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A Ligand-dependent Conformational Change of the Na^+/Galactose Cotransporter of *Vibrio parahaemolyticus*, Monitored by Tryptophan Fluorescence

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Abstract. Purification and reconstitution of the active *Vibrio parahaemolyticus* Na^+/galactose transporter (vSGLT) enables us to do protein chemistry studies on a representative member of this class of membrane transporters. By measuring intrinsic tryptophan (Trp) fluorescence, conformational changes on the binding of substrates could be investigated. Trp fluorescence increased by 6% on the addition of saturating levels of both Na^+ and D-galactose, with a $K_{0.5}$ for D-galactose of 0.6 mM. No change was seen on the addition of Na^+ alone or by adding D-galactose in the presence of K^+. The Trp fluorescence could be quenched by acrylamide, but not by Cs^+or I^-. In the presence of Na^+ or K^+ alone, of Na^+ or K^+ and D-galactose, of Na^+ and L-glucose, or in the absence of ligands, the fluorescence quenches by acrylamide were similar. This indicated that the tryptophan exposure to acrylamide was unchanged in the presence or absence of ligands. No shifts in λ_{em} maximum were observed. To find the Trp responsible for the change in fluorescence, Trp 448 in transmembrane helix 11 in the putative sugar-binding pocket was mutated. It was found that W448F showed a similar change in Trp fluorescence upon the addition of D-galactose in the presence of Na^+. We conclude that the Trp fluorescence properties of the purified and reconstituted Na^+/galactose cotransporter are selectively changed by the transported substrates Na^+ and D-galactose, but it is not the Trp (W448) in the sugar translocation pathway that is involved.

Key words: Na^+/galactose transporter — Tryptophan fluorescence — Conformational change — Quench reagents — Proteoliposomes

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Introduction

Members of the sodium/glucose transporter (SGLT) family are found throughout living organisms. They cotransport a wide variety of substrates (sugars, inositol, iodide, urea and proline) into the cell using the electrochemical gradient of Na^+, Li^+ or H^+. An apparent stoichiometry of 2 ions to 1 sugar is found for most members. All family members share a common topology of at least 13 transmembrane (TM) helices, with an extracellular N-terminus. It has been predicted that the N-terminal half is responsible for Na^+ binding and translocation and the C-terminal half for sugar binding and translocation (Turk & Wright, 1997; Wright et al., 1998; Wright, 2001).

The *Vibrio parahaemolyticus* Na^+/galactose cotranporter (vSGLT) is a bacterial member of the SGLT superfamily. It possesses 14 TM helices with an extracellular N-terminus and consists of 543 amino acids with a mass of 60 kDa. Sugar transport is Na^+-dependent with a preference of galactose > glucose > fucose. The stoichiometry for cotransport was predicted to be 1:1 by Hill coefficient analysis, with an apparent K_m for Na^+ of 129 mM and 158 μM for galactose (Turk et al., 2000). It was also found that Gly-151 and Gln-428 are essential for transport. MTSEA labeling of a cysteine at position 425 (Q425C) blocked transport and labeling could be blocked by the addition of Na^+ and D-glucose, but not by L-glucose. From cross-linking experiments with the split vSGLT (N7:C7) it was found that residues L149C and A423C are within 8 Å of another in the functional transporter, which means that helices 4 and 5 lie close to helices 10 and 11 (Xie, Turk & Wright, 2000).

To further characterize the structure/function of vSGLT, the protein was purified and reconstituted into liposomes in an active form in order to enable us to perform Trp fluorescence experiments on this class of transporters for the first time. By monitoring

changes in the fluorescence of the 12 tryptophans present in the cotransporter, putative conformational changes of the cotransporter may be observed upon binding of ions, sugars and quenchers. When both Na^+ and D-galactose were bound to the transporter a change in Trp fluorescence was observed but this was not accompanied by a shift in emission maximum or a change in accessibility to acrylamide. By changing Trp 448 to a phenylalanine (W448F) we tried to locate this substrate-induced change in the cotransporter, but W448F showed the same fluorescence properties as vSGLT.

Materials and Methods

Materials

Acrylamide was purchased from BDH (Garden City, NY). NaI, D-galactose, L-glucose, L-arabinose, cholesterol and *N*-acetyltryptophanamide were purchased from Sigma (St Louis, MO). CsCl was purchased from Life Technologies (Rockville, MD). Asolectin (unfractionated soy phospholipids) was purchased from Associated Concentrates (Woodside, NY) and decyl-β-D-maltoside and dodecyl-β-D-maltoside were purchased from Anatrace (Maumee, OH).

