SUGAR BINDING RESIDUE AFFECTS APPARENT NA⁺ AFFINITY AND TRANSPORT STOICHIOMETRY IN MOUSE SODIUM/GLUCOSE COTRANSPORTER TYPE 3B
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Running title: Uncoupled ion transport in SGLT3

SGLT1 is a sodium/glucose transporter that moves 2 Na⁺ ions with each glucose molecule per cycle. SGLT3 proteins belong to the same family and are described as glucose sensors rather than glucose transporters. Thus, human SGLT3 (hSGLT3) does not transport sugar, but extracellular glucose depolarizes the cell in which it is expressed. Mouse SGLT3b (mSGLT3b), although it transports sugar, has low apparent sugar affinity and partially uncoupled stoichiometry compared with SGLT1, suggesting that mSGLT3b is also a sugar sensor. The crystal structure of the Vibrio parahaemolyticus SGLT showed that residue Q428 interacts directly with the sugar. The corresponding amino acid in mammalian proteins, 457, is conserved in all SGLT1 proteins as glutamine. In SGLT3 proteins, glutamate is the most common residue at this position, although in mSGLT3b it is a glycine and in rat SGLT3b a serine. To test the contribution of this residue to the function of SGLT3 proteins, we constructed SGLT3b mutants that recapitulate residue 457 in SGLT1 and hSGLT3, glutamine and glutamate respectively. The presence of glutamine at residue 457 increased the apparent Na⁺ and sugar affinities, while glutamate decreased the apparent Na⁺ affinity. Moreover, glutamate transported more cations per sugar molecule than the wild type protein. We propose a model where cations are released intracellularly without the release of sugar from an intermediate state. This model explains the uncoupled charge:sugar transport phenotype observed in wild type and G457E-mSGLT3b compared with SGLT1, and the sugar activated cation transport without sugar transport that occurs in hSGLT3.

Members of the SLC5 cotransporter family present highly diverse functions. They are capable of cotransporting Na⁺ with glucose (SGLT1 and SGLT2), with myoinositol (SMIT), with iodide (NIS), or with choline (CHT), among other substrates (reviewed in ref. 1). In fact, there is one family member, human SGLT3, that is not a transporter but is a glucose sensor (2, 3).

The crystal structures of Na⁺ symporters from different families reveal that they share a core of 10 transmembrane segments composed of an inverted repeat of 5 segments (4). The crystal structure of Vibrio parahaemolyticus SGLT (vSGLT) clearly resolved the residues of the sugar binding site (5). One residue that directly interacts with the sugar is glutamine 428, which is equivalent to amino acid 457 in mammalian SGLT proteins. Notably, the identity of the amino acid at position 457 in human SGLT1 (hSGLT1) has proven to have a dramatic effect on the function of the protein. Structure-function studies on hSGLT1 suggest that residue 457 interacts with sugar (6) and mutations of this residue cause glucose-galactose malabsorption (7, 8).

The crystal structure of vSGLT did not reveal the position of the single Na⁺-binding site. SGLT1 and other transporters like LeuT, and presumably SGLT3, have two Na⁺-binding sites. Based on structural homology to LeuT whose two Na⁺ binding sites were identified, Na1 and Na2 (9), a Na⁺-binding site in vSGLT approximately 10 Å away from the sugar binding site was proposed and corresponds to Na2 in LeuT (5). The location of the other Na⁺ binding site in SGLT has not been identified, but it may correspond to Na1 in LeuT. These Na⁺-binding sites are likely to be conserved in SGLT1 and SGLT3 proteins.
Despite ~70% amino acid identity between hSGLT3 and hSGLT1, there are significant differences in their function, besides hSGLT3’s inability to transport sugar. HSGLT3 also has a weaker apparent glucose affinity, no visible pre-steady-state currents and a different tissue distribution than SGLT1 (2, 10). In terms of amino acid sequence, all SGLT1 and SGLT2 proteins have a glutamine at residue 457 while most SGLT3 have a glutamate. There are two exceptions, glycine in mouse SGLT3b and serine in rat SGLT3b (Fig. 1).

Because of the strict conservation of amino acid 457 among the SGLT1 and SGLT2 glucose transporters and the difference with the SGLT3 glucose sensors, we explored whether the identity of this residue plays a major role in the differences in function between the different proteins. Specifically, we looked at the effect of modifying this residue in mSGLT3b. We sought to learn whether the identity of residue 457 in mSGLT3b would lead to significant changes in function. We made two mutations, G457Q-mSGLT3b and G457E-mSGLT3b, to recapitulate the residue in SGLT1 and human SGLT3, respectively. We then compared the characteristics of each of the mutants with the wild type protein.

