg of protein). A similar increase was also observed in the glucose-induced Na⁺ transport. The reason for this effect is unknown.

MTSEA treatment similarly increased glucose transport by L147C (49%) and L149C (21%), whereas variable results were obtained with A423C vSGLT. The Na⁺/glucose transport activity of Q425C was inhibited 62% by MTSEA (8 ± 1 versus 21 ± 1 nmol/min/mg of protein). The glucose-induced Na⁺ transport of Q425C was also inhibited by MTSEA. Q425C was then chosen for further studies on right-side-out membrane vesicles.

Glucose transport into membrane vesicles was assayed in a buffer containing 10 mM Na⁺ and 105 μM glucose. Pretreatment of Q425C vesicles with 10 μM MTSEA for 1 min reduced the Na⁺-dependent glucose transport by 44%. Increasing the MTSEA concentration to 1 mM resulted in more than 90% inhibition. Treating the vesicles with 10 mM dithiothreitol for 1 min reversed this inhibition. In contrast, 1 mM MTSEA did not inhibit glucose transport into vesicles expressing C-less vSGLT. These results demonstrated that the MTSEA inhibition is specific for the residue Q425C.

To study the effect of ligands on MTSEA inhibition, Q425C vesicles were pretreated with Na⁺ and/or glucose for 1 min followed by MTSEA (10 μM) treatment for 1 min. The presence of 10 mM Na⁺ resulted in a slight protection (12%) from MTSEA inhibition (Fig. 2, Na⁺ (34%) versus K⁺ (56%) inhibition). Pretreatment with 250 mM L-glucose and 10 mM Na⁺ showed a level of protection similar to Na⁺ alone. However, pretreatment with 250 mM D-glucose and 10 mM Na⁺ resulted in essentially complete protection from MTSEA inhibition (Fig. 2, Na⁺ + D-gluc; no significant difference between glucose transport with and without MTSEA treatment, p > 0.10).

A Split vSGLT Maintains Na⁺-dependent Glucose Transport Activity—A split vSGLT was generated by co-expression of the N- and C-terminal seven-transmembrane helical proteins in E. coli JM1100 cells. Na⁺-dependent glucose transport (3.6 ± 0.1 nmol/min/mg of protein) was observed from cells co-expressing N7 and C7 vSGLT fragments. The Na⁺-dependent glucose transport activity of the split vSGLT was 5–10% that of the intact protein, but Western blot analysis suggested that these cells only contained about 10% of the normal amount of full-length vSGLT protein in the plasma membrane (Fig. 3A, lanes 1 and 2). Thus, the low activity of split vSGLT could be attributed to the fact that less protein was present in the plasma membrane.

C-less and C-substituted split vSGLTs were generated, and their Na⁺/glucose transport activities were determined as shown in Table III. All cysteine-substituted split vSGLTs were generated on the C-less background. C-less split vSGLT had glucose transport activity comparable with that of wild-type split vSGLT. The relative activities of single cysteine-substituted Glycophila cotransporter were compared with that of wild-type split vSGLT.
**Table III**

**Glucose transport by split vSGLT protein**

Glucose transport in JM1100 cells co-transformed with plasmids encoding split vSGLT protein were measured in NaCl (+Na, 12.5 mM Na⁺) and choline chloride (−Na) assay buffer containing 94 μM glucose. N₇ represents the N-terminal seven-transmembrane helices, C₇ represents the C-terminal seven-transmembrane helices with a C411I substitution. Shown is the mean of three experiments. The errors are S.E.

<table>
<thead>
<tr>
<th>Split vSGLT</th>
<th>C₇ Glucose transport</th>
<th>Na⁺/min/mg protein</th>
<th>−Na⁺/min/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>N₇</td>
<td>C₇</td>
<td>3.1 ± 0.1</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>L147C</td>
<td>C₇</td>
<td>1.2 ± 0.1</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>L149C</td>
<td>C₇</td>
<td>3.7 ± 0.1</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>G151C</td>
<td>C₇</td>
<td>0.5 ± 0.1</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>N₇</td>
<td>A423C</td>
<td>6.8 ± 0.3</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>N₇</td>
<td>Q425C</td>
<td>1.8 ± 0.1</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>N₇</td>
<td>Q428C</td>
<td>0.6 ± 0.1</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>L149C</td>
<td>A423C</td>
<td>3.7 ± 0.1</td>
<td>0.8 ± 0.1</td>
</tr>
</tbody>
</table>

These treatments affect the mobility of C₇ fragment in membranes containing double cysteine-substituted split vSGLTs, L149C/A423C did induce the cross-linking of these N7 and membranes containing double cysteine-substituted split vSGLT. Only one of the double cysteine-substituted split vSGLTs, L149C/A423C, showed Na⁺/glucose transport activity comparable with that of C-less split vSGLT.