Site-directed Mutagenesis

vSGLT with a C-terminal six-histidine tag (VNH6A) (Turk et al., 2000) was mutagenized by PCR with oligonucleotide primers to generate the W448F mutation. This construct was made using four primers (Life Technologies, Rockville, MD) and pVNH6A as a template. The PCR product from primers 5′GCC CGC TCT TCG GAA GAA TAA GCC AAG TAA GAA TAC 3′ and 5′ TGG TAT TGC CAG GTA TTG CCG C 3′ when cut with SapI and PvuII yielded insert 1. That from primers 5′ GCC CGC TCT TCG TTC AAG AAA ACA ACC AGT AAA GG 3′ and 5′ GAA CAA TGT ATA TAA CAC TGC 3′ when cut with SapI and ClaI yielded insert 2. Both insert and vector (pVNH6A cut with PvuII and ClaI) were combined, yielding pVW448FH6.

Expression and Purification

VNH6A and VW448FH6 were expressed in *Escherichia coli* and purified as described (Turk et al., 2000). The bacteria were grown overnight at 30°C after expression of the protein with 33 μM and 13 μM L-arabinose for VNH6A and VW448FH6, respectively. The purity of the proteins, determined by Ag^+ stains and SDS-PAGE electrophoresis, was ~90%.

Reconstitution of VNH6A and VW448FH6

Lipids and proteoliposomes were prepared as described before (Turk et al., 2000) using 90 mg of Asolectin soy lecithin and 10 mg of cholesterol. Liposomes were made at 22°C. 10% decyl-β-D-maltoside, 90 μl of lipids, 15 μl of buffer (10 mM Tris, 10 mM Tricine, 10 mM dithiothreitol, 0.01% dimethylsulfoxide and 0.1 mM Na_{12} phytate) and 350 μl of 2 × KG buffer (in mM: 20 Tris, 20 HEPES, 200 Choline·Cl, 300 KCl and 20% glycerol) were mixed and incubated for 5 min. The sample was centrifuged to remove undissolved lipids. 500 μg of purified VNH6A or VW448FH6 (238 μl) was added, mixed and incubated for 5 min. 7 μl of CM buffer (100 mM $MgCl_2$ and 100 mM $CaCl_2$) was added, mixed and incubated for 5 min. Proteoliposomes were formed upon the addition of 378 μl of CD2 buffer (60 mM β-cyclodextrin, 1 × KG buffer and 15% formamide) added in 20 μl aliquots, mixing after each addition. The mixture was centrifuged to remove debris and desalted on a 7-ml bed of Superdex 30 using 50 mM potassium phosphate buffer pH 7 at 0.5 ml/min. Proteoliposome fractions were pooled and centrifuged at 400,000 × g for 1 hr at 4°C. Pellets were resuspended in 200 μl potassium phosphate buffer with a 28 gauge needle and stored at −80°C.

Sodium-dependent D-galactose transport assays of vSGLT (VNH6A) and W448F (VW448FH6), reconstituted into liposomes (Turk et al., 2000), demonstrated that both the wildtype and mutant proteins were active. The initial transport rate for vSGLT was 0.2 nmol/mg/min and a comparable number was found for mutant W448F. The level of W448F protein reconstitution was found to be 6 to 7 times lower than for VNH6A.

Tryptophan Fluorescence Measurements

For part of the experiments it was necessary to remove K^+ from the proteoliposomes. They were thawed and then freeze/thawed three more times in liquid N_2. The proteoliposomes were then dialyzed for 16 hr at 4°C in a 500 times excess of 50 mM Tris·Cl buffer pH 7.2. The buffer was renewed twice during dialysis. Fluorescence was measured on an Aminco-SLM 4800 spectrofluorometer (Urbana, IL) at 22°C. The tryptophan residues in vSGLT and W448F were excited at 288 nm and the emission was recorded from 300 to 400 nm with slit widths of 4 nm. In a 100 μl nominal volume quartz cuvette, 6 μl proteoliposomes (7.2:1 lipid to protein ratio) were added to 120 μl buffer (50 or 200 mM sodium phosphate pH 7.3 or 7.0 respectively, 50 mM potassium phosphate pH 7.2, or 50 mM Tris-Cl buffer pH 7.2 for the dialyzed vSGLT) and the tryptophan fluorescence was recorded. D-galactose or L-glucose was added at different concentrations up to 10 mM, from a 1 M stock solution, and NaCl at a concentration of 100 mM from a 4 M stock solution. Quencher (acrylamide, NaI or CsCl) was added up to 100 mM, from 3 M stock solutions. The volume increase upon these additions was no more than 5%. 1 mM $Na_2S_2O_3$ was added to the NaI solution to prevent formation of I_3^-. The soluble Trp analog *N*-acetyltryptophanamide (NATA) was used at a concentration of 0.12 mM. The quenching of Trp fluorescence by acrylamide was measured in 200 mM sodium phosphate buffer in the presence or absence of 0.005% dodecyl-β-D-maltoside following excitation at 288 nm. All fluorescence spectra were corrected for the blank, dilution effects and for the absorbency of acrylamide in buffer. The liposome background was less than 15% of the total reconstituted liposome fluorescence.