We found that the mutations changed the apparent affinity for sugar, as expected for being part of the sugar binding site, and surprisingly, also changed the apparent affinity of Na⁺. In addition, we found that residue 457 affects the charge:sugar cotransport ratio indicating that it contributes to the uncoupled phenotype that occurs in SGLT3. We propose a mechanism for SGLT that explains the uncoupled transport that occurs in wild type mSGLT3b, G457E and also in human SGLT3.

**EXPERIMENTAL PROCEDURES**

**Mutagenesis**- Mouse SGLT3b cDNA in pGH19 was used as a template for site-directed mutagenesis using QuikChange kit (Stratagene). The oligonucleotide primers used were, for G457E-mSGLT3b, G457E-sense, 5’-CTCTTCCACTATATTGAGTCAGTTTCTAG CTAC-3’; and G457E -antisense, 5’- GTAGCTAGAAACTGACTCAATATAGTGGAAGAG-3’. The underlined letters represent the nucleotides changed. The gene was sequenced from start codon to stop codon to ensure that only the desired mutation was present.

**Expression of proteins in Xenopus laevis oocytes**- Plasmids containing the wild type and mutant cDNAs were linearized with XhoI and RNAs were transcribed and capped in vitro using the T7 RNA promoter (MEGAscript kit, Ambion). Xenopus laevis oocytes were injected with 50 ng of cRNA encoding each protein. Oocytes were maintained at 18 °C in OR2 supplemented with penicillin (10,000 U/ml)/streptomycin (10 mg/ml) for 5-8 days before experiments.

**Electrophysiology**- Electrophysiology experiments were performed using two-electrode voltage-clamp with a rapid perfusion chamber. Oocytes were bathed in Na⁺ solution composed of (in mM): 100 NaCl, 2 KCl, 1 MgCl₂, 1 CaCl₂, 10 Hepes/Tris, pH 7.4, and in Na⁺-free solution, with choline-Cl replacing NaCl. To measure sugar-induced current, the membrane potential was held at -50 mV and stepped for 100 ms from -150 mV to +50 mV or to +110 mV in 20 mV increments. The sugar-dependent current was defined as the difference between the current recorded in sugar and the previous record in Na⁺ solution alone. Experiments were controlled and data were acquired using pClamp software (Axon Instruments). Apparent Na⁺ affinity (KNa 0.5) was calculated by recording currents in 150 mM αM-glucose and in progressively increasing Na⁺ concentrations (0 to 100 mM). Apparent sugar affinity (Ks 0.5) was obtained in 100 mM Na⁺ with increasing sugar concentrations (0 to 150 mM). The steady state currents at each membrane potential were fit to Equation [1]:  

\[
I = \frac{I_{max}[S]}{K_{s 0.5} + [S]},
\]

where \(I_{max}\) is the maximal current, \([S]\) is the substrate concentration, \(K_{s 0.5}\) is the substrate concentration for one-half \(I_{max}\) and \(n\) is the Hill coefficient. For fitting of sugar dose response curves \(n\) was set to 1.

**Pre-steady-state currents**- The “Off” currents were fit with double exponentials and the membrane capacitive component obtained
from the fit was subtracted from the current record, leaving the currents attributable to the transporter. The pre-steady-state transient charge, $Q$, was determined by integration of this transient Off current with time, and the distribution of the charge moved as a function of membrane voltage ($V$) was calculated by fitting the data with the Boltzmann equation: 
\[
\frac{Q - Q_{\text{hyp}}}{Q_{\text{max}}} = \frac{1}{1 + \exp \left[ \frac{z \cdot (V - V_{0.5}) \cdot F}{RT} \right]},
\]
where $Q_{\text{max}} = Q_{\text{dep}} - Q_{\text{hyp}}$ ($Q_{\text{dep}}$ and $Q_{\text{hyp}}$ are $Q$ at depolarizing and hyperpolarizing limits, respectively), $F$ is Faraday’s constant, $R$ is the gas constant and $T$ is the absolute temperature. $V_{0.5}$ is the membrane potential where there is 50% charge transfer, and $z$ is apparent valence of the movable charge. Fits of data with equations were performed using Sigma Plot 9.0.

Sugar transport- Oocytes were incubated in 50 μM, 1 mM, or 6 mM of αM-glc (as indicated in each experiment) with traces of 14C-αM-glc for one hour in Na+ solution or in Na+-free solution. After the incubation, the oocytes were washed with cold Na+-free solution, and individually solubilized. Sugar uptake was determined by using a scintillation counter. Sugar uptake in non-injected oocytes from the same batch of oocytes was used as control.

Stoichiometry experiments- Oocytes, clamped at -90 mV, were perfused with 5 mM αM-glc with traces of 14C-αM-glc for one hour in Na+-free solution and Na+-free solution. After the incubation, the oocytes were washed with cold Na+-free solution, and individually solubilized. Sugar uptake was determined by using scintillation counting. Sugar uptake into non-injected oocytes from the same batch of oocytes was used as control. 