Double cysteine-substituted split vSGLTs were generated by pairing one single cysteine substitution in the connecting loop between helices 4 and 5 and one in the connecting loops between helices 10 and 11. Only one of the double cysteine-substituted split vSGLTs, L149C/A423C, showed Na⁺/glucose transport activity comparable with that of the C-less split vSGLT. The other eight combinations of double cysteine-substituted split vSGLT (L147C/A423C, L147C/Q425C, L147C/Q428C, L149C/Q425C, L149C/Q428C, G151C/A423C, G151C/Q425C, and G151C/Q428C) showed very low or no glucose transport activity.

**Residues L149C and A423C Are within 8 Å—** Since double cysteine-substituted split vSGLT L149C/A423C showed glucose transport activity comparable with that of a wild-type split vSGLT, this pair of cysteine residues was subjected to chemical cross-linking analysis using the homobifunctional, sulfhydryl-reactive cross-linkers with flexible spacer arm lengths of 16 Å (BMH), 11 Å (BMB), and 8.0 Å (BMOE). When membranes containing the C-less split vSGLTs were subjected to Western blot analysis, the SGLT antibody recognized a single band of 26 kDa (Fig. 3A, lane 2). This band was not detectable in the membranes containing intact vSGLT (Fig. 3A, lane 1). Applying BMH, BMB, or BMOE to membranes containing either wild-type or C-less split vSGLT did not affect the mobility of the C₇ in SDS-polyacrylamide gel electrophoresis. Neither did these treatments affect the mobility of C₇ fragment in membranes containing single cysteine-substituted split N₇/A423C (Fig. 3A, lanes 4–6). Application of BMH, BMB, or BMOE to membranes containing double cysteine-substituted split vSGLT L149C/A423C did induce the cross-linking of these N₇ and C₇ fragments, as evidenced by the appearance of a band at 46 kDa (Fig. 3A, lanes 8–10). Since BMOE has the shortest spacer arm (8 Å) among these three cross-linkers, the distance between residues L149C and A423C is within 8 Å. When membranes containing split vSGLT L149C/A423C were subjected to oxidation with iodine before Western blot analysis, more than 60% of the split vSGLT cross-linked (Fig. 3A, lane 11). This result provided supporting evidence that residues L149C and A423C are in close proximity.

**Discussion**

There are some disadvantages of animal heterologous expression systems for studying mammalian SGLTs. These include the fact that modified proteins are frequently not trafficked properly to the plasma membrane (13–17) and that it is costly to produce sufficient amounts of protein for structural studies. We reasoned that vSGLT should be an excellent candidate for structure/function studies because: 1) it is a protein smaller than the mammalian homologues (543 versus 664 amino acids), 2) it can be expressed in E. coli, and 3) it can easily be rendered cysteine-less. The advantages of bacterial expression systems for membrane proteins include their possession of machinery for the co-translational insertion of membrane proteins directly into the plasma membrane (34) and well-established protocols available for the study of recombinant transporters in cells, plasma membrane vesicles, and proteoliposomes (18). Our results from studies in cells and membrane vesicles confirm that vSGLT is a Na⁺/sugar co-transporter with properties similar to its mammalian cousins and, additionally, established that vSGLT is suitable for cysteine-scanning studies (19).

These results demonstrate that similar to SGLT1, both D-glucose and D-galactose are transported by vSGLT. These two sugars, along with D-fucose, each induced Na⁺ transport, confirming previous results obtained by Sarker et al. (4, 5). Although α-MDG is a good substrate for SGLT1, it was not transported by vSGLT, as determined by radioactive tracer and Na⁺ transport experiments. It has been reported that excess cold α-MDG inhibited [¹⁴C]glucose and [¹⁴C]galactose transport by 80% and 57%, respectively (35, 36). Together, these results suggest that α-MDG binds to but is not transported by vSGLT. Similar blocking effects on SGLT1 have been observed with substrate analogues, such as phlorizin, β-D-glucopyranosylphenylisothiocyanate (GPITC), p-nitrophenyl-β-D-glucopyranoside (PNG), and 4-β-D-glucopyranosylaminobenzensulfonylamide (GSA) (37).

Phlorizin is a potent inhibitor of SGLT1, and the affinity (Kᵣ) of human SGLT1 for phlorizin is 0.2 μM (27). However, 1 μM phlorizin inhibited glucose transport of vSGLT by only 27%. Thus, the affinity of vSGLT for phlorizin is at least 1,000-fold lower than that of SGLT1. Similar, deoxyborpodin was a less effective inhibitor of vSGLT than of SGLT1. The affinity of human SGLT1 for deoxyborpodin is 0.1 μM but 0.1 μM deoxyborpodin had no effect on glucose transport by vSGLT.