Kinetic Analysis

The acrylamide-quenching data were analyzed using the Stern-Volmer equation (Lakowicz, 1999):

$$F_0/F = 1 + K_{SV}[Q] \quad (1)$$

where F_0 and F are the fluorescence intensities in the absence and presence of acrylamide (Q), respectively and K_{SV} is the Stern-Volmer quenching constant.

The saturation fraction of ligand was determined from the variation in fluorescence intensity upon the binding of D-galactose (Guixé, Rodríguez & Babul, 1998):

$$\text{Saturation fraction} = (F_0 - F)/(F_0 - F^\infty) \quad (2)$$

where F_0 represents the fluorescence intensity in the absence of D-galactose, F^∞ the intensity in the presence of the saturating con-

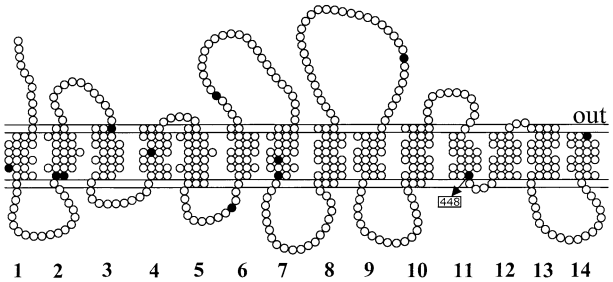

Fig. 1. Topology of vSGLT. Schematic diagram showing the location of the 12 tryptophan residues (depicted in black) in vSGLT. The N_7 terminal half contains 9 tryptophan residues of which 7 are in the transmembrane helixes. Trp29 in the first helix, Trp53 and Trp54 in the second helix, Trp89 in the third helix, Trp 134 in helix four and Trp257 and Trp264 in helix seven. One tryptophan (Trp187) is located in the intracellular loop between helix 5 and 6 and Trp217 is located in the extracellular loop between helix 6 and 7. The C_7 terminal half contains 3 tryptophans, one located in helix 11 (Trp450) and one in helix 14 (Trp543), and Trp341 is located in the extracellular loop between helix 8 and 9.

centration of D-galactose and F the intensity at a given concentration of D-galactose.

Analytical Methods

Protein concentration was determined by using the BCA protein assay (Pierce) with bovine serum albumin as a standard. SDS-PAGE (Schägger & von Jagow, 1987) and Ag^+-stained (Heukeshoven & Dernick, 1985 & 1988) gels were performed as previously described.

Results

The locations of the 12 tryptophan residues of the vSGLT protein are shown in Fig.1. Nine tryptophans are located in the N-terminal half of the protein. Trp29, Trp53 and Trp54, Trp89, Trp134, Trp257 and Trp264 are located in transmembrane helices one, two, three, four and seven respectively. Trp187 is located in the intracellular loop between helix 5 and 6 and Trp217 is located in the extracellular loop between helix 6 and 7. The C-terminal half contains only three tryptophans; Trp448 and Trp543 in transmembrane helices eleven and fourteen respectively and Trp341 in the extracellular loop between helix 8 and 9.