RESULTS

Wild type mSGLT3 (WT-mSGLT3b) and the mutant proteins G457E and G457Q were expressed in oocytes to test for differences in their function. First we tested if glucose induced any current in the proteins. The oocytes were clamped at -50 mV and glucose induced currents were recorded at different voltages, ranging from -150 mV to +50 mV. Figure 2 shows examples of induced currents recorded from WT-mSGLT3b (Fig. 2B), G457E (Fig. 2C), and G457Q (Fig. 2D) at those voltages, bathed in different glucose concentrations. The large currents that some of the glucose concentrations induced illustrate that there was robust expression of the three different clones in the oocytes. In addition, while the currents at any given sugar concentration through WT-mSGLT3b were approximately ohmic, there appeared to be increased voltage dependence in G457Q, and more dramatically in G457E, indicating that the nature of the residue at 457 affects the voltage dependence of the protein.

Because a mutation at amino acid 457 in human SGLT1 can lead to the disease glucose-galactose malabsorption due to the lack of sugar transport (7, 8) and the equivalent residue already present in the human SGLT3 appears to prevent glucose transport (3), we tested whether the mSGLT3b mutants have altered glucose transport properties. Figure 3 shows that in the presence of 50 μM of αM-glc for one hour, αM-glc transport increased approximately 4-fold in G457Q (36 ± 5 pmols; n=4) compared to WT-mSGLT3b (9 ± 0.2 pmols; n=4) but was reduced to non-injected control levels (2.5 ± 0.2 pmols; n=4) in G457E (2 ± 0.1 pmols; n=4). Additionally, we found that sugar uptake in WT-mSGLT3b and G457Q was Na+-dependent, since removal of Na+ from the media abolished uptake. These results indicate that in 50 μM αM-glc, glutamine at residue 457 in SGLT3b increases the sugar transport while glutamate decreases sugar transport.

Apparent sugar affinities ($K_{0.5}$) were calculated for WT-mSGLT3b and for the mutants G457E and G457Q. Table 1 presents the means of the apparent affinities for glucose and αM-glc measured at -90 mV. The apparent affinities of WT-mSGLT3b for αM-glc and glucose were 62 ± 5 mM (n=5) and 77 ± 3 mM (n=6) respectively. These apparent sugar affinities are low compared with SGLT1 or SGLT2 but agree with the previously published
G457E has very similar apparent sugar affinities to WT-mSGLT3b: $K_{0.5} \alpha$-M-glc was 65 ± 0.1 mM (n=2), and $K_{0.5}$ for glucose was 59 ± 4 mM (n=4). However, when residue 457 was a glutamine, the apparent affinities were higher: 9 ± 0.6 mM (n=4) and 6 ± 0.2 mM (n=4) for $\alpha$-M-glc and glucose, respectively. It was previously reported that the apparent affinity of WT-mSGLT3b for 6-deoxy-glucose (6DO-glc) is stronger than for glucose (12). Notably, here we found that the apparent affinity of G457Q for 6DO-glc (4 ± 0.3 mM) was not significantly different than for glucose (Table 1). Thus, when residue 457 is glycine (WT) the –OH group of C6 of the pyranose ring prevents the sugar from binding strongly, but when it is glutamine (G457Q) that hydroxyl group does not affect the sugar binding and glucose and 6DO-glc interact strongly and similarly. This is interesting because the crystal structure of vSGLT shows that there is a direct interaction between the residue equivalent to the mammalian 457, Q428 in \textit{vibrio}, and the hydroxyl group of the C6 of the galactose (Fig. 4, ref. 5). When the residue is short, glycine, the interaction is better when there is no –OH group at C6, and, when the residue is longer, glutamine, the interaction is not affected if there is no –OH group. Thus, the difference in apparent affinities between glucose and 6DO-glc in mSGLT3b and G457Q could be due to allosteric alteration of the binding site, to differences in formation of hydrogen bonds, or both.

Figure 5 shows the $K_{0.5}$s for glucose at different voltages in individual oocytes expressing each of the three proteins, demonstrating that WT-mSGLT3b and G457E had the same apparent affinity at all the voltages tested while G457Q resulted in stronger apparent affinity.

Next we calculated the apparent Na$^+$ affinity, $K_{0.5}$, for G457Q and G457E. The $K_{0.5}$ for WT-mSGLT3b is ~ 20 mM (12). While the residue analogous to 457 in vSGLT is known to interact directly with the sugar molecule (5), we found that mutations at this residue in mSGLT3b affected the apparent affinity for Na$^+$. Figure 6A shows sugar-induced currents at different Na$^+$ concentrations from WT-mSGLT3b, G457Q and G457E at -50 mV.