Characterization of two cysteine-less (C411I and C411V) vSGLTs showed that their Na⁺/glucose transport activities are comparable with those of the wild-type vSGLT. The single cysteine-substituted vSGLTs, L147C, L149C, and A423C, maintained Na⁺/glucose transport activities at a level similar to wild-type vSGLT; Q425C vSGLT had a modest level of Na⁺/glucose transport activity; and two single cysteine-substituted vSGLTs, G151C and Q426C, lost their Na⁺/glucose transport activities. Since the amount of the mutant proteins in the plasma membrane was similar to the wild-type vSGLT, this suggests that Gly-151 and Gln-428 are essential for a functional vSGLT. Only the cysteine at position 425 (Q425C) was sensitive to MTSEA, and this inhibition of transport was blocked by the presence of Na⁺ and D-glucose but not D-glucose (Fig. 2). This indicates that, similar to SGLT1 (30), conformational changes of the vSGLT protein are involved in Na⁺/glucose transport and that Gln-425 plays a critical role in glucose binding and/or translocation.

Characterization of double cysteine-substituted vSGLTs showed that, as predicted from the activities of single cysteine-substituted vSGLTs, two pairs (L147C/A423C and L149C/A423C) maintained Na⁺/glucose transport, three pairs (L147C/Q425C, L149C/Q425C, and G151C/A423C) retained modest levels of Na⁺/glucose transport, and four pairs (L147C/Q428C,
L149C/Q428C, G151C/Q425C, and G151C/Q428C) were inactive.

Although a high resolution structure determination remains a goal for elucidating the coupling mechanism of Na\(^+\)/glucose cotransport, low resolution structure information, such as helical packing in membranes, is essential to advancing our knowledge of structural/functional relationships. It has been shown that co-expression of the lactose permease in two fragments leads to functional complementation (25, 38, 39). The site-directed thiol cross-linking of co-expressed permease fragments has been successfully used to determine helix proximity (18, 25). The characterization of C-less, cysteine-substituted, and split vSGLT in this study (Tables II and III) demonstrates the feasibility of using this approach in obtaining structural information about Na\(^+\)/glucose cotransporters.

Among three cross-linker applied, BMOE was the one with highest cross-linking efficiency (Fig. 3A). BMH and BMB have longer spacer arms (16 Å and 11 Å) than BMOE (8 Å), so their efficiency in cross-linking two close sulphydryl groups may be expected to be lower. A close proximity of L147C and A423C was also indicated by iodine oxidation analysis.

The experiments reported here with both MTS and cross-linking reagents provide direct evidence about the secondary structure and transmembrane packing of vSGLT. First, the fact that the quite impermeable MTSEA reagent inhibits Na\(^+\)/glucose cotransport by Q425C vSGLT in right-side-out membrane vesicles indicates that the hydrophilic loop between transmembrane helices 10 and 11 is on the external surface of the plasma membrane (3). Second, the cross-linking between residues L149C and A423C in the split vSGLT (N\(_{7}/C\(_{7}\)) with cross-linkers (BMOE and iodine, Fig. 3) demonstrates that the two residues are within 8 Å of one another in the functional transporter. This latter result further supports that the placement of the hydrophilic loop between helices 4 and 5 is on the external surface of the plasma membrane (3) and indicates that helices 4 and 5 lie close to helices 10 and 11 (Fig. 3).

The characterization of a split vSGLT in this study represents the first report of successful expression of a functional split SGLT protein. With the availability of cross-linkers with different lengths of spacer arms, it should be possible now to determine the distances between loops and transmembrane helices and thereby obtain a helical packing model of vSGLT.

Acknowledgment—We acknowledge Dr. Bruce Hirayama for many productive discussions and critical suggestions. We thank Drs. H. Ronald Kaback, Johannes Le Coutre, Philip F. Gao, and Jianhua Wu for their help in developing sugar and Na\(^+\) transport assays, the membrane vesicle preparation, and the expression of split vSGLT. We also thank Jason Lam at UCLA and Dr. Karl Hager at Keck Biotechnology Laboratory at Yale University for their assistance with DNA sequencing and Drs. Kaback, Hirayama, and Quick for their advice on the manuscript.

REFERENCES
Characterization of the Vibrio parahaemolyticus Na⁺/Glucose Cotransporter: A BACTERIAL MEMBER OF THE SODIUM/GLUCOSE TRANSPORTER (SGLT) FAMILY

Zhiyi Xie, Eric Turk and Ernest M. Wright

doi: 10.1074/jbc.M002687200 originally published online June 13, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M002687200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 39 references, 18 of which can be accessed free at
http://www.jbc.org/content/275/34/25959.full.html#ref-list-1