The tryptophans of reconstituted vSGLT were excited at 288 nm and the emission maximum was observed at 327 ± 0.5 nm (Figure 2*A*, *B* and *D*). When 10 mM D-galactose was added to vSGLT in 100 mM Na^+, an increase in total fluorescence was seen (Fig. 2*A*). On average the sugar-dependent increase in Trp fluorescence was 6 ± 2% (n = 11). This change in fluorescence was not seen on the addition of 10 mM L-glucose in the presence of 100 mM Na^+ (Fig. 2*B*). This enhanced fluorescence was not accompanied by a shift in the λ_{max}. The time course of the D-galactose-induced change in fluorescence is shown in Fig. 2*C*. The Trp fluorescence increased within the 8 seconds that were required to add D-galactose to the cuvette. The increase in Trp fluorescence was only seen in the presence of Na^+. In the presence of 100 mM K^+ (Fig. 2*D*), the addition of D-galactose had no effect. No change was observed in the Trp fluorescence signal of vSGLT on the addition of 100 mM Na^+ without sugar (*data not shown*). Figure 2*E* shows that the increase in fluorescence with increasing galactose concentration in 100 mM Na^+ was hyperbolic. When fitted to the equation $V = F_{max}$ [S]/($K_{0.5}$ + [S]), where $K_{0.5}$ was the D-galactose concentration where the fluorescence intensity was half of the maximal intensity, the $K_{0.5}$ for D-galactose was 0.61 mM. A second experiment gave a $K_{0.5}$ of 0.58 mM D-galactose.

To study the influence of quenchers on the Trp fluorescence of vSGLT acrylamide, I^- or Cs^+ was added. When 100 mM acrylamide was added in the presence of 10 mM D-galactose and 100 mM Na^+, there was a $12 \pm 2\%$ ($n = 9$) quench in fluorescence (Fig. 3*A*). Similar quenches were observed in the presence of 10 mM L-glucose and 100 mM Na^+ ($14 \pm 1\%$ ($n = 4$)) (Fig. 3*B*); in the presence of 10 mM D-galactose and 100 mM K^+ ($13 \pm 1\%$ ($n = 4$)) (Fig. 3*C*); in the presence of either 100 mM Na^+ or 100 mM K^+ without sugar ($13 \pm 3\%$ ($n = 5$) and $13 \pm 1\%$ ($n = 2$) respectively); and in the absence of sugars and cations ($9 \pm 2\%$ ($n = 6$)). Table I summarizes the effect of ligands and acrylamide on the Trp fluorescence of vSGLT. There was no significant difference ($P > 0.1$) between the acrylamide quenches in the presence or absence of ligands. Figure 4 shows the Stern-Volmer plot of the fluorescence quenches of vSGLT by acrylamide up to 100 mM. The Stern-Volmer quenching constant was 1.7 M^{-1}. Above a concentration of 100 mM acrylamide the plot was non-linear and curved towards the X-axis. Neither Cs^+ nor I^- produced a change in Trp fluorescence, either in the presence of D-galactose and Na^+ (Fig. 5*A* and 5*B* for Cs^+ and I^- respectively) or in the presence of Na^+ or K^+ alone (*data not shown*).

For vSGLT solubilized in detergent the protein showed an emission λ_{max} of 329 nm. Acrylamide and Cs^+ at a concentration of 100 mM quenched the protein fluorescence to 46% and 25% respectively. When the Trp fluorescence was quenched by 0–100 mM acrylamide the Stern-Volmer plot showed a quenching constant of 2.6 M^{-1} (*data not shown*).

To more closely investigate the D-galactose-enhanced Trp fluorescence, Trp 448 was mutated into a phenylalanine, giving W448F (Fig. 1) and reconstituted into liposomes. The emission maximum was 326 ± 0.5 nm and upon the addition of 10 mM D-galactose in the presence of 100 mM Na^+ the Trp fluorescence increased 18% and 19% in two identical experiments. A 10% quench was observed when