The lowest apparent Na$^+$ affinity belonged to G457E and the highest to G457Q, while WT-mSGLT3b had an intermediate apparent affinity. We also calculated these apparent Na$^+$ affinities across a range of voltages (Fig. 6B) and found that the apparent affinity for G457Q ranged from 11 ± 2 mM at -150 mV to 21 ± 4 mM at -10 mV (data are the mean ± SEM of 4 different experiments). Comparing these results with the WT-mSGLT3b apparent affinities (data from ref. 12), the apparent affinity of Na$^+$ for G457Q is between 2-3 times higher than for WT-mSGLT3b. This change may be due to higher sugar affinity, since $K_{0.5}$ is measured by sugar-induced currents at different [Na$^+$]. For G457E-mSGLT3b, it was not possible to calculate the $K_{0.5}$ at voltages between -150 mV and -70 mV because the currents did not saturate and could not be accurately fit to a Hill equation. The data obtained at more depolarizing voltages indicated that the $K_{0.5}$s are higher than the $K_{0.5}$ obtained for the WT-mSGLT3b protein, and the $K_{0.5}$s are even higher at hyperpolarizing voltages. Since the apparent sugar affinity was higher in G457Q than in WT-mSGLT3b, the differences shown in $K_{0.5}$ may be an indirect effect of the differences in sugar affinity. However, the apparent sugar affinities for WT-mSGLT3b and G457E were similar, thus the differences observed between their $K_{0.5}$s were not due to different sugar affinities.

Since the apparent affinities of sugar were higher for G457Q than for G457E and WT-mSGLT3b, we thought this might underlie the large difference we observed in the magnitude of sugar uptake between them (Fig. 3). Therefore, we repeated the sugar transport experiments at sugar concentrations that were ~10% of the $K_{0.5}$ of each of the respective proteins (Fig. 7). In these experiments, the external sugar concentrations were 6 mM for WT-mSGLT3b and G457E, and 1 mM for G457Q. At these concentrations, the sugar transport was similar in the WT-mSGLT3b and in G457Q expressing oocytes. These data, compared with the data obtained with 50 $\mu$M sugar concentration, clearly show that apparent sugar affinities account for the differences in amount of sugar uptake within these transporters when performing the experiment with low concentrations of the substrate (Fig. 3) in WT-
mSGLT3b and G457Q. However, the transport for G457E was still low, only ~15% that of WT-mSGLT3b, indicating that G457E has less sugar transport capability.

The question remains as to why the sugar transport in G457E was so much lower than the WT-mSGLT3b? We therefore analyzed the mutant G457E in more depth.

We analyzed pre-steady-state currents of WT-mSGLT3b, G457Q and G457E. Pre-steady-state currents represent partial reactions of the transport cycle that are voltage dependent. By analyzing those currents, we obtained information on the distribution of the transporter in different states. Figure 8A shows currents of one G457E expressing oocyte in the presence of Na+ and without sugar. The figure shows the currents of an oocyte clamped at -50 mV and when the voltage was jumped from -150 mV to +50 mV in +20 mV increments. WT-mSGLT3b, G457E and G457Q pre-steady-state currents were analyzed to obtain the charge transfer (Q) at each voltage and to fit those data with a Boltzmann equation. Charge/voltage obtained for G457E is presented in Figure 8B (down triangle). For comparison, we also plotted data from WT-mSGLT3b to show how G457E data were shifted to depolarizing voltages. The same Q/V plot and analysis were done with G457Q (not shown). Data obtained from the fit to the Boltzmann equation (dotted line) in G457E and G457Q along with the data for WT-mSGLT3b (from ref. 12) are presented in Table 2. $V_{0.5}$ in G457E was +36 mV while in G457Q it was +3 mV and this shift in $V_{0.5}$ is even greater when we compare it to the $V_{0.5}$ of WT-mSGLT3b, -17 mV. These data indicate that at the same membrane potential, the three proteins have different distributions among the different states of the protein cycle.

Apart from the $V_{0.5}$, the other kinetic parameters obtained from analyzing the pre-steady-state currents were similar in the three proteins: Turnover numbers, calculated as $I_{\text{max}}$ divided by $Q_{\text{max}}$ (as previously described for other SGLT proteins (13)) were 76, 64 and 75 cycles per second in G457E, G457Q and WT-mSGLT3b respectively, and the $Q_{\text{max}}$ were 17, 25 and 25 nC. These $Q_{\text{max}}$ values indicate that the transport levels are comparable and that decreased expression levels do not explain the lower sugar uptake in G457E.