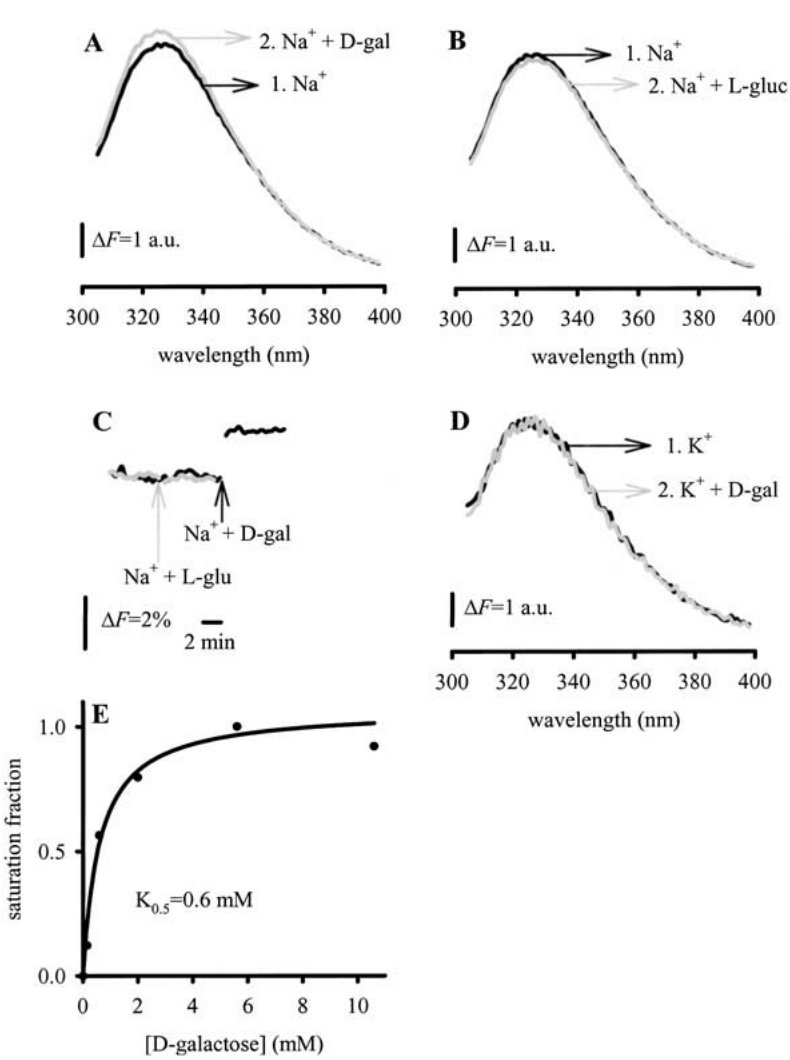

Fig. 2. The effect of sugars on the tryptophan fluorescence of vSGLT in liposomes in the presence of Na^+ or K^+. Proteoliposomes were added to 50 mM sodium phosphate buffer pH 7.3 (*A*, *B*, *C* and *E*) or 50 mM potassium phosphate buffer pH 7.2 (*D*). 10 mM D-galactose (*A*) or 10 mM L-glucose (*B*) was added to proteoliposomes in 50 mM sodium phosphate buffer and the emission spectra were recorded from 300 to 400 nm with excitation at 288 nm. All spectra were taken with an average of 100 samples/data point. (*C*) Fluorescence tracing of proteoliposomes in 200 mM sodium phosphate buffer pH7 was recorded at 327 nm following excitation at 288 nm. The recordings were stopped (breaks) to add 10 mM D-galactose (black) or 10 mM L-glucose (gray). All spectra were taken with an average of 10 samples/data point. 10 mM D-galactose was added to proteoliposomes in 50 mM potassium phosphate buffer (*D*) and the emission spectra were recorded from 300 to 400 nm with excitation at 288 nm. All spectra were taken with an average of 10 samples/data point. (*E*) The increase in tryptophan fluorescence of vSGLT in liposomes in 50 mM sodium phosphate buffer pH 7.3 at increasing concentrations of D-galactose. The saturation fraction $(F_0-F)/(F_0-F^\infty)$, where F,F_0 and F^∞ represent the fluorescence intensity at a given concentration of D-galactose, in the absence of D-galactose and at the saturating concentration of D-galactose respectively, was plotted against the concentration of D-galactose.

100 mM acrylamide was added (*data not shown*). Again, there was no shift in the emission maximum upon the addition of D-galactose. The fluorescence for W448F was 6 to 7 times lower compared to vSGLT, which was consistent with the amount of protein incorporated into the liposomes.

Discussion

Previously it was found that Na^+ binding induces a conformational change in SGLT1 from rabbit, which enhances the cotransporter's affinity for sugar (Hirayama, Loo & Wright, 1997; Loo et al 1998). Until recently we were unable to study this conformational change directly on the protein, but with the purification and reconstitution of vSGLT we are now able to do so. In this study we have examined the intrinsic fluorescence of vSGLT in the presence and absence of ligands. We found that a change in Trp fluorescence was observed on the formation of a Na^+/galactose/cotransporter complex, which reflects a conformational change in the protein, and that this change was

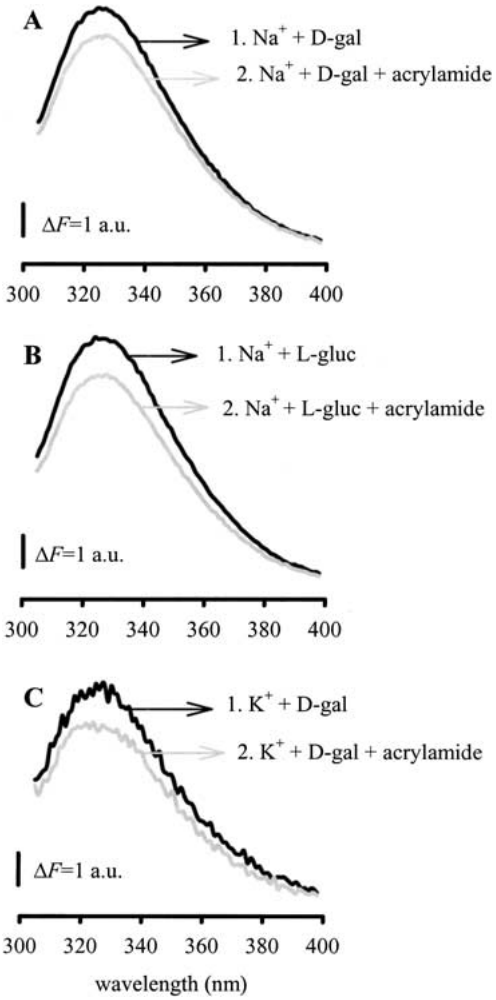

Fig. 3. Fluorescence emission spectra of the effect of acrylamide on the tryptophan fluorescence of vSGLT in liposomes in the presence of Na^+ or K^+ and 10 mM sugar. Emission spectra of vSGLT in liposomes added to 50 mM sodium phosphate buffer pH 7.2 (*A* and *B*) or 50 mM potassium phosphate buffer pH 7.2 (*C*) were recorded from 300 to 400 nm after excitation at 288 nm. 100 mM acrylamide was added to the liposomes in the presence of 50 mM sodium phosphate buffer and 10 mM D-galactose (*A*) or 10 mM L-glucose (*B*). (*C*) 100 mM acrylamide was added to the liposomes in the presence of 50 mM potassium phosphate buffer and 10 mM D-galactose. All spectra were taken with an average of 10 samples data point.

induced only by the simultaneous presence of the transported substrates Na^+ and D-galactose.

It was determined that the maximum λ_{ex} for vSGLT reconstituted into liposomes was 288 nm. An excitation of 295 nm is often used to measure Trp fluorescence to avoid excitation of tyrosines (Tyr) (λ_{ex} is 275 nm). Throughout the vSGLT protein there are 25 Tyr residues and if these residues were excited at 288 nm, a peak in the emission spectra around 300 nm would be expected. This was not observed in the emission spectra of vSGLT. When the protein was excited at 288 nm it exhibited a peak emission at 327 ± 0.5 nm. The spectrum is similar to that for the

Table 1. The effect of ligands and acrylamide on the Trp fluorescence of vSGLT

Additions	Change in ΔF (%)
Na^+ + D-galactose	$+6 \pm 2$
Na^+ + acrylamide	-13 ± 3
Na^+ + D-galactose + acrylamide	-12 ± 2
Na^+ + L-glucose + acrylamide	-14 ± 1
K^+ + D-galactose + acrylamide	-13 ± 1
Acrylamide	-9 ± 2

ΔF is the change in intrinsic protein fluorescence at 327 nm. It shows a mean of 4–11 experiments ± the standard deviation. The Trp fluorescence of vSGLT was recorded following excitation at 288 nm. Emission spectra were recorded from 300 to 400 nm. vSGLT was added to 50 mM sodium- or potassium-phosphate buffer. D-galactose or L-glucose was added at a final concentration of 10 mM and finally acrylamide was added at a concentration of 100 mM.