Figure 8C shows the average sugar-induced current in G457E and WT-mSGLT3b expressing oocytes (mean ± SEM). At -150 mV, the sugar induced current in G457E was the same as in WT-mSGLT3b. However, at more depolarizing voltages, the induced currents were lower in G457E than in the WT-mSGLT3b protein. These lower currents could explain, at least partially, why the sugar transport in G457E expressing oocytes is lower than in WT-mSGLT3b, since the resting potential of the expressing oocytes is between -30 and -50 mV, and Figure 7 represents sugar transport measured in non-clamped oocytes.

Finally, we tested the ratio of charge to sugar transport (Fig. 8D). SGLT1 transports 2 sodium ions with each sugar (14). The WT-mSGLT3b ratio was 2.6 charges per sugar molecule (data from ref. 12) showing that this transport was not as tightly coupled as in SGLT1. In G457E, we found that charge:sugar transport was even more uncoupled, with an average of 4.5 charges per sugar molecule. Thus, the same magnitude of sugar-induced current in G457E as in WT-mSGLT3b will correspond with less sugar transported by G457E compared to WT-mSGLT3b. Thus, the lower rate of sugar transport observed in G457E (Fig. 7) can be due to either the decreased sugar-induced currents at resting potentials (Fig. 2 and Fig. 8C), to the uncoupled charge:sugar transport phenotype (Fig. 8D), or to a combination of both.

**DISCUSSION**

The goal of this study was to understand the functional differences between SGLT1 and SGLT3. The advantage of studying mSGLT3b instead of human SGLT3 is that we can analyze pre-steady-state currents and calculate the apparent affinities of Na+ (typical recordings of hSGLT3 do not show pre-steady-state currents (3) and to date, the apparent Na+ affinity in hSGLT3 has not been resolved). We studied the mSGLT3b mutants G457E and G457Q, which recapitulate the amino acids at residue 457 in hSGLT3 and SGLT1, respectively. Thus, we investigated the modifications in apparent sugar and Na+ affinities, ability to transport sugar, the
distribution of the protein in different states, and
the charge-to-sugar stoichiometry to suggest a
mechanistic model for current transport
uncoupled from sugar transport.

**Apparent Na\(^+\) affinity.** We found that
G457E has lower apparent Na\(^+\) affinity than
WT-mSGLT3b while both have the same
apparent sugar affinity. Can a different
distribution of G457E between inward and
outward facing states explain this lower Na\(^+\)
affinity? A simplified six-state ordered kinetic
model that described the states of SGLT1 was
earlier proposed (15, 16). Half of the states have
the binding site of the protein facing the
extracellular space (C1, C2 and C3 of Fig. 9)
and half the intracellular space (C4, C5 and C6).
By introducing glutamate at residue 457 we
added an extra negative charge to an already
voltage dependent protein. Analyzing the pre-
steady-state currents in G457E, we observed that
the \(V_{0.5}\), the voltage at which the charge is
equally distributed between inward and outward
facing conformations, is more positive than in
the WT-mSGLT3b protein (Fig. 8A, 8B, Table
2). This means that at physiological voltages a
higher percentage of the protein will be facing
out. Thus we would expect stronger apparent
affinities for sugar and Na\(^+\), the opposite of what
we observed. The extra negative charge in the
protein weakens the apparent affinity for Na\(^+\)
while leaving the apparent sugar affinity
unaffected. Hence the favorable distribution of
G457E towards the outward facing conformation
compared with WT-mSGLT3b does not explain
the weaker \(K_{0.5}^{\text{Na}}\) of the protein, since that
distribution would cause a stronger affinity.

The introduction of a negative charge at
457 weakened the apparent affinity of Na\(^+\) while
a neutral mutation (G457Q-mSGLT3b) significantly strengthened it (although this could be
due to a higher glucose affinity in G457Q). In
vSGLT one Na\(^+\) binding site was proposed to be
approximately 8-10 Å from the bound sugar
based on structural homology to LeuT. This
would correspond with the Na2 site in LeuT. In
dGLT the Na1 site was not detected, but in
LeuT the octahedral coordination for that Na1 includes one leucine carboxy oxygen (4, 5, 9).
We found that glutamate at residue 457, lowered
the apparent Na\(^+\) affinity. This suggests that
mutations at 457 may change the geometry of a
nearby Na\(^+\) binding site, Na2, but that residue
457 is not part of the Na\(^+\) binding site. However,
we cannot rule the possibility that in SGLT3b
one Na\(^+\) binds to the sugar, similarly to Na1 in
LeuT, so that alteration of the sugar binding will
directly affect that Na\(^+\) binding.