melibiose transporter (Mus-Veteau & Leblanc, 1996), the P-glycoprotein multidrug transporter (Lui, Siemiarczuk & Sharom, 2000) and the hexose transporter (Pawagi & Deber, 1990). These emission spectra reflect an average of all the tryptophans in a protein, some of which are in a polar environment ($\lambda_{max} \approx 350$ nm) and others that are in a nonpolar environment ($\lambda_{max} \approx 310$–$324$ nm). (Pawagi & Deber, 1990). The intrinsic fluorescence of the purified protein is selectively modified by the transported substrate D-galactose, but only in the presence of Na^+, giving an increase in fluorescence of $6 \pm 2\%$. There was no change in vSGLT fluorescence upon the addition of D-galactose in the absence of Na^+, in the presence of K^+, or upon the addition of the nontransported sugar L-glucose in the presence of Na^+. Neither was there a change upon the addition of Na^+ in the absence of sugar. This negative result would still be consistent with a Na^+-induced conformation change, which does not alter the environment of the tryptophans within the protein. Furthermore, the D-galactose-induced increase in vSGLT fluorescence in the presence of Na^+ became saturated as the D-galactose concentration increased, and the apparent D-galactose affinity was determined to be 0.6 mM. For Na^+/D-galactose transport by vSGLT in proteoliposomes (Turk et al., 2000) the apparent affinity for D-galactose was 0.2 mM. Neither a change in the tryptophan emission spectrum nor a change in accessibility to quench reagents such as acrylamide, Cs^+ and I^- accompanied the Na^+-dependent D-galactose-induced increase in vSGLT fluorescence. The acrylamide quench was similar in the presence or in the absence of ligands. It is possible that a change in conformation, in which one Trp gets more or less exposed to the solvent, might be undetectable in a background of 12 tryptophans. Another explanation could be that the substrate-induced increase in fluorescence is caused not by a change in solvent exposure

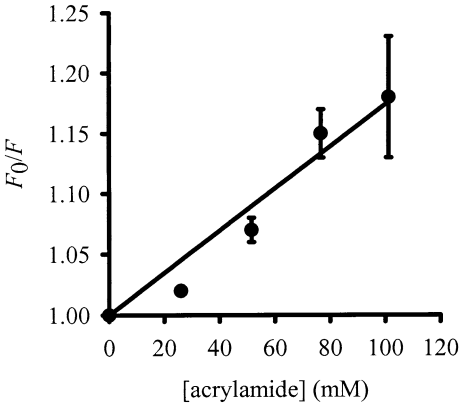

Fig. 4. Stern-Volmer plot of the effect of acrylamide on the tryptophan fluorescence of vSGLT in liposomes. The tryptophan fluorescence of vSGLTl in liposomes was measured in 50 mM sodium phosphate buffer pH 7.3 at increasing concentrations of acrylamide. F/F_0, where F is the fluorescence intensity at increasing concentrations of acrylamide and F_0 is the intensity in the absence of acrylamide is plotted against the acrylamide concentration.

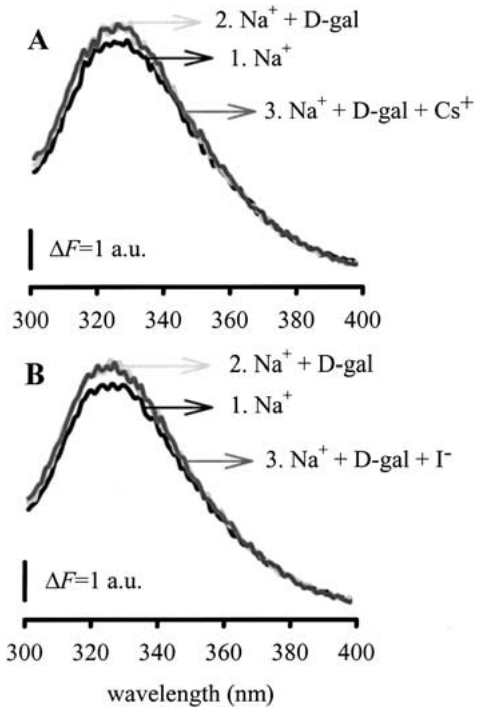

Fig. 5. Fluorescence emission spectra of the effect of quenchers on the tryptophan fluorescence of vSGLT in liposomes in the presence of Na^+ and D-galactose. The emission spectra of vSGLT in liposomes in 50 mM sodium phosphate buffer pH 7.3 were taken after excitation at 288 nm. 10 mM D-galactose was added and finally 100 mM CsCl (*A*) or 100 mM NaI (*B*) was added. All spectra were taken with an average of 10 samples/ data point.