**Uncoupled current to sugar transport.**
According to the existing 6-state kinetic model
developed for SGLT1 (16, 17), empty outward
facing proteins (C1) first bind two sodium ions
(C2), then bind one sugar molecule (C3) and
undergo a subsequent conformational change
that transitions the protein from outward facing
to inward facing (C4) where the ligands are
released (C5 and C6) before returning to the
outward facing empty conformation (C1).
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that transitions the protein from outward facing
to inward facing (C4) where the ligands are
released (C5 and C6) before returning to the
outward facing empty conformation (C1).
Among SGLT clones that transport with
stoichiometries close to 2:1, all bind Na\(^+\) with
apparent affinities in the 10-30 mM range
(SGLT1 (14); mSGLT3b (12); pig SGLT3 (11)).
In contrast, G457E had weaker Na\(^+\) affinity and
an uncoupled stoichiometry 4.5:1 (Fig. 8D),
while the apparent affinity of human SGLT3 for
Na\(^+\) remains undetermined. Applying the 6-state
model to all SGLT proteins, one simple
explanation for progressively more uncoupled
stoichiometries is a greater fraction of proteins
that undergo the C2-C5 transition, thereby
releasing internal Na\(^+\), but without any sugar
binding or transport. However, this explanation
cannot account for uncoupled current in its
entirety. In particular, in human SGLT3 the
current is sugar dependent yet none of the
protein appears to reach state C4. What then is
the determinant of sugar transport or non-
transport in SGLT proteins?

The apparent affinity of sugar does not
appear to determine sugar transport, as WT-
mSGLT3b and G457Q transport equally well
when the sugar concentration was normalized to
their respective \(K_{0.5}^{\text{Na}}\) values (Fig. 7). However,
G457E, with the same apparent sugar affinity
but lower Na\(^+\) affinity than WT-mSGLT3b,
transports sugar less efficiently (Fig. 7), and
more uncoupled from the charge transport (Fig.
8D). These data suggest that the ability or failure
to transport sugar depends upon completion of
the conformational change after sugar binding.
In other transporters, the existence of
intermediate states between C3 to C4 has been
proposed (18). In SGLT1, although intermediate
states have been described between C6 and C1 (19), none has been proposed between C3 and C4.

**Intermediate conformational states.** The order of release of Na\(^+\) and sugar by SGLT is not yet well established. Equilibrium simulations performed in vSGLT suggest that Na\(^+\) is released intracellularly very quickly and before the sugar (20). Moreover, using molecular dynamics simulations in LeuT it was proposed that one Na\(^+\) is released before the other Na\(^+\) and the substrate (21). Thus, in our proposed model for SGLT (Fig. 9), one Na\(^+\) is first released inside from an intermediate state (C3\') between C3 and C4, while sugar and the other Na\(^+\) are occluded. Then, there is a conformational change that expose the occluded Na\(^+\) and sugar (C4) followed by their release (C5 and C6). Uncoupled stoichiometries could be explained by the transition from C3\' to C3'a, where the first Na\(^+\) is released but the conformational change to expose Na\(^+\) and sugar does not occur. The side chain at residue 457 may be responsible for preventing the progression (C3’a to C4) towards the conformation that favors intracellular release of the other Na\(^+\) and sugar, thus the transporter goes back towards the outward-facing conformation (C3’a to C3’b to C3). In fact, it was recently proposed that charge and polarity of residue 457 affect the conformational changes that occur after Na\(^+\) and sugar binding (22). Human SGLT3, which transports ions but not sugar, would then be the most extreme manifestation of this failure to complete the translocation step, shifting the protein towards C3’a and with no protein completing the C3’a to C4 transition.

An alternative explanation for the failure to complete C3’a to C4 transition could be due to the premature release of the Na\(^+\) when apparent Na\(^+\) affinity is low. In addition, our data do not differentiate whether only one or both Na\(^+\) ions are released during sugar-dependent ion leak.

In conclusion, the nature of the side chain at amino acid 457 has an effect on the apparent sugar and Na\(^+\) affinities and, moreover, on the uncoupled charge transport. Sugar affinity is likely to be affected by a direct effect of the sugar binding site, but the Na\(^+\) affinity may be affected by allosterically altering the Na\(^+\) binding site. Uncoupled stoichiometries may reflect the existence of an intermediate state from which the protein can release one Na\(^+\). This must be followed by difficulties in progressing to states where the other Na\(^+\) and sugar can be released. The degree of uncoupling in transport would then be dependent on the energetic balance between transition to the fully inward facing state, where the other Na\(^+\) and sugar could be released, versus returning to an outward facing conformation.

**REFERENCES**


FOOTNOTES

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FIGURE LEGENDS

Fig. 1. Amino acid alignment of several SGLT proteins. All the SGLT1 and SGLT2 proteins present a glutamine at position 457 while the residue at this position in SGLT3s are glutamate, glycine or serine. h: human, m: mouse, r: rat, TM11: transmembrane segment 11.

Fig. 2. Glucose-induced currents in control and WT-mSGLT3b, G457E and G457Q expressing oocytes. Steady-state currents induced by various concentrations of glucose at voltages ranging from -150 mV to +50 mV were recorded from oocytes injected with RNA and expressing each of the constructs. Results from individual oocytes are shown.

Fig. 3. Residue 457 affects sugar transport. Sugar uptake in non-injected oocytes or those expressing WT-mSGLT3b, G457E or G457Q in the presence or absence of Na⁺. Oocytes expressing WT-mSGLT3b transport more sugar than control oocytes. The mutation G457E abolished sugar transport while G457Q dramatically enhanced sugar transport in these experimental conditions (100 mM Na⁺ and 50 μM αM-glc).

Fig. 4. Interaction between galactose (Gal) and residue Q428 in vSGLT (5). The figure shows amino acid Q428, which corresponds to G457 in mSGLT3b, interacting with the galactose molecule at hydroxyl group from C6 and the oxygen (O5) in the pyranose ring. Glucose differs from galactose only in the orientation of the hydroxyl group of the C4. Red spheres are oxygen molecules, blue are nitrogen and dotted lines represent hydrogen bonds.

Fig. 5. Residue 457 has an effect on glucose affinity in mSGLT3b. Glucose Kₐ was calculated based on fits of equation [1] to sugar-induced currents in single oocytes expressing WT-mSGLT3b, G457Q or G457E. Kₐ for glucose in WT-mSGLT3b and the mutants G457Q and G457E as a function of voltage. Note that the apparent affinities of WT-mSGLT3b and G457E are very similar. However, G457Q showed much higher apparent affinities. Average data from different oocytes at -90 mV are presented in Table 1.
**Fig. 6.** Residue 457 influences the apparent Na⁺ affinity. A. Apparent Na⁺ affinities were measured in WT-mSGLT3b, G457Q or G457E expressing oocytes at -50 mV. Raw currents at different [Na⁺] were fit with the Hill equation (dotted lines). Data shown were normalized between the minimum and maximum as defined by the fit in order to display the shifts in K_{Na}^{0.5}. For the individual experiments shown, K_{Na}^{0.5} in WT-mSGLT3b was 30 ± 1.3 mM, G457Q was 11 ± 0.2 mM and G457E was 71 ± 11 mM. B. Average K_{Na}^{0.5} from WT-mSGLT3b (n=4), G457Q (n=4), or G457E (n=4). In G457E we were only able to fit the data between -10 mV and -70 mV and the data suggests that glutamate increases the K_{Na}^{0.5}.

**Fig. 7.** Sugar transport is decreased in G457E. Uptake of αM-glc in WT-mSGLT3b, G457Q and G457E as percentage of WT-mSGLT3b uptake. The concentration of sugar in the bath for each protein was adjusted to 10% of the K_{0.5} for each respective protein (6 mM for WT and G457E, 1 mM for G457Q). The average sugar uptake of WT-mSGLT3b (1,243 pmols) was normalized to 100%. The uptake into comparably incubated non-injected oocytes has been subtracted in each case. Under these conditions WT and G457Q showed very similar amounts of uptake, while G457E remained transport deficient. Data shown as mean ± SEM, n=10.

**Fig. 8.** Voltage dependence and stoichiometry of G457E. A. Currents were recorded in the absence of sugar from oocytes expressing G457E, held at -50 mV and subjected to 100 ms test pulses from -150 mV to + 50 mV. B. Charge movement in G457E was found by integrating the non-capacitive pre-steady-state component of the “Off” currents. The V_{0.5} was depolarized compared to the V_{0.5} for WT-mSGLT3b, suggesting that at rest the Na⁺ and sugar binding sites are more likely to be facing outward. C. Average sugar-induced currents of WT-mSGLT3b (n=6) and G457E (n=7) as a function of voltage. D. Stoichiometry of charge movement to sugar uptake, measured by simultaneously recording currents and sugar uptake under voltage clamp. G457E was significantly uncoupled compared to WT-mSGLT3b (12), which is itself slightly uncoupled compared to SGLT1 (2:1 ref. 14).

**Fig. 9.** Intermediate conformational states in the transport cycle of SGLT proteins. Cartoon adapted from the transport model previously described for SGLT proteins (modified from ref. 15), that includes intermediate states between C3 and C4. Intermediate states between C6 and C1 earlier described are represented as multiple arrows joining C6 and C1 (19). The simplified kinetic model suggests that the empty protein facing the outside of the cell (C1), binds first 2 Na⁺ (C2) and then sugar (C3). Then, the protein reaches an intermediate state where one Na⁺ is exposed (C3’) and then released (C3’a) while the other Na⁺ and the sugar are occluded. An additional conformational change exposes the sugar and Na⁺ (C4), and they are then released (C5 and C6). The empty transporter (C6) faces again the extracellular compartment (C1). From the state where the first Na⁺ is released, C3’a, if the sequence of the protein does not allow the next conformational change that exposes the other Na⁺ and sugar (C4), the protein will go into conformation C3’b, facing outward again. Protein will be back in conformation C3 if one Na⁺ is bound. This alternate cycle (***) will result with Na⁺ transport with no sugar transport. Thus, in SGLT1s, the protein will tend to follow the cycle *. However, if the ratio of charge to sugar transport is greater than 2:1, in addition to cycle *, a percentage of the protein will follow cycle ** which we call sugar-dependent ion leak. Circles represent Na⁺ ions and pentagon the sugar.
### TABLES