of vSGLT tryptophans, but is perhaps due to decreases in interactions between the indole rings and adjacent polar residues in the transporter. Similar results have been reported for the effect of ligands on the *E. coli* melibiose (Mus-Veteau & Leblanc, 1996; Mus-Veteau, Poucher & Leblanc, 1995) and the human hexose transporter (Pawagi & Deber, 1990) reconstituted into liposomes. In the case of the hexose transporter, which has 6 Trp residues, D-glucose specifically decreased the fluorescence by 10% and this decrease was accompanied by small decreases in the fluorescence quenches produced by I^- and acrylamide. The Na^+/melibiose cotransporter contains 8 tryptophans, and here the addition of Na^+ induced a 2% quench in fluorescence. However, the addition of melibiose in the presence of Na^+ enhanced Trp fluorescence up to 20%. Both the sugar specificity and the concentration dependence of the increase agree closely with those for sugar cotransport. Mutation of the two tryptophans (W299 and W342) in the C-terminal half of Mel permease to phenylalanines (Mus-Veteau et al., 1995) did not alter the cation quench of the remaining 6 Trp residues in the N-terminal half of the protein, but the sugar-induced fluorescence increase varied from mutant to mutant in a sugar-specific manner. It was concluded that W299 and W342 in helices IX and X are at, or close to, the sugar-binding site.

Neither iodide nor cesium quenched vSGLT Trp fluorescence in proteoliposomes and this suggests that none of the 12 Trp residues are readily accessible to the external polar environment. Of these 12 tryphophans, Trp217 and Trp341 are on extracellular hydrophilic loops, and Trp 187 is on an intracellular loop, thus one might have expected a 10–20% iodide or cesium quench depending on the orientation of the reconstituted protein. In detergent, however, the Trp fluorescence of vSGLT showed a 25% quench upon the addition of 100 mM Cs^+, suggesting that detergent solubilization perturbs the native conformation of the protein in the bilayer. The collisional quencher acrylamide, however, does quench vSGLT Trp fluorescence. The quench in proteoliposomes was $12 \pm 2\%$ at a concentration of 100 mM acrylamide, which is comparable to that observed for P-glycoprotein (Lui et al., 2000), but at higher concentrations the quench becomes less efficient. This indicates heterogeneity in the accessibility of the 12 vSGLT tryptophans to this collisional quencher (Eftink & Ghiron, 1976). Strikingly the Trp fluorescence quench of vSGLT in detergent by 100 mM acrylamide was 40% and the quench constants of the Stern-Volmer plots were 1.7 M^{-1} and 2.6 M^{-1}, for vSGLT in liposomes and detergent respectively. For the soluble Trp analog *N*-acetyltryptophanamide the quench constants were 24.6 M^{-1} in buffer and 18 M^{-1} in detergent. This indicates that even in detergent, most of the tryptophans that contribute to protein fluorescence are buried in vSGLT.

Which tryptophans are involved in sugar transport by vSGLT? Previous studies on the SGLT1 protein suggest that the C-terminal domain-containing helices 10–14 form the sugar translocation pathway through the cotransporter: 1) the sugar

selectivity and affinity of the cotransporter are determined by the residues in this C-terminal portion of the protein (Panayotova-Heiermann et al., 1996); 2) the truncated protein, when expressed in oocytes or when it is purified and reconstituted into proteoliposomes, behaves as a glucose uniporter (Panayotova-Heiermann et al., 1997 & 1999); and 3) Q457 on helix 11 of SGLT1 (Loo et al., 1998) and Q425 of the vSGLT cotransporter (Xie at al., 2000) dramatically control glucose translocation. There are only two tryptophans in this C-terminal region of vSGLT, W448 on helix 11 and W543 at the C-terminal of helix 14 (Fig. 1). The tryptophan that we deemed most likely to respond to the addition of D-galactose in the presence of Na^+ was W448 because it is close to Q425 in vSGLT (Xie at al., 2000), which resides in a position equivalent to Q457 in SGLT1 (Loo et al., 1998). When this tryptophan was mutated into a phenylalanine, the reconstituted transporter still showed enhanced Trp fluorescence upon the formation of a Na^+/galactose/vSGLT complex. This suggests that the tryptophan in the sugar-translocation domain is not responsible for the sugar-induced increase in fluorescence.

The data presented here suggest that the binding of Na^+ and D-galactose induce a conformational change in the Na^+/galactose cotransporter. We did not elucidate the part of the protein where this change takes place, since W448F still exhibits a ligand-induced fluorescence increase. Future approaches to this question will include the use of a distance-reporter-group method (Pierce & Wright, 1987) where tryptophan is used as an energy donor to an extrinsic fluorescent reporter group, e.g., anthracene bound to specific cysteines in the N- or C-terminal halves of the protein. The Förster critical distance, R_o, for tryptophan to anthracene is 21 Å, which indicates that anthracene is an efficient reporter of tryptophans within 15–30 Å. This, combined with tryptophan mutagenesis, is a powerful method to identify specific tryptophans associated with ligand-induced conformational changes of the cotransporter.

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