**Table I** Apparent affinities in mM for glucose and $\alpha$M-glc for WT-mSGLT3b, G457E and G457Q, measured at -90 mV in oocytes. Values shown are mean ± SEM, and the number of experiments varies between 2 and 6.

<table>
<thead>
<tr>
<th>Sugar</th>
<th>WT-mSGLT3b</th>
<th>G457E</th>
<th>G457Q</th>
</tr>
</thead>
<tbody>
<tr>
<td>glucose</td>
<td>77 ± 3</td>
<td>59 ± 4</td>
<td>6 ± 0.2</td>
</tr>
<tr>
<td>$\alpha$M-glc</td>
<td>62 ± 5</td>
<td>65 ± 0.1</td>
<td>9 ± 0.6</td>
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**Table II** Summary of kinetic parameters. Parameters obtained from analyzing pre-steady-state currents on G457Q and G457E. The table includes values for WT-mSGLT3b from ref. 12. These data were derived from fits of the Boltzmann equation to Q-V distributions as shown in Figure 8. $Q_{\text{max}}$ is the total charge movement by the protein. $V_{0.5}$ is the voltage at which the charge is equally distributed between inward and outward facing conformations, and turnover is the rate at which the protein cycles. All parameters, except $V_{0.5}$, were similar between both mutants and similar to WT-mSGLT3b. $V_{0.5}$ in G457E is more depolarized than in G457Q. Data is shown as mean ± SEM, n= 6 to 8.

<table>
<thead>
<tr>
<th></th>
<th>WT-mSGLT3b</th>
<th>G457E</th>
<th>G457Q</th>
</tr>
</thead>
<tbody>
<tr>
<td>$I_{\text{max}}$ (nA)</td>
<td>1,800 ± 250</td>
<td>1,200 ± 130</td>
<td>1,600 ± 330</td>
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<tr>
<td>$Q_{\text{max}}$ (nC)</td>
<td>25 ± 4</td>
<td>17 ± 2</td>
<td>25 ± 4</td>
</tr>
<tr>
<td>$V_{0.5}$ (mV)</td>
<td>-17 ± 1</td>
<td>36 ± 2</td>
<td>3.3 ± 4</td>
</tr>
<tr>
<td>Turnover (cycles sec$^{-1}$)</td>
<td>75 ± 3.4</td>
<td>76 ± 8</td>
<td>64 ± 6</td>
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<tr>
<td>rSGLT3b</td>
<td>QNGQLFHYIESFSSYIGPP</td>
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</tbody>
</table>

457
Figure 2

A  Control

B  WT-mSGLT3b

C  G457E-mSGLT3b

D  G457Q-mSGLT3b
Figure 3

pmols αM-glC uptake/hour

Na⁺  Na⁺-free

control  WT  G457E  G457Q

mSGLT3b
Figure 4
**Figure 5**

![Graph showing the relationship between membrane voltage and glucose concentration for G457Q, WT, and G457E](image)

- **G457Q**
- **WT**
- **G457E**

**Axes:**
- **X-axis:** Membrane voltage, mV
- **Y-axis:** $K_{0.5}$ glucose, mM
Figure 6

A

\[
\text{\% maximal current vs. [Na], mM}
\]

- G457Q
- WT
- G457E

B

\[
\text{K}_{0.5} Na, mM vs. Membrane voltage, mV}
\]

- G457Q
- WT
- G457E
Figure 7

WT G457E G457Q

mSGLT3b

% WT uptake/hour
Figure 8

A

B

C

D

Membrane voltage, mV

0.0 0.2 0.4 0.6 0.8 1.0

Q(nC)

-150 -100 -50 0 50 100

Membrane voltage, mV

-150 -100 -50 0 50 100

Charge/\alpha\text{-}M\text{-}glc uptake

WT G457E

I, \mu A

-2.5 -2.0 -1.5 -1.0 -0.5 0 0.5

WT G457E
Sugar binding residue affects apparent Na+ affinity and transport stoichiometry in mouse sodium/glucose cotransporter type 3B
Ana Diez-Sampedro and Stephanie Barcelona

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